7. The neutralization titer per milliliter is the product of the reciprocal of the highest dilution of the antiserum which completely neutralizes a sample of interferon and the titer of that sample of interferon expressed in reference units per milliliter.
8. I. A. Braude, C. Tarr, A. D. Sagar, P. B. Sehgal, in preparation.
9. S. L. Berger, M. J. M. Hitchcock, K. C. Zoon, C. S. Birkenmeier, R. M. Friedman, E. H. Chang, *J. Biol. Chem.* **255**, 2955 (1980).
10. A. D. Sagar *et al.*, *Nucleic Acids Res.* **9**, 149 (1981).
11. P. B. Sehgal and A. D. Sagar, *Nature (London)* **287**, 95 (1980).
12. Provided by W. E. Stewart II, originally prepared by Dr. K. Fantes.
13. Provided by Y. H. Tan.
14. I. A. Braude, L. S. Lin, W. E. Stewart II, *Biochem. Biophys. Res. Commun.* **89**, 612 (1979).
15. We thank Dr. I. Tamm for discussions and I. Augenzucker and R. Biehl for technical assistance, Dr. G. C. Tarr for help with the IFN-α production, and Drs. Y. H. Tan and W. E. Stewart II for providing us some of the antiserums to interferon used. Supported in part by grant NIAID AI-16262 (to P.B.S.), by a predoctoral institutional training grant from the National Institutes of Health (to A.D.S.), and by a junior faculty research award from the American Cancer Society (P.B.S.).

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Detection of Circulating Metallothionein in Rats Injected with Zinc or Cadmium

Abstract. *Circulating metallothionein was measured by radioimmunoassay over a 13-day period in male Sprague-Dawley rats that received a sequence of three intraperitoneal injections (at 3-day intervals) of either 5 milligrams of zinc or 0.8 milligrams of cadmium per kilogram of body weight. These amounts of zinc and cadmium produced metallothionein concentrations in the range of 2 to 5 nanograms per milliliter of serum (zinc) and 2 to 15 nanograms per milliliter of serum (cadmium). In control rats given saline injections over the same period the metallothionein concentration ranged from 1 to 3 nanograms per milliliter of serum.*

Numerous investigators, using physicochemical methods, have detected metallothionein (Mt) in rat and mouse plasma (*1–3*). However, the accurate measurement of low concentrations of Mt or its precursor thionein in the serum of animals exposed to metals (for example, Zn, Cd, Cu, Hg) for short periods has not been achieved to date because of the lack of a sensitive measurement technique (*4*). The recent development of a radioimmunoassay (RIA) for Mt with a detection limit of less than 100 pg now permits accurate measurements of circulating Mt (*5*). We used this method to measure Mt in the serum of rats given intraperitoneal injections of zinc and cadmium.

Male Sprague-Dawley rats (275 to 325 g) received three injections (spaced 3 days apart) of either $ZnSO_4$ in saline (5 mg of zinc per kilogram of body weight) or $CdCl_2$ in saline (0.8 mg of cadmium per kilogram of body weight); controls received saline only. The protocol was established by conducting dose-response experiments (*6*) and comparing our findings with those of others (*7, 8*). Blood, approximately 1.5 ml per sample, was obtained retro-orbitally at intervals of 18 or 24 hours (with a few instances of 6- or 12-hour intervals). The samples were allowed to clot, the serum was removed and centrifuged at 600*g* for 10 minutes to completely remove the cells, and the samples were frozen. Subsequently, four 50-μl portions from each sample were radioimmunoassayed for Mt.

Serum concentrations of Mt were determined by the use of simultaneously prepared logit-log regressions (*9*) derived from standardizing data relating the concentration of unlabeled Mt to the percentage of bound protein (the ^{125}I-labeled Mt used in the RIA). Significance levels of differences of means were evaluated on the assumption of a two-tailed Student *t* distribution and populations of unequal variances (*10*).

The curves (Fig. 1) relating Mt concentration to time after the initial injection are based on the values associated with blood samples being obtained at intervals of 18 or 24 hours; a few 12-hour values are included (*11*). The mean concentrations for the controls in each injection period are not statistically distinguishable; this encourages the belief that the injection and bleeding protocols did not induce Mt production independent of metal injection. The fiducial limits at 99 percent confidence (0.60 to 2.98 ng of Mt per milliliter) of the mean control concentration over the 13-day period are in accord with values previously found for Mt concentrations in the serum of humans with no history of significant exposure to heavy metals (*12*).

The nature of the response to the multiple injections of zinc (sequential relative maxima showing an increase with each injection) was characteristic of responses to multiple injections of zinc at nontoxic levels in the preliminary dose-response experiments (*6*). This behavior accords with results obtained by others (*13, 14*). The return of Mt concentration to control levels in about 4 days after the last injection is in accord with the reported short biological half-life of zinc and of the Mt it induces (*13, 15*).

In contrast to the slight initial response to the 5-mg/kg dose of zinc, the response to 0.8 mg of cadmium per kilogram led to a rapid increase in Mt concentrations (about 7 ng/ml per day) during the first 12 hours after injection (*16*), this peaking at about 48 hours and gradually subsiding with brief interruptions after the second and third injections. The response remained relatively constant at about four times the control levels in the terminal period (9 to 13 days); this response appears to be in accord with the reported long biological half-life of cadmium (*17, 18*).

The difference in character of the response to nontoxic doses of zinc and of cadmium in a sequence of multiple injections (a succession of increasing relative maxima followed by a return to control levels contrasted to an initial maximum followed by a gradual decrease to a level about four times control concentrations) is certainly in part dose-related (*6, 16*), but it must also reflect a difference between the two metals in the combined processes of initial Mt induction and the subsequent kinetics of metal retention, recirculation, and repeated Mt induction.

From our results on induced Mt concentrations in serum and the results of others on cadmium and zinc concentrations in plasma or serum after intraperitoneal or subcutaneous injections of these metals (*18–21*), we may arrive at some conclusions regarding Mt in its role as a metal-binding protein. Typical measurements at 1 to 30 minutes after the injection of cadmium indicate plasma concentrations about one-fifth of the injected whole-body concentration, these decreasing rapidly during the first few hours, becoming about 10^{-4} of the injected dose after 24 to 48 hours, and remaining essentially constant for the next few days. On this basis, our initial (total) injection of about 250 μg per rat would have led in minutes to the appearance of about 50 μg (total) of cadmium in serum, this decreasing to about 25 ng (total) at 24 to 48 hours after injection. The observed increase in Mt concentration after the initial injection implies a maximum Mt concentration in the first few hours of less than 1 ng/ml or less than 20 ng (total) in serum. Even if saturated with cadmium this amount of Mt could bind only about 2 ng of cadmium and could not be a major carrier of serum cadmium in this period. The situation is even more extreme for zinc. The results are thus in accord with experiments which indicate

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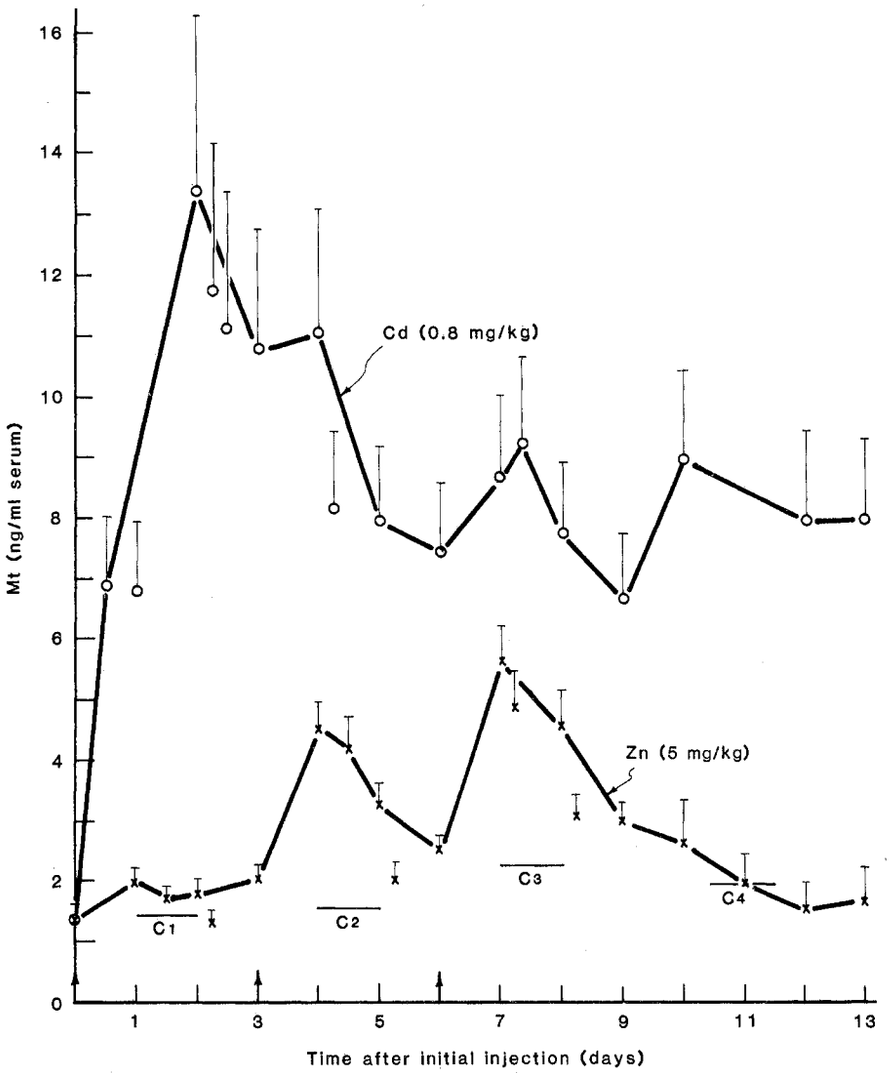

Fig. 1. Concentrations of Mt in the serum of rats given three intraperitoneal injections of zinc ($ZnSO_4$ in saline; 5 mg of zinc per kilogram of body weight per injection) or cadmium ($CdCl_2$ in saline; 0.8 mg of cadmium per kilogram of body weight per injection). Plotted are polygonal curves of circulating Mt as a function of time after the first of three injections (given at 3-day intervals) into male Sprague-Dawley rats (275 to 325 g). In each experiment, $N = 5$. Arrows indicate times of injection. Indicated values are means with their standard errors, the latter including the errors arising from the assay standardization process. About one-half (two-thirds) of the magnitudes of the indicated errors in the case of zinc (cadmium) treatments stem from the regressions used to obtain the unknown concentrations. Concentrations associated with 6-hour intervals between blood withdrawal (and one associated with a 12-hour interval) are left isolated [see (*11*)]. Also shown are the mean Mt concentrations for the controls ($N = 2$) [bled and injected (saline) on the same schedule as the treated rats] for each injection period (labeled C1 through C4: 0 to 3, 3 to 6, 6 to 9, and 10 to 13 days, respectively). The control means (in nanograms of Mt per milliliter of serum) are: C1 = 1.43 ± 0.46; C2 = 1.55 ± 0.28; C3 = 2.24 ± 0.40; and C4 = 1.95 ± 0.46. The initial value for the group of rats used later as controls or in metal treatment is 1.35 ± 0.25. The mean concentrations for C1 through C4 are not statistically distinguishable ($P < .30$ for the largest differences). The mean value for the control concentration for the entire 13-day period is 1.79 ± 0.41. The fiducial limits at 95 percent confidence are 0.92 and 2.65 (0.60 and 2.98 at 99 percent confidence). The initial injection of zinc produced a maximum Mt response not distinguishable from the 0- to 3-day mean control level ($P < .30$), but the second and third injections led to maxima readily distinguishable from their respective control levels ($P < .01$ in both cases). The relative maxima at 1 and 4 days and at 1 and 7 days are significantly different ($P < .01$ and $P < .02$, respectively). With respect to the Mt response to cadmium injections, the following points are pertinent. The initial maximum is significantly different from the mean control level in the 0- to 3-day period ($P < .02$), and from the maximum response after the second injection of zinc ($P < .05$). The individual measurements of Mt in the 9- to 13-day period differ significantly from the mean control level, C4 (P ranges from $< .02$ to $< .01$). However, the standard errors in the measurements in the 6- to 13-day period are such that no significance can be attached to the fluctuations in that period, although the increases after the second and third injections conform qualitatively to expectations.

that the initial metal-binding proteins in serum or plasma after cadmium or zinc injections are not Mt but are principally α-2-macroglobulin and albumin (*18, 21–23*). The estimated 25 ng of cadmium in serum at 24 to 48 hours after injection could, in theory, be bound by the observed maximum amount of Mt then present (13 ng/ml, or a total of about 250 ng in serum) if this Mt were a cadmium-saturated thionein. However, it is more probable that this Mt is a (cadmium, zinc)-thionein, with both cadmium and zinc bound to thionein (*24*). The Mt could now be a major carrier of serum cadmium, although not the only carrier present. This accords with experiments on cadmium-binding proteins in serum after cadmium injections (*1, 2, 20, 23, 25*).

Two additional matters with implications for future investigations may be mentioned. First, the capability of the assay to measure Mt concentrations in serum in the range 1 to 3 ng/ml makes it feasible to include Mt in the kinetic models (*26*) which attempt to describe quantitatively the metabolism of metals in the body. Second, the observed appearance of Mt in serum at concentrations significantly different from control levels a few hours after injections of either cadmium or zinc is pertinent to the resolution of the controversy regarding the kinetics of metal and Mt metabolism after such injections. Any acceptable theory must be compatible with our observation that subtoxic doses of these metals lead rapidly to increased plasma concentrations of Mt. This Mt is capable of acting as a transport protein for cadmium or zinc, and it is not necessary that there be overt renal damage, as maintained by some investigators, before Mt appears in measurable amounts in plasma.

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References and Notes

1. G. F. Nordberg, M. Piscator, M. Nordberg, *Acta. Pharmacol. Toxicol.* **30**, 289 (1971).
2. R. A. Goyer, M. G. Cherian, L. Delaquerriere-Richardson, *Cadmium 77* (Proceedings of the First International Cadmium Conference, San Francisco) (Metal Bulletin Ltd., London, 1978), pp. 183–185.
3. Z. A. Shaikh and K. Hirayama, *Environ. Health Perspect.* **28**, 267 (1979).
4. J. H. Kägi and M. Nordberg, Eds., *Metallothionein* (Proceedings of the First International Meeting on Metallothionein and Other Low Molecular Weight Metal-Binding Proteins), *Experientia Suppl. No. 34* (1979). As of 1978, Mt had not been detected in human plasma (pp. 133–134).
5. R. J. Vander Mallie and J. S. Garvey, *J. Biol. Chem.* **254**, 8416 (1979); C. C. Chang, R. J. Vander Mallie, J. S. Garvey, *Toxicol. Appl. Pharmacol.* **55**, 94 (1980). The development and early application of the RIA is described in these papers, including measurement of Mt in plasma

in concentrations of a few nanograms per milliliter. Note that the assay does not distinguish between Mt or thionein.
6. We established that the ranges 5 to 10 mg of zinc and 0.5 to 1.0 mg of cadmium per kilogram were nontoxic and led to the production of readily measurable Mt.
7. See M. Webb, Ed., *The Chemistry, Biochemistry and Biology of Cadmium* (Elsevier/North-Holland, Amsterdam, 1979), for general information on cadmium toxicity.
8. A. V. Colucci, D. Winge, J. Krasno, *Arch. Environ. Health* **30**, 153 (1975); M. Webb, in (*7*), chap. 6, pp. 229–230; G. P. Samarawickrama, in (*7*), chap. 9, pp. 341–343.
9. See W. Vogt, P. Sandel, Ch. Langfelder, M. Knedel, *Clin. Chim. Acta* **87**, 101 (1978) for techniques in expressing RIA data. The standardization process described by Vander Mallie and Garvey and Chang *et al.* (*5*) characteristically yields regressions (logit-log or linear-log) with correlation coefficients between −.97 and −.99. See also D. R. Winge, B. L. Geller, J. S. Garvey, *Arch. Biochem. Biophys.* **208**, 160 (1981).
10. R. G. D. Steel and J. H. Torrie, *Principles and Procedures of Statistics* (McGraw-Hill, New York, ed. 2, 1980), chap. 5. The use of effective degrees of freedom (Satterthwaite's derivation) characteristically reduced available degrees of freedom by 50 percent in the calculations herein of the significance of the difference between means.
11. The 18- or 24-hour intervals between blood withdrawal were established on the basis of certain characteristics of the rat [See. D. H. Ringler and L. Dabich, in *The Laboratory Rat*, vol. 1, *Biology and Diseases*, H. J. Baker, J. R. Lindsey, S. H. Weisbroth, Eds. (Academic Press, New York, 1979), p. 108; C. E. Yale and J. B. Torhorst, *Lab. Anim. Sci.* **22**, 497 (1972); O. W. Schalm, N. C. Jain, E. J. Carroll, *Veterinary Hematology* (Lea and Febiger, Philadelphia, ed. 3, 1975), p. 1]. Blood is normally 7 percent of body weight (about 18 to 20 ml in volume); withdrawal of about 40 percent at one time is lethal. Blood was withdrawn at a few 6-hour and 12-hour intervals to obtain more time-dependent information. Withdrawal at 6-hour intervals produced Mt values about 10 percent less than expected (presumably because of the inflow of interstitial fluid and the dilution of serum constituents) if the values associated with withdrawal at 24-hour intervals are used as a guide. The magnitude of the observed concentrations may also have been influenced by the production of Mt in response to bleeding and injection stresses, but the kinetics are uncertain [see S. H. Oh, J. T. Deagen, P. D. Whanger, P. H. Weswig, *Am. J. Physiol.* **234**, E282 (1978); P. D. Whanger and S. H. Oh, in (*4*), pp. 287–289].
12. C. C. Chang, R. Lauwerys, A. Bernard, H. Roels, J. P. Buchet, J. S. Garvey, *Environ. Res.* **23**, 422 (1980).
13. R. W. Chen, P. D. Whanger, P. H. Weswig, *Biochem. Med.* **12**, 95 (1975).
14. M. P. Richards and R. J. Cousins, *J. Nutr.* **106**, 1591 (1976).
15. M. Webb, in (*7*), chap. 6, p. 235; S. S. Feldman and R. J. Cousins, *Biochem. J.* **160**, 583 (1976); P. D. Whanger and S. H. Oh, in (*4*), pp. 281–291; R. J. Cousins, in (*4*), pp. 293–301; summary comments in (*4*), pp. 71, 74–75.
16. A single injection of 0.5 mg per kilogram of cadmium produced an Mt response similar, though decreased in magnitude, to the initial response to the 0.8 mg/kg dose. The return toward control levels was more abrupt, and measurement errors precluded assigning significance to differences between control levels and induced Mt response by day 3. Multiple injections (2; at 48-hour intervals) of 20 mg of zinc produced an Mt response which attained a maximum of about 18 ng/ml by day 4. The experiment was then terminated because of the obvious toxicity of the dose.
17. B. Engström and G. F. Nordberg, *Toxicology* **13**, 215 (1979); *Acta Pharmacol. Toxicol.* **45**, 315 (1979); G. P. Samarawickrama, in (*7*), chap. 9, pp. 351, 366–367.
18. G. F. Nordberg, *Environ. Physiol. Biochem.* **2**, 7 (1972).
19. H. M. Perry and M. Erlanger, *Am. J. Physiol.* **220**, 803 (1971); A. D. Johnson and W. J. Miller, *J. Reprod. Fertil.* **21**, 395 (1970); M. Nordberg, *Environ. Res.* **15**, 381 (1978).
20. G. P. Samarawickrama, in (*7*), chap. 9, pp. 348–349.
21. Z. A. Shaikh and O. J. Lucis, *Arch. Environ. Health* **24**, 411 (1972).
22. S. R. Watkins, R. M. Hodge, D. C. Cowman, P. P. Wickham, *Biochem. Biophys. Res. Commun.* **74**, 1403 (1977); N. T. Davies, I. Bremmer, C. F. Mills, *Biochem. Soc. London Trans.* **1**, 984 (1973); T. A. Gasiewicz and J. C. Smith, *Biochim. Biophys. Acta* **428**, 113 (1976); I. Bremner (*23*); M. Webb, in (*7*), chap. 6, p. 230, and chap. 8, p. 294.
23. I. Bremner, in (*7*), chap. 5, pp. 178–180.
24. F. O. Brady, M. Panemangalore, F. A. Day, in (*4*), pp. 261–271; M. Webb, in (*4*), pp. 313–320.
25. We were unable to justify with confidence a reduction factor for zinc (to compare with that of 10^{-4} calculated for cadmium) and make no estimate of the probable percentage of circulating zinc bound to Mt at times of maximum Mt concentration.
26. T. Kjellström and G. F. Nordberg, *Environ. Res.* **16**, 248 (1978); G. F. Nordberg and T. J. Kjellström, *Environ. Health Perspect.* **28**, 211 (1979).
27. Supported by grant ES 01629 from the National Institutes of Health.

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Phototherapy-Induced Hypocalcemia in Newborn Rats: Prevention by Melatonin

Abstract. *When young rats are exposed to white fluorescent light the concentration of calcium in their serum decreases. This effect is prevented by shielding the occiput, by inhibiting corticosterone synthesis, and by exogenous melatonin. Furthermore, the expected hypocalcemic response to cortisol injection is prevented by melatonin. Light-induced hypocalcemia may result from increased calcium uptake by bone when the blocking effect of melatonin decreases after pineal inhibition by transcranial illumination.*

Jaundice is a common problem in newborn infants, and the risk of brain damage from the cytotoxic effects of bilirubin justifies efforts to prevent or ameliorate excessive concentrations of bilirubin in the plasma. Exposure to intense light (phototherapy) accelerates the formation of photobilirubin, a metastable geometric isomer efficiently excreted in bile. Approximately 2.5 percent of all babies born in the United States are so treated (*1*). Both white and blue fluorescent lights are used (*2*).

Premature infants undergoing phototherapy with white light have an increased incidence of hypocalcemia compared to coeval controls under standard nursery conditions (*3*). Blue light, though effective in reducing hyperbilirubinemia, produces no significant change in serum calcium (*4*). These findings suggest that phototherapy-induced hypocalcemia is not mediated by bilirubin metabolites. Since all infants wear eye shields during phototherapy, the hypocalcemic effect of light probably involves extraoptic pathways. We have studied this phenomenon in newborn rats, and our results suggest that white light affects calcium homeostasis by inhibiting pineal secretion of melatonin.

Rats aged 6 hours to 14 days were exposed to white fluorescent light (*2*) delivering a spectral irradiance of 5 μW

Table 1. Change in serum calcium concentration after 3 hours of phototherapy. The results are expressed as means ± standard error. N.S., not significant.

Group	Age	Serum calcium (mg/dl)				Change in calcium (mg/dl)
		Shade	*N*	Phototherapy	*N*	
Control	6 hours	7.0 ± 0.13	4	6.2 ± 0.05	5	−0.8*
Control	18 to 24 hours	7.9 ± 0.11	13	7.1 ± 0.13	14	−0.8*
Control	1.5 to 2.5 days	8.6 ± 0.06	15	7.9 ± 0.06	19	−0.7*
Control	4 to 14 days	9.2 ± 0.12	29	8.3 ± 0.07	29	−0.9*
Gunn, icteric	2.5 days	7.5 ± 0.15	8	6.6 ± 0.24	8	−0.9*
Gunn, nonicteric	2.5 days	7.5 ± 0.13	8	6.6 ± 0.14	8	−0.9*
Jacket	18 to 24 hours	7.9 ± 0.18	4	7.1 ± 0.18	4	−0.8*
Jacket	1.5 days	9.4 ± 0.04	5	8.7 ± 0.10	6	−0.7*
Blindfold	1.5 days	8.7 ± 0.05	10	7.9 ± 0.08	9	−0.8*
Blindfold	4 days	9.5 ± 0.09	6	9.0 ± 0.11	6	−0.5*
Enucleated	1 to 4 days	8.9 ± 0.16	12	8.4 ± 0.13	13	−0.5†
Hood	1.5 days	9.4 ± 0.04	5	9.4 ± 0.07	12	0.0
Cap	1 to 2.5 days	8.3 ± 0.11	13	8.2 ± 0.09	13	0.0
Cap	4.5 days	9.0 ± 0.10	14	8.9 ± 0.10	13	0.0
Melatonin	3 to 4 days	8.7 ± 0.06	13	8.7 ± 0.07	24	0.0
Vehicle	3 to 4 days	8.8 ± 0.10	12	8.0 ± 0.09	22	−0.8*
Metyrapone	3 to 10 days	8.8 ± 0.17	11	8.6 ± 0.12	11	−0.2(N.S.)
Vehicle	3 to 10 days	8.4 ± 0.10	6	7.3 ± 0.08	7	−1.1
Blue light	4 to 5 days	8.8 ± 0.07	20	8.7 ± 0.07	17	−0.1(N.S.)

*$P < .01$. †$P < .05$ (Student's *t*-test).

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