Precipitated Withdrawal by a Benzodiazepine Receptor Antagonist (Ro 15-1788) After 7 Days of Diazepam

Abstract. *Baboons implanted with intragastric catheters were given diazepam (10 milligrams per kilogram of body weight) twice daily for 45 consecutive days. On days 7 and 35, they were given intramuscular injections of the benzodiazepine receptor antagonist Ro 15-1788. Mild and intermediate withdrawal signs, including retching and vomiting, were observed after 7 days of diazepam, and more frequent and intense withdrawal signs, including tremor and convulsion, occurred after 35 days of diazepam. With the termination of the diazepam injections after 45 days, a mild to intermediate withdrawal syndrome was observed over the next 15-day period.*

The benzodiazepines, which are among the most widely used of all prescribed drugs, are given in the therapeutic management of anxiety, insomnia, muscle rigidity and spasticity, and convulsions. Treatment with benzodiazepines frequently extends for periods of months or years, and there have been numerous clinical reports of a withdrawal syndrome upon the abrupt cessation of drug administration after such long-term treatment. The incidence of withdrawal is controversial. Although a review of the literature suggests a relatively low incidence of withdrawal reaction (*1*), other studies suggest that, if patients are evaluated carefully, the incidence of withdrawal may be considerably higher, even among those maintained at therapeutic dose levels (*2*). The signs and symptoms of withdrawal resemble those that occur with alcohol and barbiturates (*1*). The benzodiazepine withdrawal syndrome has also been documented in animal studies. Cessation of drug administration after oral dosing of diazepam or chlordiazepoxide to monkeys for four or more weeks produced a typical barbiturate-like withdrawal syndrome (*3*).

The discovery of specific, high-affinity benzodiazepine receptors in the mammalian brain (*4*) has spurred considerable research relating to the site of central action of these compounds (*5*). High correlations obtained between the potencies of a series of benzodiazepines in binding the receptor and their potencies as anxiolytics, muscle relaxants, and anticonvulsants strongly suggest that these receptors mediate the therapeutic actions of the benzodiazepines. Recently, a specific benzodiazepine receptor antagonist was discovered (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5-a)(1,4)benzodiazepine-3-carboxylate, or Ro 15-1788) that blocks numerous pharmacological effects of the benzodiazepines (*6*). This antagonist provides a unique and potentially powerful tool for further investigating benzodiazepine withdrawal effects. We now report that Ro 15-1788 precipitates an acute withdrawal syndrome when administered to baboons that have received diazepam for as short a period as 7 days.

Four adult male baboons (*Papio anubis*) weighing between 25 and 31 kg were surgically implanted with intragastric catheters (*7*) and placed in a harness-tether restraint system. One baboon (JE) was drug-naive; the other three had his-

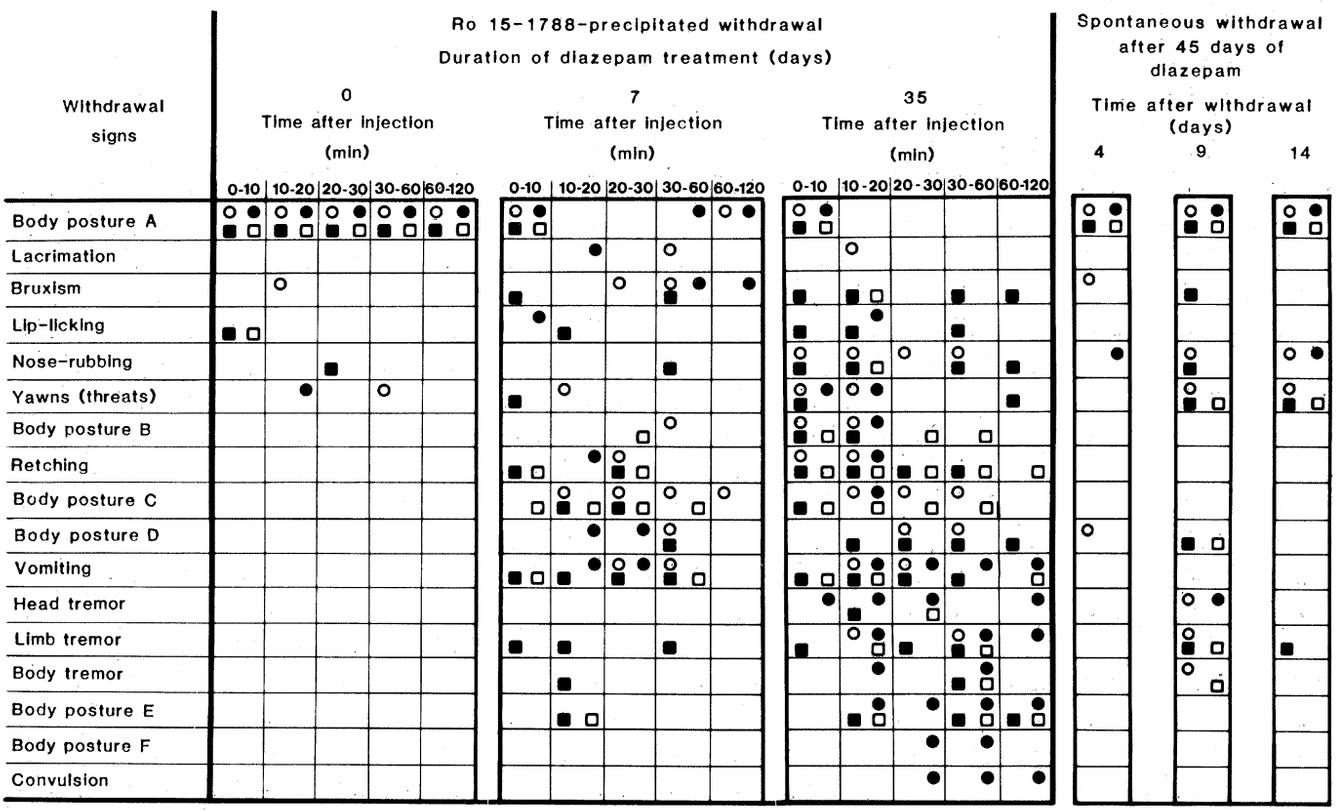

Fig. 1. Data on Ro 15-1788–precipitated withdrawal and spontaneous withdrawal in diazepam-treated baboons. Data for day 0 represent control data obtained after administration of Ro 15-1788 (5.0 mg/kg, intramuscularly) alone. The antagonist Ro 15-1788 was also given on days 7 and 35 of continuous diazepam administration (20 mg/kg per day, intragastrically). We assessed spontaneous withdrawal by terminating diazepam administration after 45 consecutive days. Symbols indicate that the withdrawal sign occurred one or more times in the time block except for yawns for which four or more were required. The data shown for the spontaneous withdrawal were for 10-minute observation periods at 12 noon on days 4, 9, and 14. Body postures are depicted in Fig. 2. Individual subjects are indicated by different symbols: PH, ○; AL, ●; SA, ■; and JE, □.

0036-8075/82/0917-1161$01.00/0
Copyright © 1982 AAAS

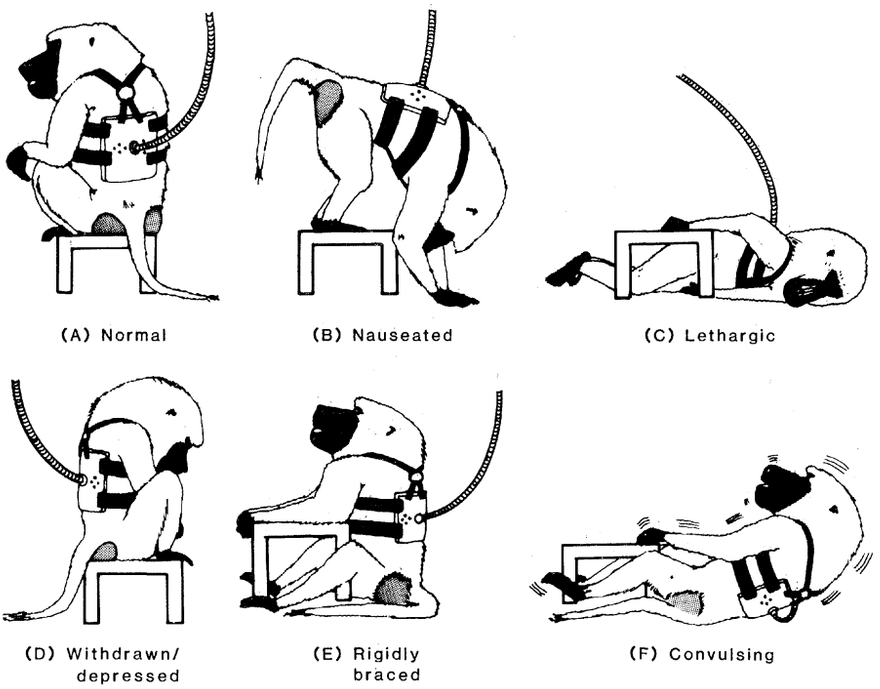

Fig. 2. Baboon body postures observed during control conditions and during benzodiazepine withdrawal. The ratings in Fig. 1 were conducted on the basis of the observation of body postures and not on the basis of the interpretative labels that appear below each posture sketch. The baboons are wearing harness-tether vests. The rigidly braced posture may be a preconvulsive state.

tories of intermittent exposure to various drugs, none of which were benzodiazepines. Diazepam was put into suspension (*8*) and administered at a dosage of 10 mg/kg, intragastrically, twice daily (9:00 a.m. and 5:00 p.m.) for 45 consecutive days (*9*). At days 7 and 35 of diazepam administration, Ro 15-1788 was administered (5.0 mg/kg, intramuscularly) at 11:00 a.m. (*10*), and the subjects were continuously observed for signs of withdrawal over a 2-hour period. Subjects were also observed for withdrawal after receiving control injections of Ro 15-1788 (5.0 mg/kg, intramuscularly) either prior to diazepam administration (subjects SA and JE) or 6 weeks after the termination of diazepam administration (subjects PH and AL). All subjects were observed for withdrawal after receiving vehicle control injections on days 6 and 34 of diazepam administration. Approximately 30 percent of these withdrawal observations were conducted by observers who were blind to the experimental condition. Diazepam dosing was continued until day 45, at which time injections were stopped and the subjects were observed for signs of spontaneous withdrawal over the next 15 days (*11*).

Subjects were sedated, ataxic, and hypokinetic but remained ambulatory during the early days of diazepam administration. In addition, in three of the baboons a loss of facial muscle tone was evidenced by a characteristic droop of the lower lip. Subjects developed virtually complete tolerance to these sedative effects over the course of diazepam administration (*12*). One of the baboons (subject PH), however, continued to display the "lip-droop" throughout the diazepam treatment. There were no obvious changes in eating or drinking behavior during the period of diazepam administration.

No unusual changes in behavior were noted after the control injections of Ro 15-1788 (day 0 in Fig. 1) or after injections of the Ro 15-1788 vehicle on days 6 and 34 of diazepam administration. Normal eating, drinking, and grooming predominated for the next hour. The withdrawal signs noted after injection of Ro 15-1788 in baboons treated with diazepam for 7 days included bruxism, retching, vomiting, and several abnormal body postures suggesting nausea, lethargy, and withdrawal/depression (Figs. 1 and 2). One subject also exhibited body and foot tremors. Withdrawal effects peaked 10 minutes after the Ro 15-1788 injection and lasted about 2 hours. In comparison to that observed after 7 days, the frequency of withdrawal signs precipitated by Ro 15-1788 was increased after 35 days of diazepam administration. Also, these withdrawal signs persisted for 4 to 6 hours after the injection. In addition to body and limb tremors, head tremors were observed in two subjects and grand mal convulsions in one subject (Figs. 1 and 2).

After 45 days of diazepam administration, we examined spontaneous withdrawal by stopping the diazepam injections. No clear signs of withdrawal appeared until day 7, after which signs progressively developed, peaked on days 9 and 10, and subsided thereafter (Fig. 1). Food intake was suppressed to approximately 25 percent of control levels beginning on day 8 and remained at a low level throughout the 15-day period.

To verify that the observed precipitated withdrawal signs reflected physical dependence and not an idiosyncratic interaction between diazepam and Ro 15-1788, an additional control experiment was conducted 8 weeks after termination of the diazepam in three subjects. In this experiment Ro 15-1788 was given at a dose of 5.0 mg/kg, intramuscularly, 1 hour after the subjects had received a single dose (20 mg/kg) of diazepam intragastrically. Diazepam produced signs of sedation and ataxia which were completely reversed by the antagonist. No withdrawal signs were observed during a 4-hour postinjection period.

The results of this study demonstrate that physical dependence to diazepam, as revealed by precipitated withdrawal, can develop in as few as 7 days of diazepam administration. However, the degree of withdrawal after 7 days was not maximal since after 35 days more intense and frequent withdrawal signs were observed, including grand mal convulsions. This finding is consistent with an earlier study (*3*) that demonstrated that the intensity of withdrawal produced by diazepam increases with longer exposure.

Although the dose of diazepam used in this study was relatively large (*13*), it was by no means behaviorally toxic. Subjects remained ambulatory throughout and, although signs of sedation were evident during the early days of diazepam administration, almost complete tolerance developed to these effects.

The withdrawal induced by Ro 15-1788 had a rapid onset, was very intense, and lasted only a few hours. Spontaneous withdrawal, in contrast, had a slow onset, was relatively mild, and lasted for a number of days. These differences between Ro 15-1788–precipitated and spontaneous withdrawal are quite likely related to the kinetics of diazepam (or metabolites) receptor binding. Diazepam and its active metabolite, *N*-desmethyl-

diazepam, are only slowly eliminated from the body (*14*); thus, the rate at which the benzodiazepine receptors are left unoccupied is relatively slow, thereby producing a gradual change in the functional status of the central nervous system. On the other hand, by virtue of its ability to displace diazepam from the benzodiazepine receptor, Ro 15-1788 produces a relatively rapid change in the central nervous system. A similar phenomenon has been identified for the opioid 1-α-acetylmethadol (LAAM). This opioid has long-acting metabolites (*15*) that delay the onset of abstinence signs after spontaneous withdrawal (*16*). Immediate and intense withdrawal effects, however, were observed after LAAM-treated animals received the opioid receptor antagonist naloxone (*16*).

The clinical significance of the relatively rapid development of physical dependence to diazepam observed in our study is unclear. Several clinical case reports indicate that withdrawal signs and symptoms have been observed after relatively short-term administration (2 weeks to 4 months) of diazepam, even at therapeutic doses (*17*). The low frequency of such reports, however, has led some investigators to conclude that the risk of significant benzodiazepine withdrawal is very low if patients have been taking diazepam for long periods at therapeutic doses for 6 months or less (*18*). On the other hand, it has been argued that benzodiazepine withdrawal may be missed or underreported because anxiety is the cardinal symptom of both benzodiazepine withdrawal and of the original condition for which the drug was prescribed (*19*). Carefully conducted clinical studies will be necessary to determine the minimum dose and treatment interval after which significant benzodiazepine withdrawal occurs.

A major implication of this study is that benzodiazepines may produce clinically relevant functional changes in the central nervous system more rapidly than heretofore expected. The procedure used in this experiment of exploring the development of physical dependence on benzodiazepines with antagonist-precipitated withdrawal will be useful in investigating biochemical, neurophysiological, and behavioral aspects of benzodiazepine physical dependence.

SCOTT E. LUKAS
ROLAND R. GRIFFITHS
Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

References and Notes

1. J. Marks, *The Benzodiazepines: Use, Overuse, Misuse, Abuse* (University Park Press, Baltimore, 1978).
2. H. Petursson and M. H. Lader, *Br. Med. J.* **283**, 643 (1981); P. Tyrer, D. Rutherford, T. Huggett, *Lancet* **1981-I**, 520 (1981); B. M. Maletzky and J. Klotter, *Int. J. Addict.* **11**, 95 (1976); N. Kemper, W. Poser, S. Poser, *Dtsch. Med. Wochenschr.* **105**, 1707 (1980).
3. T. Yanagita and S. Takahashi, *J. Pharmacol. Exp. Ther.* **185**, 307 (1973).
4. R. F. Squires and C. Braestrup, *Nature (London)* **266**, 732 (1977); H. Möhler and T. Okada, *Science* **198**, 849 (1977).
5. J. F. Tallman, S. M. Paul, P. Skolnick, D. W. Gallager, *Science* **207**, 274 (1980); W. Haefely, L. Pieri, P. Polc, R. Schaffner, *Handbook of Experimental Pharmacology* (Springer-Verlag, Berlin, 1981), vol. 55.
6. W. Hunkeler, H. Möhler, L. Pieri, P. Polc, E. P. Bonetti, R. Cumin, R. Schaffner, W. Haefely, *Nature (London)* **290**, 514 (1981); A. Darragh, R. Lambe, M. Scully, I. Brick, C. O'Boyle, W. W. Downie, *Lancet* **1981-II**, 8 (1981); H. Möhler, W. P. Burkard, H. H. Keller, J. G. Richards, W. Haefely, *J. Neurochem.* **37**, 714 (1981); P. Polc, J.-P. Lawrent, R. Scherschlicht, W. Haefely, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **316**, 317 (1981).
7. S. E. Lukas, R. R. Griffiths, L. D. Bradford, J. V. Brady, L. Daley, R. Delorenzo, *Pharmacol. Biochem. Behav.*, in press.
8. The suspending agent was Agent K (BioServ, Inc., Frenchtown, N.J.) at a concentration of 4.0 g/liter.
9. There was one exception to this procedure. Subject PH received a continuous infusion with a peristaltic pump of the suspended drug at the same daily dose (20 mg/kg).
10. There were several exceptions to this procedure. Subject AL was evaluated after the administration of 10 mg/kg, intragastrically, on day 7; subject PH was evaluated at day 14 (rather than day 7) at 10 mg/kg, intragastrically, and again at day 35 at 10 mg/kg, intramuscularly. The Ro 15-1788 was dissolved in a propylene glycol–alcohol–water vehicle in ratios of 40:10:43.5, respectively, the remaining percentage being buffers and preservatives (Valium vehicle) and administered in a volume of 2 ml per injection.
11. Observations were carried out for 10 minutes every 3 hours during the peak of withdrawal (days 9 to 10) and at least once daily (12 noon) on all other days.
12. In addition to these observational data, additional behavioral data were collected for subject AL. During daily sessions this animal responded on a lever which produced a 1-g food pellet after every 50th response. Response rates, which were progressively depressed over the first 4 days of diazepam treatment, gradually returned to predrug control levels by day 15 of diazepam administration.
13. Although daily therapeutic doses of diazepam are commonly in the range of 0.1 to 0.5 mg/kg, diazepam is apparently not infrequently abused at doses in excess of 3 mg/kg, and occasionally doses of 15 to 20 mg/kg have been reported [M. L. Stitzer, R. R. Griffiths, A. T. McLellan, J. Grabowski, J. W. Hawthorne, *Drug Alcohol Depend.* **8**, 189 (1981)].
14. J. A. F. DeSilva, B. A. Koechlin, G. Bader, *J. Pharm. Sci.* **55**, 692 (1966); M. Mandelli, G. Tognoni, S. Garattini, *Clin. Pharmacokinet.* **3**, 72 (1978).
15. S. E. Lukas, J. E. Moreton, N. Khazan, *J. Pharmacol. Exp. Ther.* **215**, 382 (1980).
16. G. A. Young, G. F. Steinfels, N. Khazan, *Pharmacol. Biochem. Behav.* **10**, 585 (1979).
17. H. H. Barten, *Am. J. Psychiatry* **121**, 1210 (1965); G. E. Woody, C. P. O'Brien, R. Greenstein, *Int. J. Addict.* **10**, 843 (1975); A. Rifkin, F. Quitkin, D. F. Klein, *J. Am. Med. Assoc.* **236**, 2172 (1976); P. Agrawal, *Can. Psychiatr. Assoc. J.* **23**, 35 (1978).
18. Proceedings of the Symposium at Georgetown University School of Medicine, *Drug Therapy* (supplement) (Biomedical Information Corp., New York, 1981).
19. H. Petursson and M. H. Lader, *Br. J. Addict.* **76**, 133 (1981).
20. We thank B. Bailer and E. Cook for technical assistance and K. Arvin for secretarial assistance. Diazepam and Ro 15-1788 were generously supplied by Hoffmann–La Roche Inc., Nutley, N.J. This research was supported by National Institute on Drug Abuse grant DA-01147 and contract 271-80-3718. S.E.L. is a recipient of National Institute on Drug Abuse national research service award DA-05186.

10 March 1982; revised 26 April 1982

Mammalian Tyrosinase Catalyzes Three Reactions in the Biosynthesis of Melanin

Abstract. *The biosynthesis of melanin is initiated by the catalytic oxidation of tyrosine to dopa by tyrosinase in a reaction that requires dopa as a cofactor. Tyrosinase then catalyzes the dehydrogenation of dopa to dopaquinone. The subsequent reactions can proceed spontaneously in vitro. Tyrosinase, purified from murine melanomas and the skins of brown mice, has now been shown to catalyze a third reaction in mammalian melanogenesis, namely the conversion of 5,6-dihydroxyindole to melanochrome. This reaction requires dopa as a cofactor and is inhibited by tyrosine. Conversely, 5,6-dihydroxyindole inhibits the oxidation of tyrosine to dopa, so that the relative concentrations of tyrosine and 5,6-dihydroxyindole within the mammalian pigment cell are capable of regulating melanogenesis in a previously unrecognized fashion. Tyrosinase has the unusual property of catalyzing three distinct reactions within a single biochemical pathway: the hydroxylation of a monophenol, the dehydrogenation of a catechol, and the dehydrogenation of a dihydroxyindole. The first and third of these reactions require dopa as a cofactor; in the second reaction, dopa is a substrate.*

Melanin is a biopolymer found throughout the animal and plant kingdoms. Regulation of melanin biosynthesis has been under investigation for more than 80 years, and the intermediate chemical reactions, known as the Mason-Raper pathway, were determined in the 1920's (*1*). The pathway is initiated by the conversion of tyrosine to dihydroxyphenylalanine (dopa), then of dopa to dopaquinone. Both steps are catalyzed by the enzyme tyrosinase (E.C. 1.14.18.1), and, in mammals, dopa is a cofactor for the oxidation of tyrosine to dopa. Melanotropin (MSH) causes a marked increase in tyrosinase activity and a concomitant increase in melanin formation (*2*). Until recently it appeared

0036-8075/82/0917-1163$01.00/0 Copyright © 1982 AAAS