Phase response curve to anisomycin in *tau* mutant hamsters

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Received 27 January 1992; accepted 18 May 1992

Abstract. Administration of the protein synthesis inhibitor, anisomycin, to wild type hamsters produces phase shifts in their circadian rhythms that have similarities to shifts produced by non-photic behavioral stimulation. A mutation that shortens the period of rhythms in hamsters results in altered responsiveness to non-photic input. However, responses of the mutants to anisomycin are unaffected: their phase response curve (PRC) for anisomycin is similar to that of wild types. This suggests that 1) anisomycin is not acting on mechanisms specifically involved in non-photic behavioral phase shifting, and 2) the mutation affects the non-photic input pathway or the pacemaker itself at a point that is upstream from anisomycin's site of action.
Key words. Activity; anisomycin; hamster; non-photic; phase response curve; protein synthesis; rhythms.

The *tau* mutation in golden hamsters results in a shortening of the circadian period from about 24 h in wild types to about 20 h in homozygous mutants. Heterozygous animals have intermediate periods near to 22 h [1]. These phenotypes were characterized by experiments with animals kept in constant environmental conditions. They therefore reflect differences in the period of an endogenous oscillator. However, these clock mutants also differ in their reaction to external inputs to their endogenous oscillators. An important feature of circadian systems is the phase response curve (PRC): rhythms may advance, delay, or remain unshifted, depending on when during the cycle a stimulus is given. Light is often used as the phase-shifting stimulus but PRCs can also be generated with certain non-photic stimuli. It has been found that the *tau* mutation in hamsters alters the shape of their PRCs. This is the case both for light PRCs [2] and those for non-photic events [3].

The alteration in the non-photic PRC provides a tool for studying phase-shifting mechanisms. At circadian time (CT) 24 the mutants respond with sizeable phase advances to non-photic events such as running induced by being put in a novel wheel [3]; at this time wild type hamsters show small phase delays following non-photic stimulation. A number of pharmacological manipulations appear to mimic the effects of novelty-induced wheel running in wild type hamsters [4]. It can therefore be asked whether any of these manipulations activate steps in the same neurochemical pathways to the clock that are activated by novelty-induced running. If this is the case, then responses to these manipulations should be altered in the *tau* mutant in a manner that conforms to the shape of its non-photic PRC. This possibility was tested using the protein synthesis inhibitor, anisomycin.

In wild types, the anisomycin PRC [5] is generally similar in shape to the PRC for novelty-induced wheel running [3] and for triazolam which is now known to be mediated by the activity (or some correlate) that this benzodiazepine induces in hamsters [6, 7]. However, because these PRCs differ in some respects, such as amplitude, comparisons between anisomycin and behavioral PRCs in wild types do not provide a clear indication of whether these manipulations phase shift through different or common mechanisms. To obtain unambiguous information bearing on this point, we turned to the mutant hamster because its non-photic PRC is clearly different from the wild type's anisomycin PRC. If anisomycin activates mechanisms involved in such behaviorally produced phase-shifts, then when given to mutant hamsters it should produce a PRC resembling the mutant's PRC to novelty-induced wheel running; in particular anisomycin should produce advances when administered to mutants between CT 22 and CT 2.

Materials and methods

Male heterozygous *tau* mutant and wild type hamsters came from our breeding colony in Toronto. All were from lines outbred to a wild type strain (LAK:LVG); additional wild types were obtained from Charles River (Quebec). At age 26–41 days, they were placed in metal-walled cages [8] equipped with running wheels and kept in a light: dark cycle (LD) 14:10 h. Activity was recorded on an Esterline Angus recorder; for some animals actograms were also printed from a Dataquest III (Minimitter, Oregon) system run in parallel. Purina chow pellets and water were available ad libitum. After 14–15 days in LD, the animals were transferred to a room kept constantly dark (DD). Room temperatures were 22 ± 3 °C.

After 10–15 days in DD, animals were given injections s.c. of 12 mg/animal of anisomycin (Sigma). The anisomycin was dissolved in saline, with the aid of drops of HCl to bring the pH to about 7; volume given was ca. 0.4 ml. All heterozygotes (N = 16) received a second injection 21–27 days after the first administration; 13 of these animals provided enough data to calculate phase shifts for both injections. No data for the other 3 heterozygotes could be included because of apparatus problems or low activity levels associated with poorly defined rhythms.

Sixteen wild type hamsters were tested. Of these, 6 received 2 injections of anisomycin and 10 received one. Of

the latter 10, 6 animals were left in their home cages at the time of their second injection, to provide control data, and 4 were given anisomycin only at the time scheduled for the second injection; these 4 animals had previously been given an activity pulse in another experiment [3]. Apparatus problems resulted in loss of data for 4 wild types; 2 of these were for animals given 1 anisomycin injection, and 2 were for animals given 2 anisomycin injections. Thus a total of 13 animals contributed 18 data points to the wild type PRC. No individual contributed more than two points to the final PRCs. No cage change was done within 10 days before or after an injection.

The methods for calculation of phase shifts were the same as described in detail by Mrosovsky et al. [3]. Onsets of wheel-running were defined objectively [8], the 3 post-injection onsets excluded allowance for transients, and pre and post manipulation regression lines were each based respectively on the preceding and subsequent 7 cycles. If < 5 onsets were available, for whatever reason, for either line, then no phase shift was calculated. For two of the heterozygotes, one each of their 7 post-anisomycin onsets were excluded because activity was unusually low that day.

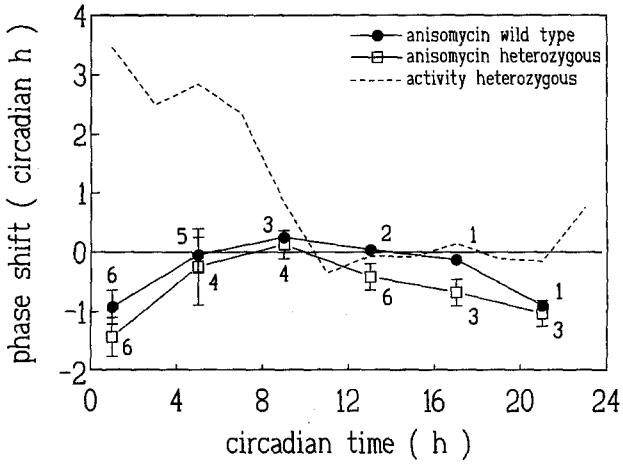

Figure 2. Comparison of phase shifts for wild type (solid squares) and heterozygous hamsters (open squares) within the present experiment for 4 h circadian time bins. Bins were chosen to provide at least one point in each time bin. Numbers beside data points show n values for shifts. Vertical lines show SEMs. Note that the shifts are given in circadian hours. Thin dashed line shows PRC for novelty-induced wheel running in heterozygotes (data from Mrosovsky et al. [3]).

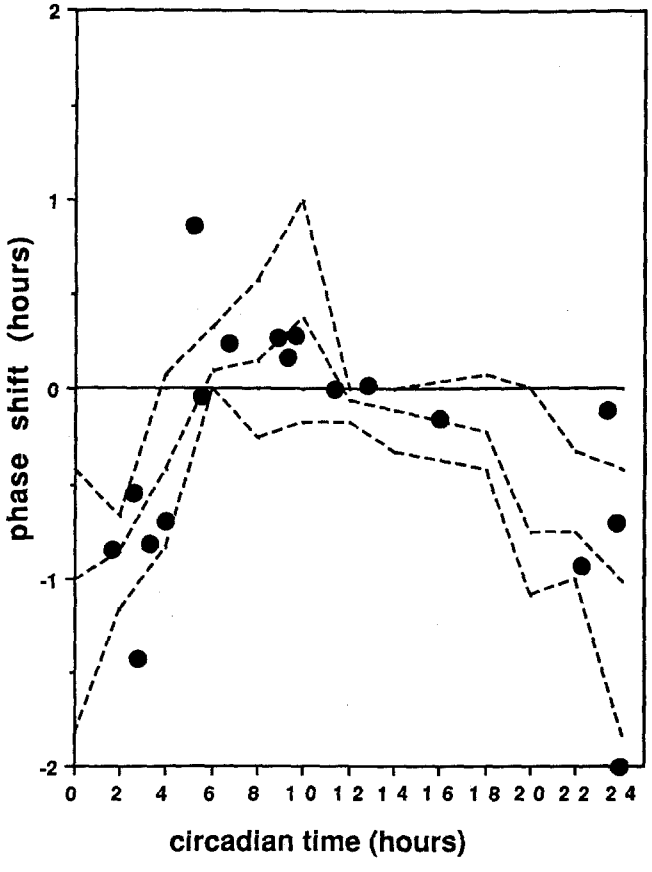

Figure 1. Phase shifts following anisomycin injections given at different circadian times: comparison between present data for wild types (circles show each shift obtained) and results from Takahashi and Turek [5]. Dashed lines show mean and range of their results for 2 h circadian time bins.

Results

Phase shifts for wild type hamsters (fig. 1) were generally within the range of those reported by Takahashi and Turek [5]. For the control animals left in their home cages, the mean shift was 0.11 h (± 0.12 SEM). Within the present experiment there were no major differences between wild types and mutants in the overall shape of their anisomycin PRCs (fig. 2). In particular, mutants did not respond between CT 22 and CT 2 with large advances, as they do to novelty-induced wheel running. On the contrary, they responded with phase delays. If anything the amplitude of phase delays was slightly larger in the mutants, but sample sizes were small. It should be noted also that the shifts in figure 2 are plotted in circadian hours (i.e. 24 ÷ free-running period); for a mutant with a 22-h period, this adds about 0.1 h for each clock-time h of shift. The main point is that the anisomycin PRC of mutants is clearly dissimilar in shape to their non-photic activity-induced PRC, and resembles more closely the anisomycin PRC of wild types.

Non-specific effects of anisomycin did not appear to be responsible for the results. At the end of the experiment, dry scabby areas with loss of hair were seen at the injection sites. This was not reported by Takahashi and Turek [5]. Possibly anisomycin affected skin cell proliferation at the injection site. However, the affected areas did not appear sensitive to touch. Most hamsters were less active for only about a day following the injections (fig. 3). A single hamster, tested in experiments other than those reported here, lost no weight after receiving 12 mg anisomycin.

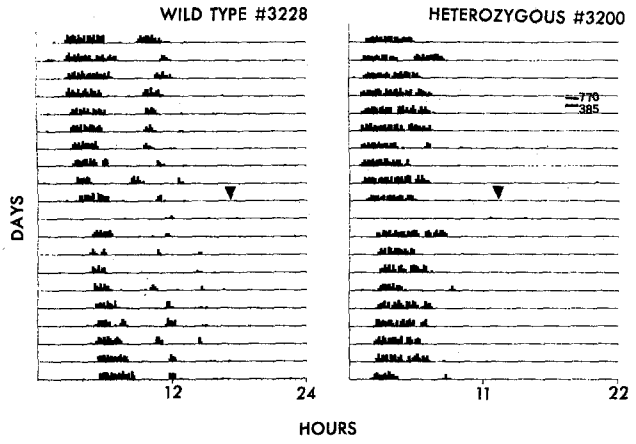

Figure 3. Actograms of wheel running showing typical phase delays obtained after anisomycin injections (downward pointing triangles) given at CT 1 ± 2 h. Wheel resolutions for each 10-min bin are plotted in one of 15 quantization levels. The scale marker shows the number of resolutions corresponding to the 7th and the 14th level (see Mrosovsky and Janik[9] for further explanation of the Dataquest III program).

Discussion

The phenomenon of phase shifting following behavioral activation means that interpretations of drug effects on pacemaker resetting should take behavioral changes into account[8]. It is conceivable, for instance, that the effects of anisomycin are mediated by behavioral activity or some correlate. This is unlikely in this case because substances such as cycloheximide and anisomycin, unlike triazolam[6,7], do not obviously induce activity in hamsters[5,10]; on the contrary, wheel running following anisomycin decreases for a day or so, as our data confirm. Moreover, preventing animals from moving after injections of protein synthesis inhibitors does not abolish phase shifts produced by these substances[11].

Although anisomycin does not itself induce activity, it is still possible that the mechanisms affected by this drug are part of a non-photic phase shifting system that can be stimulated by motor activity. The present results suggest that this is not the case since in the mutant the non-photic and the anisomycin PRC are very different. It is not that the mutant is incapable of phase delaying, as figures 2 and 3 demonstrate. Yet with novelty-induced activity, advances of a few hours occur at phases such as CT 24 when anisomycin causes delays in both heterozygotes and wild types.

Since, in the wild type, anisomycin injections direct to the suprachiasmatic nuclei (SCN) have phase-shifting effects similar to those seen after systemic injections, it is likely that phase shifts depend on the drug's action on the SCN[10]. Because effects of anisomycin appear to be similar in mutant and wild type, it is possible that the differences in their non-photic behavioral PRCs depend on differences in inputs to the SCN, or on differences at some site within the SCN or its pacemaker cells that is distal to that at which anisomycin exerts its phase shifting effects. Tests of this speculation require information about such input pathways. Such information is lacking, although it has been reported that large chemical lesions of the lateral geniculate area abolish triazolam-induced phase shifts[12].

Whatever the mechanisms involved, dividing PRCs into two families, the photic and its relatives (e.g. carbachol[4]) and the non-photic and its relatives (e.g. dark pulse, NPY, activity-induced[8,13]) is probably an oversimplification. In the mutant, at least, the PRC produced by anisomycin is different from that obtained with induced running, and both are different from the photic PRC. There are more than two countries in the atlas of PRCs[14].

Acknowledgments. We thank P. A. Salmon, D. S. Janik, O. Burack and M. H. Godfrey for help. J. S. Takahashi kindly provided advice and details of his work. Support for these experiments came from the Medical Research Council of Canada. Support for the colony of mutant hamsters came from the Natural Sciences and Engineering Research Council of Canada.

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