The cytoplasmic location of prothymosin alpha is in agreement with the known release of prothymosin alpha in blood plasma [8] and thymosin alpha 1 crossreactive material in serum [22] by an as yet unknown mechanism. It is speculated that thymosin beta 4 is also a cytoplasmic polypeptide as it has been found in human plasma at higher than prothymosin alpha levels [23].

Acknowledgments. This work was supported by a grant from the Greek Ministry of Industry Energy and Technology.

* To whom correspondence should be addressed.

1 Haritos, A. A., Blacher, R., Stein, S., Caldarella, J., and Horecker, B. L., Proc. natl Acad. Sci. USA *82* (1985) 343.
2 Pan, L.-X., Haritos, A. A., Wideman, J., Komiyama, T., Chang, M., Stein, S., Salvin, S. B., and Horecker, B. L., Archs Biochem. Biophys. *250* (1986) 197.
3 Goodall, G. J., Dominguez, F., and Horecker, B. L., Proc. natl Acad. Sci. USA *83* (1986) 8926.
4 Eschenfeldt, W. H., and Berger, S. L., Proc. natl Acad. Sci. USA *83* (1986) 9403.
5 Panneerselvam, C., Wellner, D., and Horecker, B. L., Archs Biochem. Biophys. *265* (1988) 454.
6 Haritos, A. A., Tsolas, O., and Horecker, B. L., Proc. natl Acad. Sci. USA *81* (1984) 1391.
7 Haritos, A. A., Caldarella, J., and Horecker, B. L., Analyt. Biochem. *144* (1985) 436.
8 Panneerselvam, C., Haritos, A. A., Caldarella, J., and Horecker, B. L., Proc. natl Acad. Sci. USA *84* (1987) 4465.
9 Haritos, A. A., in: Isozymes: Current Topics in Biological and Medical Research, vol. 14, p. 123. Eds M. C. Ratazzi, J. G. Scandalios and G. Whitt. Alan R. Liss Inc., New York 1987.
10 Yialouris, P. P., Evangelatos, G. P., Soteriadis-Vlahos, C., Heimer, E. P., Felix, A. M., Tsitsiloni, O. E., and Haritos, A. A., J. immun. Meth. *106* (1988) 267.
11 Haritos, A. A., Salvin, S., Blacher, R., Stein, S., and Horecker, B. L., Proc. natl Acad. Sci. USA *82* (1985) 1050.
12 Baxevanis, C. N., Reclos, G. J., Papamichail, M., and Tsokos, G. C., Immunopharmac. Immunotoxic. *9* (1987) 429.
13 Reclos, G. J., Baxevanis, C. N., Sfagos, C., Papageorgiou, C., Tsokos, G. C., and Papamichail, M., Clin. exp. Immun. *70* (1987) 336.
14 Gómez-Márquez, J., and Segade, F., FEBS Lett. *226* (1988) 217.
15 Higashi, K., Narayanan, K. S., Adams, H. R., and Busch, H., Cancer Res. *26* (1966) 1582.
16 Haritos, A. A., Goodall, G. J., and Horecker, B. L., Proc. natl Acad. Sci. USA *81* (1984) 1008.
17 Tsitsiloni, O. E., Yialouris, P. P., Heimer, E. P., Felix, A. M., Evangelatos, G. P., Soteriadis-Vlahos, C., Stiakakis, J., Hannappel, E., and Haritos, A. A., J. immun. Meth. *113* (1988) 175.
18 Stahli, C., Takacs, B., and Kocyba, C., Molec. Immun. *20* (1983) 20.
19 Auger, C., Stahli, C., Fabien, N., and Monier, J. C., J. Histochem. Cytochem. *35* (1987) 181.
20 Fabien, N., Auger, C., and Monier, J. C., Immunology *63* (1988) 721.
21 Horecker, B. L., and Morgan, J., in: Lymphokines, vol. 9, p. 15. Eds E. Pick and M. Landy. Academic Press, New York 1984.
22 McClure, J. E., Lameris, N., Wara, D. W., and Goldstein, A. L., J. Immun. *128* (1981) 368.
23 Hannappel, E., and Van Kampen, M., J. Chromat. *397* (1987) 279.

0014-4754/89/040332-03$1.50 + 0.20/0

β-Methyl carboline, a benzodiazepine inverse agonist, attenuates the effect of triazolam on the circadian rhythm of locomotor activity

R. D. Smith and F. W. Turek

Department of Neurobiology and Physiology, Northwestern University, Evanston (Illinois 60208, USA)
Received 28 October 1988; accepted 23 November 1988

Summary. The benzodiazepine triazolam, the benzodiazepine inverse agonist, β-methyl carboline (β-CCM) or both, were administered to adult male hamsters under conditions of constant light. When given alone, triazolam induced phase advances in the circadian activity rhythm of about 90 min, while β-CCM when given alone, had no effect on phase of the activity rhythm. However, when triazolam and β-CCM were given at the same time, the magnitude of the phase advances induced by triazolam were attenuated to about 30 min. These data, in conjunction with previous results, provide pharmacological evidence for a GABAergic system involved in the regulation of a central circadian pacemaker.
Key words. Benzodiazepine; circadian rhythm; gamma-aminobutyric acid; inverse agonist; suprachiasmatic nucleus; triazolam.

In the absence of environmental time cues, a single intraperitoneal injection of the relatively short-acting benzodiazepine, triazolam, can induce either phase advances or phase delays in the circadian rhythm of the onset of locomotor activity in the golden hamster. Whether an advance or a delay occurs in response to triazolam depends upon the phase of the animal's activity cycle at which the drug is administered: phase advances of the activity rhythm can be induced if an injection of triazolam is administered within 6 h before the onset of activity, while phase delays can be induced if triazolam is given 6–9 h after the onset of activity [1]. The phase-shifting effects of triazolam on the hamster activity rhythm are also dose-dependent [2] and can be blocked by Ro 15–1788, a benzodiazepine antagonist [3].

Triazolam is a member of the family of triazolobenzodiazepines that act by potentiating the effects of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) [4, 5]. A second triazolobenzodiazepine, midazolam, also induces phase advances and phase delays in

hamster activity rhythms which are similar to those observed in response to triazolam (Wee and Turek, in press). Taken together, these data suggest that the ability of the benzodiazepines to cause phase shifts in the circadian rhythm of activity in hamsters may be due to the activation of a GABA-mediated pathway which ultimately affects a central circadian pacemaker.

The benzodiazepines have been broadly characterized as anxiolytic, sedative hypnotic agents. The β-carbolines are a class of newly synthesized drugs comprising a series of esters or amides of beta-carboline-3-carboxylate, which appear to display intrinsic pharmacological efficacy opposite to that of the benzodiazepines. The methyl ester of β-carboline-3-carboxylate (β-CCM), for example, has anxiogenic and convulsant properties in various animal models[6–9]. In addition, and similar to the selective and potent benzodiazepine antagonist Ro 15–1788, β-carbolines antagonize the receptor-mediated effects of benzodiazepines[10]. Because of the intrinsic activity of these compounds which tend to result in effects opposite to the benzodiazepines, and to distinguish them from antagonists such as Ro 15–1788 which tend to be either inert or to possess very little intrinsic activity, the β-carbolines have been termed 'inverse agonists'[11].

The opposing pharmacological activities shared by the benzodiazepines and the inverse agonists raise the possibility that the inverse agonists might

1) alter the effects of triazolam on the mammalian circadian clock, and/or
2) have opposite effects on the clock than do benzodiazepines.

Thus, we examined the effects of the inverse agonist, β-CCM, alone and in conjunction with triazolam on the circadian rhythm of locomotor activity in the golden hamster.

Materials and methods. Male golden hamsters, [*Mesocricetus auratus* Lak: LVG (SYR); N = 28] 8 weeks old, were initially group housed in a temperature-controlled room (22 ± 1 °C) with a light/dark cycle of 14 h light and 10 h dark (lights on at 07.00 h). At the end of a two-week acclimation period, each animal was transferred to an individual polypropylene cage maintained under conditions of constant light (LL). The ambient illumination when measured with a UDT 350 photometer from the floor of each cage, was approximately 15 lux (range = 1–40 lux). Each cage was equipped with a running wheel connected to an Esterline Angus event recorder. A single rotation of the running wheel activated a pen deflection on continuously moving chart paper. Successive intervals of chart paper containing these pen deflections were cut every 24 h and pasted vertically and chronologically, top to bottom, to form a continuous picture of running wheel activity.

After steady-state conditions had been achieved in an animal's locomotor rhythm for at least 10 days, the animal was given an i.p. injection of either 5.0 mg β-carboline-3-carboxylate (β-CCM; Research Biochemicals, Inc., Massachusetts, USA), 0.5 mg of triazolam (tz; Upjohn, Michigan, USA), or 0.5 mg triazolam plus 5.0 mg β-CCM in cocktail. A total of 19 animals received repeated injections of either triazolam, β-CCM, or triazolam plus β-CCM, while a separate group of nine animals was given injections of vehicle alone. All drugs were administered in a volume of 0.1 cc DMSO, and at the time of the first injection, the animals weighed approximately 150 g. Activity onset was designated as circadian time 12 (CT 12) and each injection was given 6 h before the estimated time of activity onset, i.e., at CT 6. Injections of triazolam at this circadian phase of the hamster activity rhythm produce maximal phase advances[1,2]. Animals received 2–5 injections of either triazolam, β-CCM, or both, on a statistically randomized basis to control for both the effects of the order in which the drugs were administered, and for systematic trends due to individual animal (phase-shifting) characteristics in response to repeated injections. Two-way analysis of variance (ANOVA) was used to statistically assess the effects of animal characteristic or treatment (i.e., pharmacological agent) on phase change induced in the activity rhythm. In addition, values obtained for the change in phase of the activity rhythm induced by β-CCM, triazolam or triazolam plus β-CCM were plotted graphically for six animals which received each of the three pharmacological agents in sequence, so that a qualitative assessment could be used to check for systematic trends as a result of injection order or animal characteristics. Finally, all sequential injections given to the animals were separated by at least 10 days until a new steady state in the periodicity of the daily activity onsets of the individual rhythm developed. As previously described[1], phase shifts in the onset of activity were calculated by taking the difference between at least 5–7 consecutive activity onsets from both the pre- and post-injection rhythm.

Results and discussion. As expected, the administration of 0.5 mg of triazolam 6 h before the onset of activity induced clear advances in the locomotor rhythm. However, when given in conjunction with 5.0 mg β-CCM, phase-advances induced by triazolam were sharply attenuated (fig. 1). The difference between the magnitude of the phase advances induced by triazolam compared to the phase advances induced by triazolam given in conjunction with β-CCM, was statistically significant ($t = 4.5$, $df = 36$, $p < 0.001$; orthogonal, post hoc comparison). In addition, the difference between the average magnitude for phase changes induced by triazolam given in conjunction with β-CCM when compared to phase changes induced by β-CCM given alone, was statistically significant ($t = -2.84$, $df = 36$, $p < 0.01$; orthogonal, post hoc comparison). A tendency occurred for injections of β-CCM to cause phase delays in the activity rhythm; however, the average difference between groups receiving vehicle or β-CCM alone was not statistically significant (Student's t-test, fig. 2).

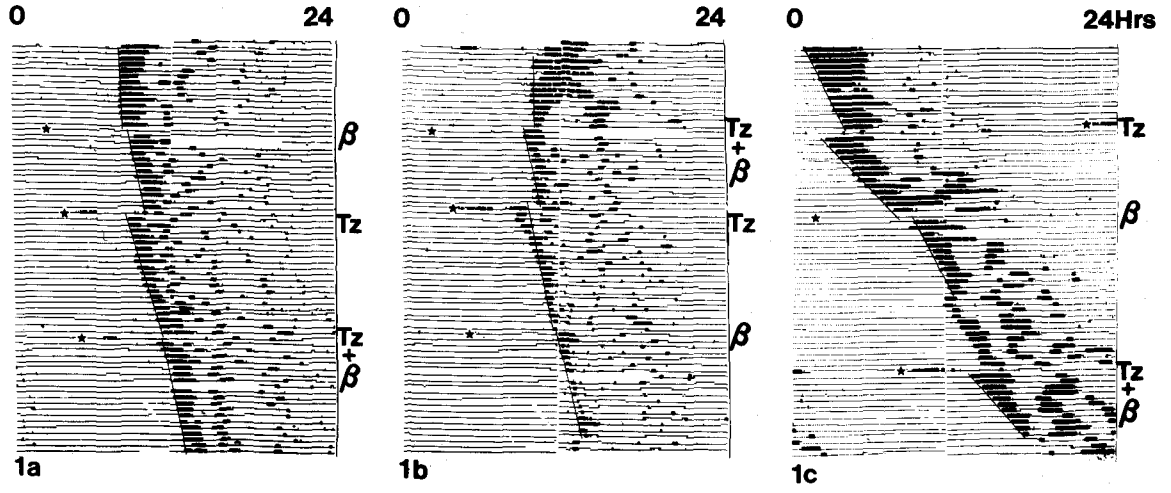

Figure 1. Representative activity records for three animals maintained in constant light (LL) are presented in panels 1a, 1b, and 1c, to illustrate the effects of injections of triazolam, β-CCM, or triazolam plus β-CCM on the free-running circadian rhythm of activity. The day of injection and the drug given, are indicated by Tz, Tz + β, or β. The actual time of injection is indicated by a star; all drugs were administered 6 h before the estimated onset of activity (CT 6). Each line represents a single 24 h period and successive days are plotted top to bottom. Either an advance (+) or a delay (−) in the phase of the rhythm of the onset of activity was estimated for panel 1a, β: −20, Tz: +120, Tz + β: +10; panel 1b, Tz + β: +40, Tz: +45, β: −10; and panel 1c, Tz: +110, β: −40, Tz + β: +75.

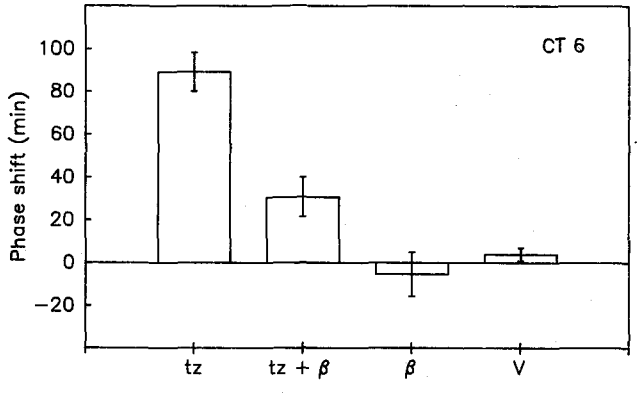

Figure 2. The histograms plot the mean (±SEM) phase shift in the hamster locomotor rhythm after triazolam (tz), triazolam plus β-CCM (tz + β), β-CCM (β) or vehicle (V) alone. The number of injections per cell were 12, 14, 11, and 9, respectively. All injections were given at CT 6. A negative value represents a phase delay of the activity rhythm while a positive value indicates an advance.

The ANOVA revealed a statistically significant main effect for drug treatment ($F = 14.48$, $df = 2,17$ $p < 0.001$), but not for individual animals. Similarly, the graphical examination of drug type and order of administration did not indicate the presence of an effect of order of administration on phase changes in the activity rhythms for individual animals. Collectively, these analyses indicate that systematic variation, as a result of sequential injections given to a single animal, did not influence the relative differences observed for the overall effects on phase of the locomotor activity rhythm induced from the administration of either triazolam, β-CCM, or triazolam plus β-CCM.

A variety of studies have established that i.p. injections of triazolam, ranging in doses between 0.5 and 2.5 mg, can induce reliable phase advances in the circadian rhythm of locomotor activity in the hamster when given during the animal's subjective day[1–3, 12, 13]. When triazolam is given 6 h before the onset of activity (CT 6), phase advances of about 90 min are usually induced. Overall, the phase shifts induced by triazolam when given in conjunction with β-CCM were diminished in size by about 67% when compared to the phase advances induced by triazolam given alone. When given alone, β-CCM had no systematic effects on phase of the activity rhythm.

A narrow dose-range limitation was found to exist for examining the effects of β-CCM on triazolam-induced changes in phase of the activity rhythm of the adult hamster. We found that hamsters could tolerate 5.0 mg β-CCM with no apparent long-lasting side effects, although at this dose, convulsive reactions usually lasting less than 1 min were observed to occur within 1–2 min following an injection. Doses below 5.0 mg were found to be ineffective in producing consistent or clear changes in phase shifts induced by triazolam in the hamster locomotor rhythm, and doses above 5.0 mg produced long-lasting (greater than 2 min) convulsive reactions in the animals. This physiological dose limitation may explain why, when compared to Ro 15–1788 which completely blocks the effects of triazolam[3], a dose of 5.0 mg of β-CCM only attenuated, rather than totally blocked triazolam-induced phase advances. In addition, from earlier, preliminary investigations with other inverse agonists, we found no clear effects on the hamster activity rhythm for either the ethyl ester of β-carboline-3-carboxylate (β-CCE) or methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), when these compounds were given alone.

In a manner similar to the triazolobenzodiazepines, such as triazolam or midazolam, diazepam has also been

found to cause phase shifts in hamster locomotor activity rhythms, the magnitude and direction of which are dependent upon the time of administration in the animal's activity cycle[14]. Diazepam also appears to block light-induced phase advances, at doses that do not block light-induced phase delays, in hamsters free-running in constant dark[15]. Alternatively, phase delays, but not advances can be blocked by bicucculine, a GABA antagonist[16]. Microinjections of muscimol, a GABA agonist, when directed to the anterior hypothalamic suprachiasmatic nucleus (SCN), a putative circadian pacemaker, causes phase shifts in blinded hamsters which are similar in direction and magnitude to phase shifts induced by single injections of triazolam given to hamsters maintained in constant darkness[17]. The magnitude of the phase shifts induced by muscimol is dependent on the proximity of the microinjection to the SCN (Smith, Inouye and Turek, in press). In addition, phase advances induced by triazolam in the circadian rhythm of locomotor activity in golden hamsters can be abolished through lesions of the ventral lateral geniculate nucleus when this area includes the intergeniculate leaflet (IGL)[18]. The IGL sends afferent projections to the SCN, and is thought to be partially involved in the entrainment of activity rhythms to light/dark cycles[19]. These results suggest that the SCN may have a role in the regulation of GABA-mediated phase shifts of the circadian rhythm of locomotor activity. Taken together these data provide further evidence for a GABA-mediated system involved in the regulation or generation of the circadian rhythm of activity in the golden hamster.

Acknowledgments. We wish to thank Sue Losee-Olson and William Olson for technical assistance. This work was supported by NIH grant HD-09885, and the Upjohn Company.

1 Turek, F. W., and Losee-Olson, S., Nature *321* (1986) 167.
2 Turek, F. W., and Losee-Olson, S., Life Sci. *40* (1987) 1033.
3 Van Reeth, O., Vanderhaeghen, J. J., and Turek, F. W., Brain Res. *444* (1988) 333.
4 Krogsgaard-Larsen, P., Medicinal Res. Rev. *8* (1988) 27.
5 Möhler, H., Schoch, P., and Richards, J. G., in: Molecular Aspects of Neurobiology, p. 91. Ed. R. Levi-Montalcini. Springer, Berlin 1986.
6 Prado de Carvalho, L., Grecksch, G., Chapouthier, G., and Rossier, J., Nature *301* (1983) 64.
7 Prado de Carvalho, L., Grecksch, G., Cavalheiro, E. A., Dodd, R. H., Chapouthier, G., and Rossier, J., Eur. J. Pharmac. *103* (1984) 287.
8 Valin, A., Dodd, R. H., Liston, D. R., Potier, P., and Rossier, J., Eur. J. Pharmac. *85* (1982) 93.
9 Venault, P., Chapouthier, G., Prado de Carvalho, L., Simiand, M. M., Dodd, R. H., and Rossier, J., Nature *321* (1986) 864.
10 Haefely, W. E., Kyberz, M., Gerecke, M., and Mohler, H., in: Advances in Drug Research, p. 171. Ed. B. Testa. Academic Press, London 1985.
11 Polc, P., Bonetti, E. P., Schaffner, R., and Haefely, W., Archs. Pharmac. *321* (1982) 260.
12 Turek, F. W., and Losee-Olsen, S., Endocrinology *122* (1988) 756.
13 Van Reeth, O., Losee-Olsen, S., and Turek, F. W., Neurosci. Lett. *80* (1987) 185.
14 Houpt, T. A., Mistleberger, R. E., and Moore-Ede, M. C., Soc. Neurosci. Abstr. *13* (1987) 422.
15 Ralph, M. R., and Menaker, M., Brain Res. *372* (1986) 405.
16 Ralph, M. R., and Menaker, M., Brain Res. *325* (1985) 362.
17 Smith, R. D., and Turek, F. W., Soc. Neurosci. Abstr. *12* (1986) 209.
18 Johnston, R., Smale, L., Moore, R. Y., and Morin, L. P., Proc. natl Acad. Sci. *85* (1988) 5301.
19 Pickard, G. E., Ralph, M. R., and Menaker, M., J. biol. Rhythms *2* (1987) 35.

0014-4754/89/040334-04$1.50 + 0.20/0

Failure of opioids to affect excitation and contraction in isolated ventricular heart muscle

H. Nawrath*, J. Rupp*, H. Jakob*, U. Sack, F. Mertzlufft and W. Dick

* *Pharmakologisches Institut, Obere Zahlbacher-Strasse 67, and Klinik für Anästhesiologie, Universität Mainz, D-6500 Mainz (Federal Republic of Germany)*
Received 29 July 1988; accepted 1 December 1988

Summary. The opioid agonists morphine (selective for μ-receptors) and ethylketocyclazocine (selective for kappa-receptors), at concentrations evoking strong effects in neuronal structures, did not significantly affect the configuration of the intracellularly recorded action potential and the force of contraction in ventricular heart muscle isolated from guinea pigs, rabbits and man. These results suggest that any changes of heart functions in vivo in response to opioid-like drugs are probably not mediated postsynaptically at the myocardial cell membrane but rather presynaptically, influencing the release of noradrenaline and/or acetylcholine from the nerve terminals.
Key words. Heart muscle; opioids; morphine; ethylketocyclazocine; cardiac function; presynaptic modification.

Morphine and other opioid-like drugs have been reported to influence hemodynamic parameters in vivo[1, 2] and to depress cardiac functions in vitro[3–5]. The discovery of different subtypes of opioid receptors[6] and the evidence for the presence of enkephalins in the heart[7] have renewed the interest in the study of opioids in this tissue[8]. Whereas the earlier in vitro studies can be best explained by the existence of postsynaptic μ-receptors, it has been suggested more recently that the sympathetic axons innervating the sinus node of the rabbit possess