REPORT DOCUMENTATION PAGE

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
		FINAL 01 Sep 94 To 31 Aug 97

4. TITLE AND SUBTITLE
CONTROL OF CIRCADIAN BEHAVIOR BY TRANSPLANTED SUPRACHIASMATIC NUCLEI AND BY THE TAU GENE

5. FUNDING NUMBERS
F49620-94-1-0356
2312/CS
61102F

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110 DUNCAN AVE ROOM B115
BOLLING AFB DC 20332-8050
DR HADDAD

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release;
distribution unlimited.

19980219 125

13. ABSTRACT *(Maximum 200 words)*

The mammalian retina was found to contain an independent circadian oscillator which regulates the synthesis of melatonin and has effects, through a presently unknown pathway, on the circadian rhythm of locomotor behavior in infact animals. Electrical recordings were successfully obtained from several brain regions of intact, behaving hamsters. The suprachiasmatic nucleus (SCN) expressed circadian rhythms of electrical activity with peak electrical activity during the animals' "day" (inactive period), and low activity during the animals' night (active period). The electrical activity in the bed nucleus of the stria terminalis was in phase with that in the SCN. All other brain regions studied showed clectrical rhythms with the opposite phase. The circadian mutation tau was found to affect the period and the temperature compensation mechanism of the oscillator in the cultured retina as well as the dynamics of c-fos induction in the SCN. Tau mutan hamsters were found to have significantly altere responese of their circadian rhythms to GABAergic pharmacological agents. A model system was developed (using the green iguana) with which it is possible, for the first time, to study the interaction of multiple, distributed circadian oscillators. This is the only available experimental model of human circadian dissociation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
DTIC QUALITY INSPECTED 2	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(UL)

FINAL TECHNICAL REPORT

September 1, 1994-August 30, 1997

CONTROL OF CIRCADIAN BEHAVIOR BY TRANSPLANTED SUPRACHIASMATIC NUCLEI AND BY THE TAU GENE

AFOSR Grant # F49620-94-1-0356

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EXECUTIVE SUMMARY

- The mammalian retina was found to contain an independent circadian oscillator which regulates the synthesis of melatonin and has effects, through a presently unknown pathway, on the circadian rhythm of locomotor behavior in intact animals.
- Electrical recordings were successfully obtained from several brain regions of intact, behaving hamsters. The suprachiasmatic nucleus (SCN) expressed circadian rhythms of electrical activity with peak electrical activity during the animals' "day" (inactive period), and low activity during the animals' night (active period). The electrical activity in the bed nucleus of the stria terminalis was in phase with that in the SCN. All other brain regions studied showed electrical rhythms with the opposite phase.
- The circadian mutation *tau* was found to affect the period and the temperature compensation mechanism of the oscillator in the cultured retina as well as the dynamics of *c-fos* induction in the SCN.
- *Tau* mutant hamsters were found to have significantly altered responses of their circadian rhythms to GABAergic pharmacological agents.
- A model system was developed (using the green iguana) with which it is possible, for the first time, to study the interaction of multiple, distributed circadian oscillators. This is the only available experimental model of human circadian dissociation.

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PUBLICATIONS

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CIRCADIAN PROPERTIES OF THE TAU MUTANT RETINA

The *tau* mutation changes the response of the circadian system to phase shifting light pulses in a way that is both dramatic and subtle. If one measures the response of the circadian rhythm of locomotor activity to single light pulses presented in the standard manner [i.e., light pulses given at different circadian times to animals that have been in constant darkness (DD) for one week], one finds no difference between the responses of *tau* mutant and wild type animals. If, on the other hand, one waits until the animals have been in DD for seven weeks, the amplitude of the *tau* mutant response increases dramatically whereas that of the wild type animals increases only slightly if at all[1] (Fig. 1). Because of our ignorance of the molecular

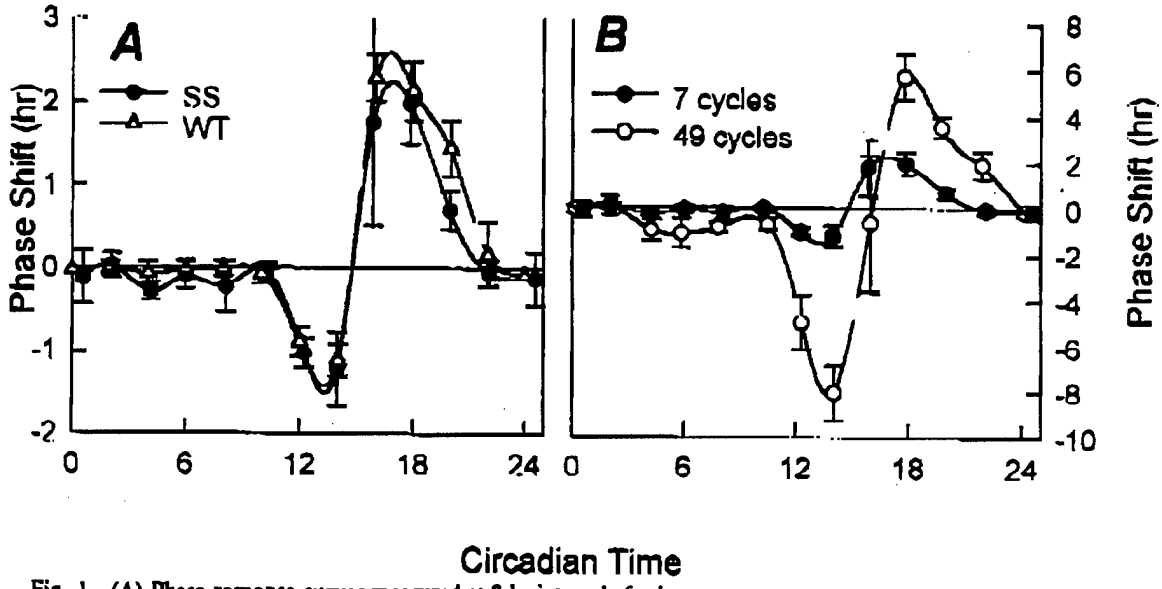

Fig. 1. (A) Phase response curves measured at 2-hr intervals for homozygous *tau* mutant hamsters (●) and wild type hamsters (Δ) after 7 days in constant darkness plotted on equivalent circadian coordinates[2]. (B) Phase response curves measured at 2 hour intervals for homozygous *tau* mutant hamsters after 7 (●) and 49 (○) days in constant darkness.

biology of the *tau* gene we do not yet know whether this change is a direct result of the mutation acting on the clock mechanism or an indirect consequence of its interaction with other genes that may modify the light response. A more immediately accessible set of questions concerns the physiological level of organization at which this effect occurs. Is it only behavior that responds in this way? Can the response be localized specifically to the SCN or perhaps to one or more of the input or modulatory pathways that are known to modulate SCN activity? We have approached the first of these questions by examining the effects of light pulses on a non-behavioral end point, *c-fos* induction.

We compared light-induced *c-fos* expression in the SCN of *tau* mutant hamsters that had been in DD for either 2 or 49 days. Photic thresholds for light-induced behavioral phase shifts, *c-fos* mRNA, and Fos immunoreactivity were closely correlated within both groups, and these thresholds were lower (more sensitive to light) after 49 than after 2 days in DD. This

correlation between phase shifting and Fos induction thresholds, under conditions where both responses are dramatically altered by the previous light history, suggests Fos may play a role in light activated signaling mechanisms leading to phase shifting. However, the maximum amplitudes of Fos induction and phase shifting were not correlated in animals that had been in DD for 2 days[3] (Fig 2). This suggests that if Fos does play such a role, its level of expression does not directly determine phase shift amplitude. We propose to approach the second question (is the effect limited to SCN oscillators?) by asking experimentally whether we can demonstrate the phenomenon in isolated retina.

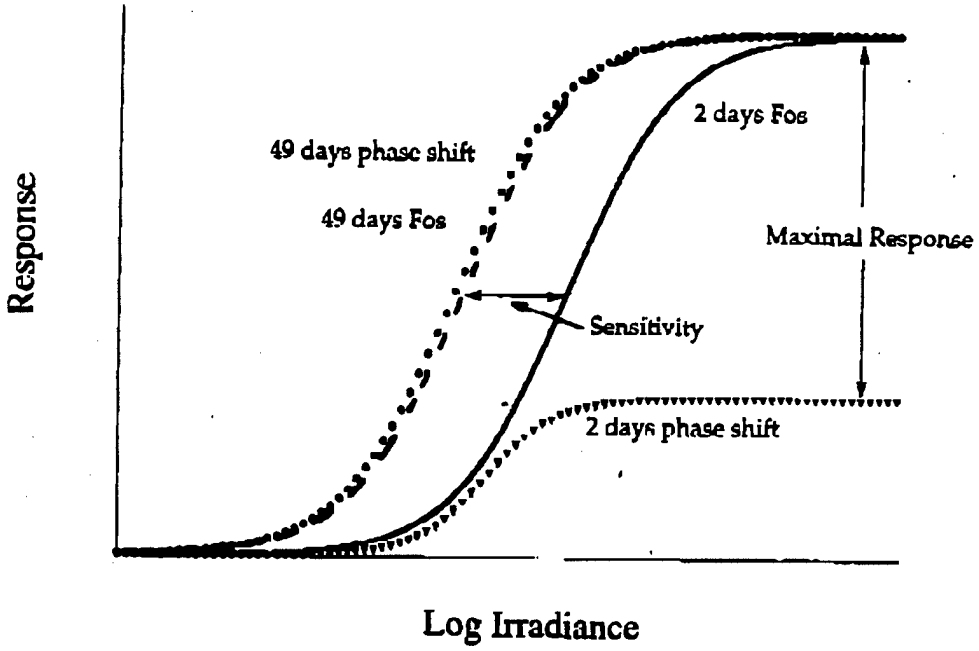

Fig. 2. Diagrammatic illustration of the changes in the relationship between Fos induction and phase shift response in hamsters held for 2 or 49 days in DD[3]. Note the discrepancy between the amplitude of the maximum phase shift and the Fos response in the 2-day DD animals.

We have recently shown that the hamster retina expresses a circadian rhythm of melatonin synthesis when it is isolated and maintained in culture. Furthermore, the period of this rhythm is determined by the genotype of the animal: retinas cultured from wild type hamsters have melatonin rhythms with periods of approximately 24 hours, whereas retinas from homozygous *tau* mutant hamsters have approximately 20 hour periods[4] (Fig. 3). This makes it clear that the mutation has affected the circadian oscillator(s) in the retina, as well as those in the SCN. Our first set of experiments will ask directly whether the *tau* mutation has produced the same kinds of changes in the response to phase shifting light pulses in the isolated retina as it has produced in the behavioral response.

In addition to affecting the period of the retinal oscillator the *tau* mutation affects the temperature compensation of the period. The circadian period of the cultured *tau* mutant retina remains temperature compensated (its Q_{10} is barely within the accepted circadian range) however the effect of temperature on period is considerably greater in mutant than in wild type retinas

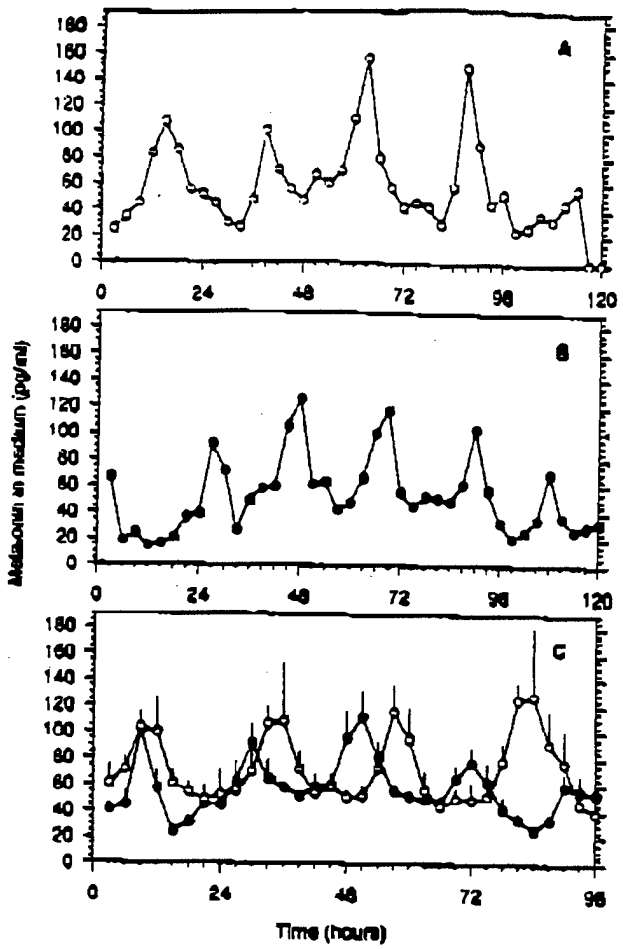

Fig. 3. Rhythms of melatonin synthesis from single neural retinas of (A) wild-type and (B) homozygous *tau* mutant hamsters cultured at 27°C in constant darkness. (C) Free-running rhythms of melatonin synthesis by wild-type and *tau* mutant retinas. The data (mean ± SEM) are from four wild-type hamster retinas (○) and four homozygous mutant hamster retinas (●), and the two data sets were normalized to the time of the first peak[4].

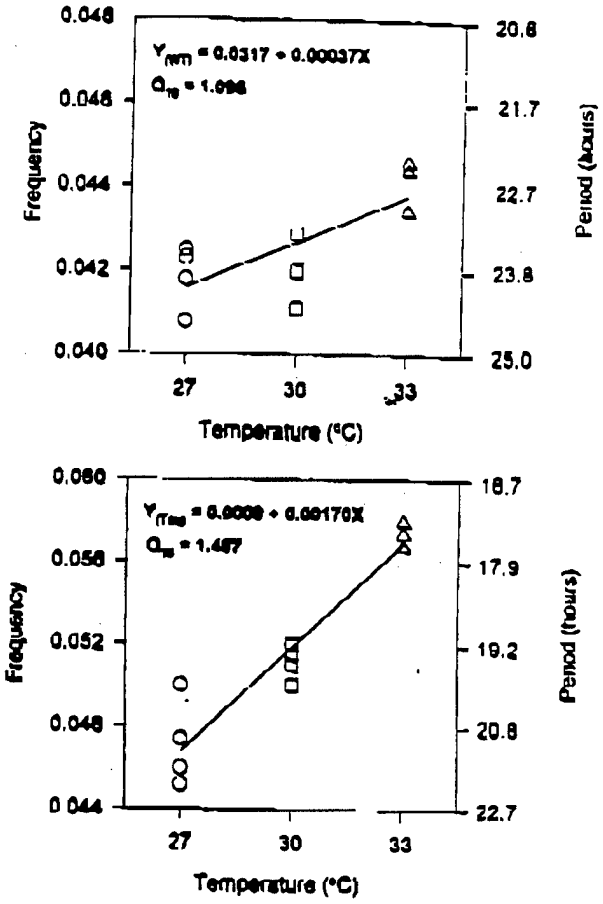

Fig. 4. The effect of culture temperature on the free-running period of the melatonin synthesis rhythm in isolated hamster retina. Data in the upper panel are from wild-type hamsters, while those in the lower panel are from *tau* mutant hamsters. Note the marked difference in Q_{10}.

(Fig.4). In a second set of experiments we will investigate the influence of culture temperature on the phase shifting effects of light pulses given to *tau* mutant and wild type retinas. In a recent paper, Barrett and Takahashi[3] found that a 3°C difference in culture temperature has a large effect on the amplitude of light-induced phase shifts in cultured chick pineal cells. They explain this (and other data) on the basis of an effect of temperature on the amplitude of the underlying circadian oscillators--the lower temperature decreases the amplitude of the oscillators, making it "easier" to phase shift and thus enabling a larger phase shift in response to a given signal. We favor a similar explanation for the effect of the *tau* mutation on phase shifts of the locomotor rhythms (during prolonged exposure to constant darkness, the amplitude of *tau* mutants' oscillators declines more than does the amplitude of the wild types' oscillators). Does

such an amplitude change occur in the hamster retina in response to culture temperature, and is the change larger (as we might expect on the basis of the locomotor data) in *tau* mutant than in wild type retinas? Note that the isolated retina is the only mammalian preparation in which the effects of both light and temperature and their interactions can be measured, as the whole animal homeostatically defends its body temperature and the isolated SCN preparation is not light sensitive. These experiments will provide direct answers to questions about the dependence of retinal oscillator properties on previous light history and culture temperature, and should also begin to localize the system level effects of prolonged dark exposure by either implicating or eliminating the retinal circadian oscillators.

INTERACTION OF THE RETINA WITH CIRCADIAN OSCILLATORS IN THE SCN

We have known for some time that circadian locomotor behavior is controlled more or less directly by oscillators in the SCN, and that in mammals phase shifting and entraining light signals arrive at the SCN only from retinal photoreceptors. We now have evidence to suggest that the retinas influence the properties of the SCN (inferred from locomotor behavior) in the absence of light. We performed a series of experiments in which properties of free running locomotor rhythms were compared between wild type hamsters enucleated at different ages and controls placed in DD at the same time. The distribution of free running periods was significantly different in the enucleated than in the DD control group, regardless of the age at which they were enucleated (1, 7 or 28 days postnatally). Raw data from the day 1 group and summary data from all three groups are shown in Figure 5. Clearly the robust effect of enucleation on the absolute value of the free running period is not a developmental effect since retinal connections in hamsters are fully formed before day 28. Our preliminary interpretation of this result is that the absolute value of the free running period--a value which critically influences adaptive phase relationships within the organism and to environmental cycles--depends on continuous interaction (coupling) between circadian oscillators in the retina and others in the SCN.

The simple experiment outlined above revealed a second effect of enucleation--an effect on the duration of the active portion of each cycle (α). In contrast to the effect on period, the effect on α appears to be developmental; it occurs in animals enucleated on day 1 or 7 but not in those enucleated on day 28 (Fig. 6). Our preliminary interpretation of this result is that enucleation before organization within the SCN has been completed, deprives the SCN of retinal input (perhaps rhythmic input) which is necessary to produce normal coupling among the circadian oscillators within the SCN. There is strong evidence supporting the idea that the duration of α depends on phase relationships among circadian oscillators. In its simplest form this idea has been elegantly articulated[6] as the dependence of α (in rodents) on the phase angle between two oscillators--E, which controls that portion of the active period that occurs in the early subjective night (Evening) and M, which controls activity occurring in the late subjective night (Morning). Since the function being regulated--activity--is under direct SCN control[7,8], it is reasonable to assume that the E and M oscillators are located within the SCN. If the above chain of reasoning is correct, then the increase in α caused by enucleation early (but not late) in development has been produced by a permanent change in the coupling of E and M oscillators within the SCN. An increase in α implies an increase in the phase angle between E and M, probably resulting from weakened coupling between them. The *tau* mutation is known to

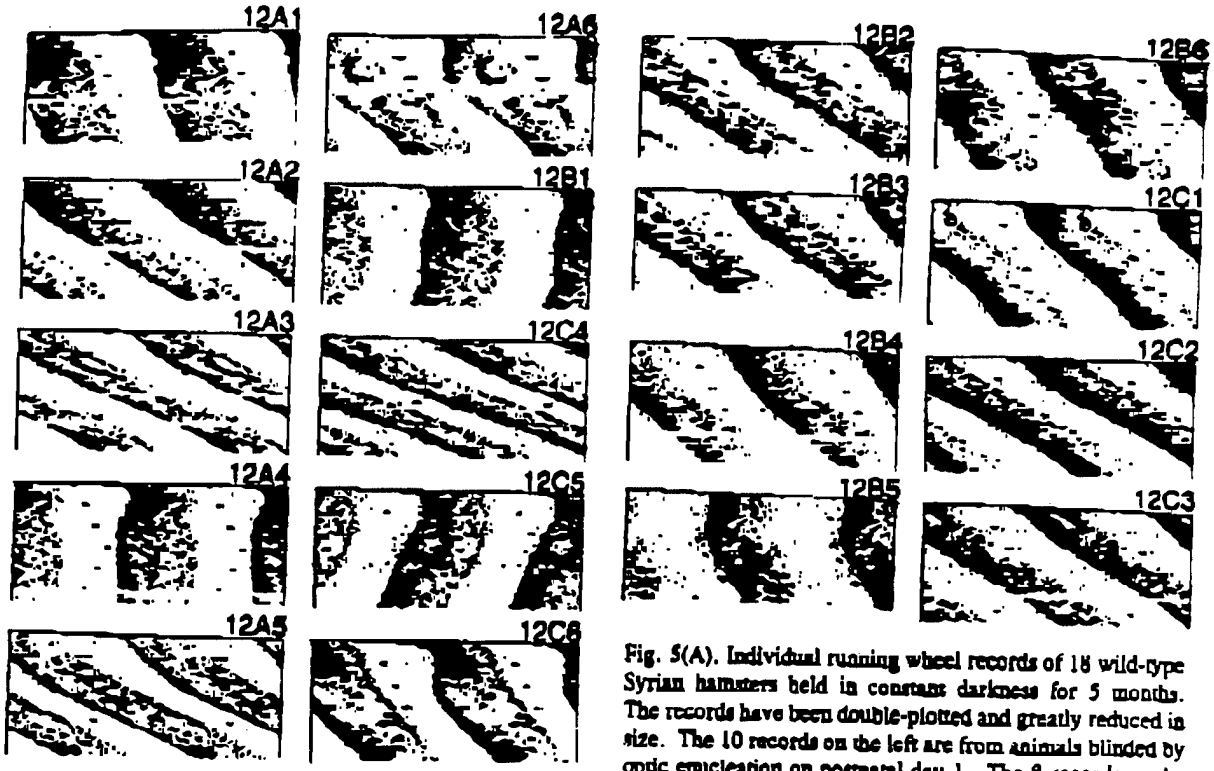

Fig. 5(A). Individual running wheel records of 18 wild-type Syrian hamsters held in constant darkness for 5 months. The records have been double-plotted and greatly reduced in size. The 10 records on the left are from animals blinded by optic enucleation on postnatal day 1. The 8 records on the right are from intact animals. Note the increased variability of free-running period in the enucleated animals.

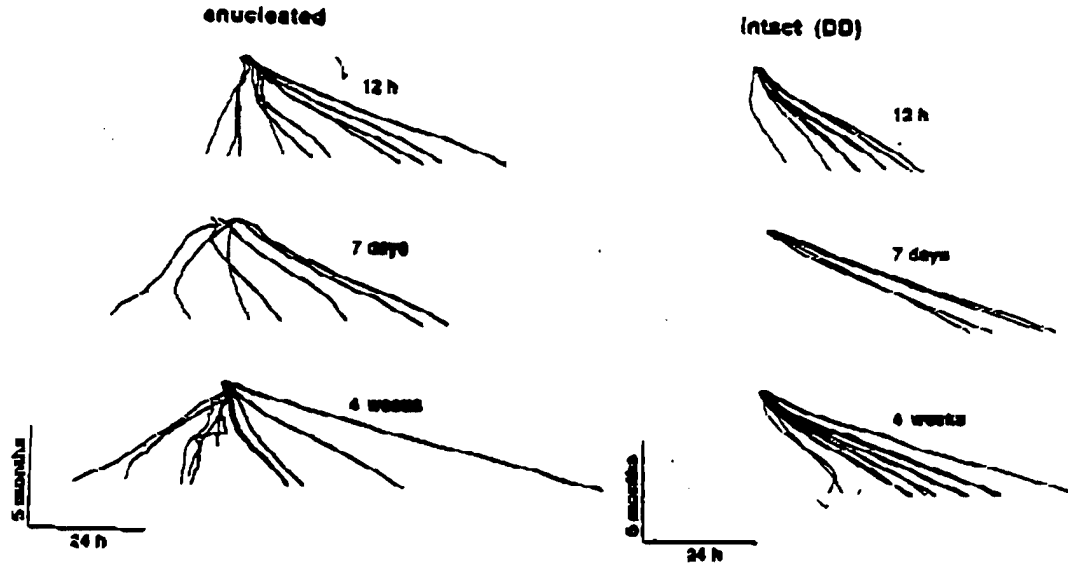

Fig. 5(B). Comparison of free-running periods of locomotor activity between hamsters enucleated at the times indicated (12h = 12 hours after birth, 7 days and 4 weeks after birth) and intact controls placed in constant darkness at the same time. Each line is a tracing of the activity onsets from a single individual. The records are approximately 5 months long.

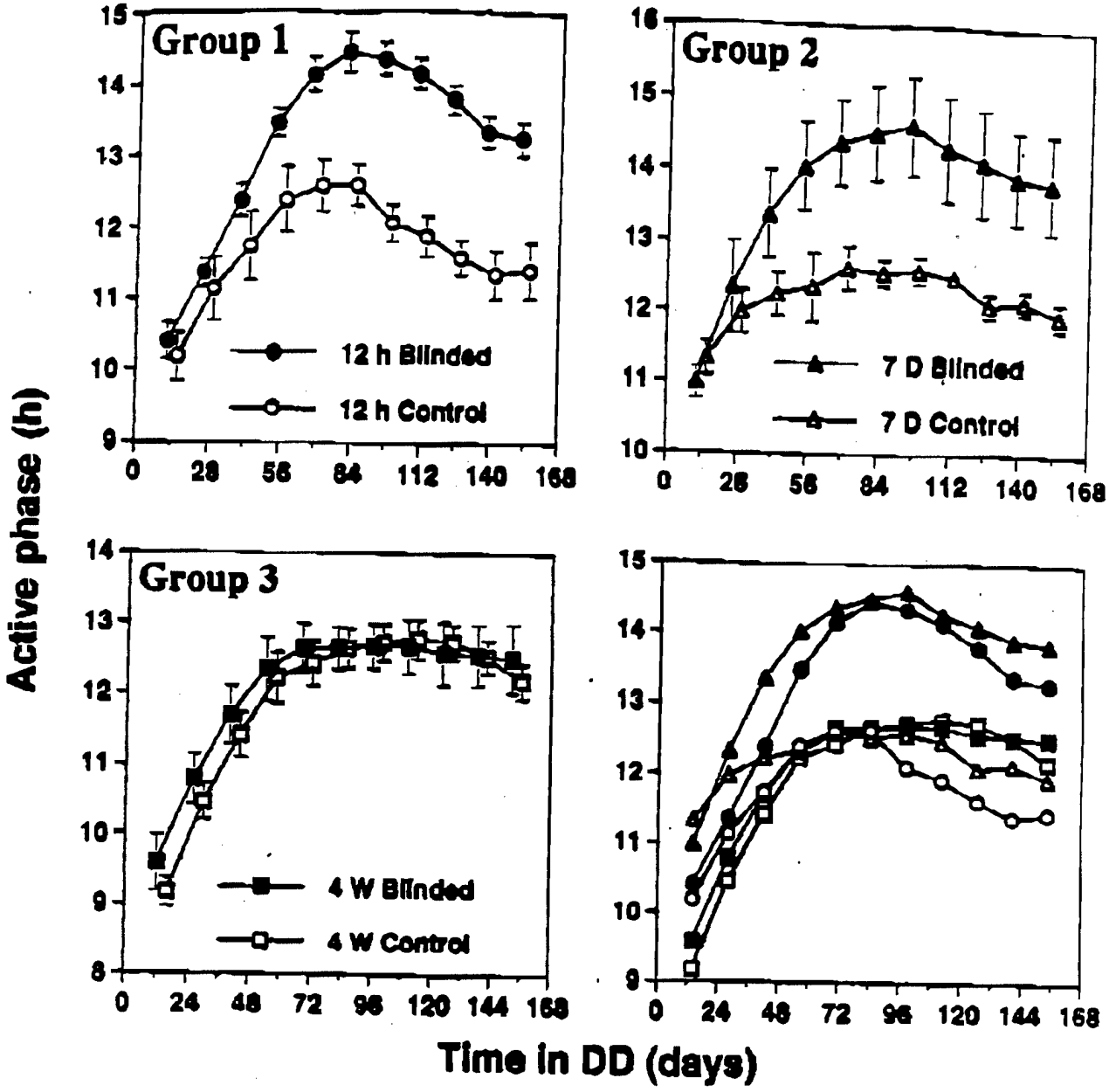

Fig. 6. The first 3 panels (labeled Groups 1, 2 and 3) plot the change with age in the absolute duration of the active phase (α) of the activity cycles of enucleated (filled symbols) and intact controls (open symbols) kept in DD. The three groups were enucleated or placed in DD at 12h, 7 days or 4 weeks after birth. The 4th panel plots all 6 curves on the same axes to emphasize the effect of early (12h, 7d) but not late (4 wk) enucleation.

destabilize the circadian system[9] perhaps by weakening the coupling among constituent circadian oscillators and therefore we expect an even more robust developmental effect of enucleation on *tau* than on wild type animals. Changes in coupling among circadian oscillators within the SCN should be reflected in the electrical activity of that hypothalamic area and perhaps in its phase relationship with its output areas elsewhere in the brain.

Our preliminary data indicates that there are robust circadian rhythms of electrical activity both within and outside the SCN in awake, behaving hamsters of *tau* as well as wild type genotype. Electrical rhythmicity recorded within the SCN has a period of ≈24 hours in wild type hamsters and ≈20 hours in homozygous mutants. Electrical activity is, as expected, highest during the animals' day when locomotor activity is lowest, and there is a one to one correlation between bouts of locomotor activity (occurring at any time of the day or night) and suppression of electrical activity in the SCN. There are also ultradian rhythms (≈15 and ≈80 minutes) that are not affected by the mutation (Fig. 7). As has been previously reported[10], there are circadian electrical rhythms present in several areas outside of the SCN that are 180° out of phase with SCN rhythms, but in addition, we have found several other brain areas that are in phase with the SCN (bed nucleus of the stria terminalis; Fig. 8). We will study the effects of early and late enucleation on the subsequent patterns of electrical activity in the brains of adult animals, first in wild type hamsters and then, if their locomotor responses to enucleation warrant it, in *tau* mutant animals. In these studies we will pay particular attention to the relationship between locomotor activity and electrical activity and to phase relationships among rhythms in different brain areas.

Enucleation causes degeneration of the optic nerves and produces damage in all the retino-recipient areas of the brain. Thus it is possible that the effects of enucleation on circadian parameters are due to damage that is too non-specific to be useful in dissecting circadian organization. At least two considerations mitigate against this conclusion: first the effects are not global; only the distribution of free running periods is affected by enucleation at any age and only α is affected developmentally; second, we have been unable to find any differences in immunocytochemical amounts or localizations of NPY, VIP, AVP, or Calbindin between the SCNs of early enucleated and control animals (we have not yet examined the brains of the animals enucleated at 28 days; Fig. 9). This suggests that there may be specific neurochemical deficits in the SCN produced by enucleation which, when uncovered, may lead us to the particular roles played by specific pathways and transmitters. At least it is clear that the SCN is not massively damaged by enucleation. We will continue to investigate the SCNs of enucleated animals, expanding our immunocytochemical search to include serotonin, GABA, somatostatin, GFAP and other peptides found in SCNs of intact animals.

EFFECTS OF GABA AGONISTS & ANTAGONISTS ON *TAU* MUTANT AND WILD TYPE HAMSTERS

The modulatory role of GABA on circadian rhythmicity has been known to be complex since the experiments 10 years ago with bicuculline, diazepam and baclofen[11,12,13]. These drugs block the phase shifting effects of light, but only at specific phases in the circadian cycle (bicuculline only in the first half of the subjective night, diazepam only in the second half). Recently the situation has become more complex with the discovery that within the SCN, GABA appears to function as an inhibitory neurotransmitter during the night, but as an excitatory

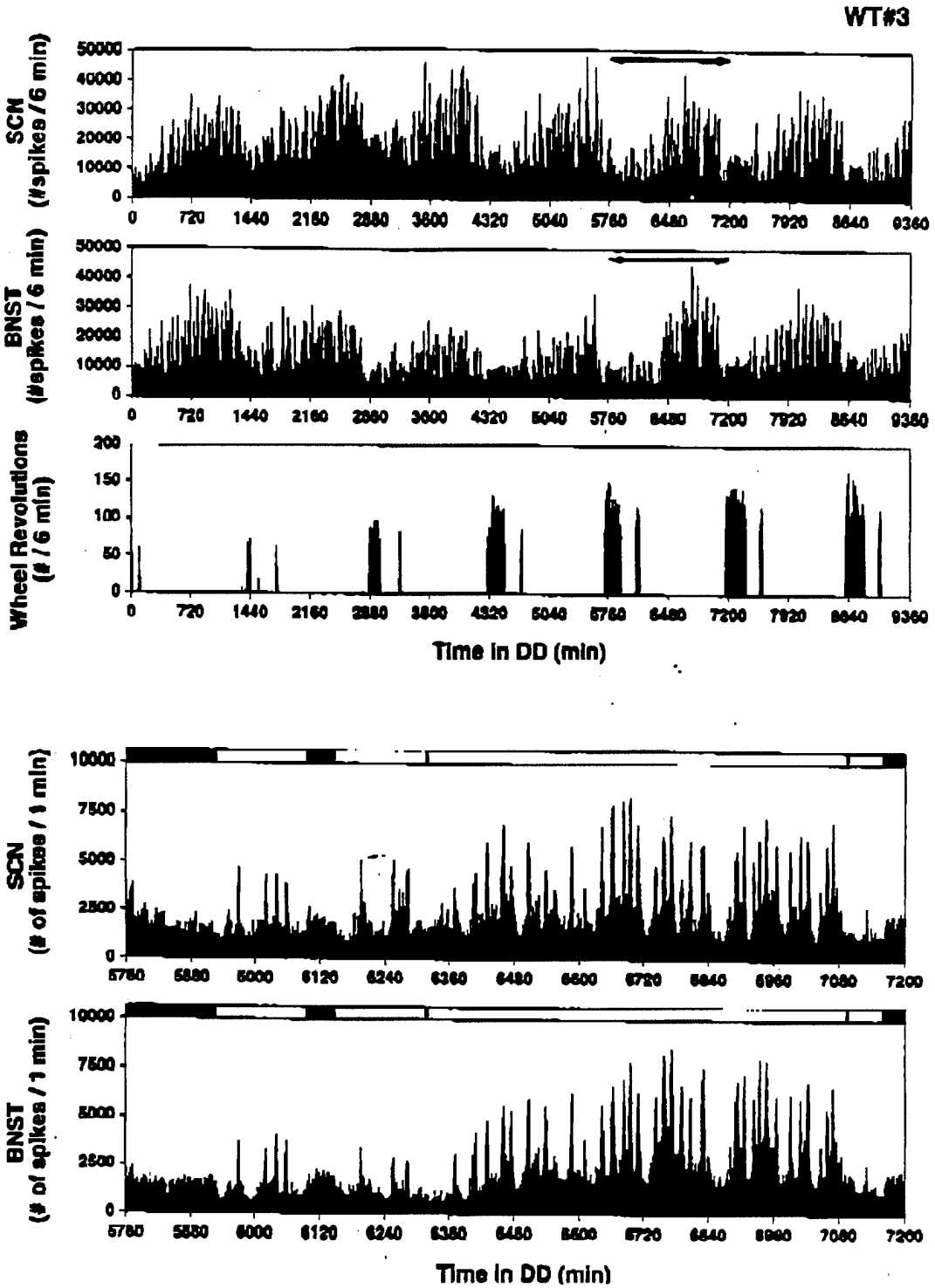

Fig.7. Neural activity records from an awake, behaving wild-type hamster. The period of the envelope of electrical activity recorded from both the SCN and the stria is approximately 24 hours, as is the period of the wheel running activity recorded simultaneously (3rd panel). The phase relationship between the rhythm in the SCN and the locomotor rhythm is, as expected, 180°. The SCN and the stria are exactly in phase. This phase relationship is unusual: most brain areas are 180° out of phase with the SCN (see Fig.8). The above phase relationships are maintained at the level of fine structure shown in the lower two panels in which a 24 hour segment of the data has been expanded (locomotor activity is here indicated by the black bars along the top of each panel).

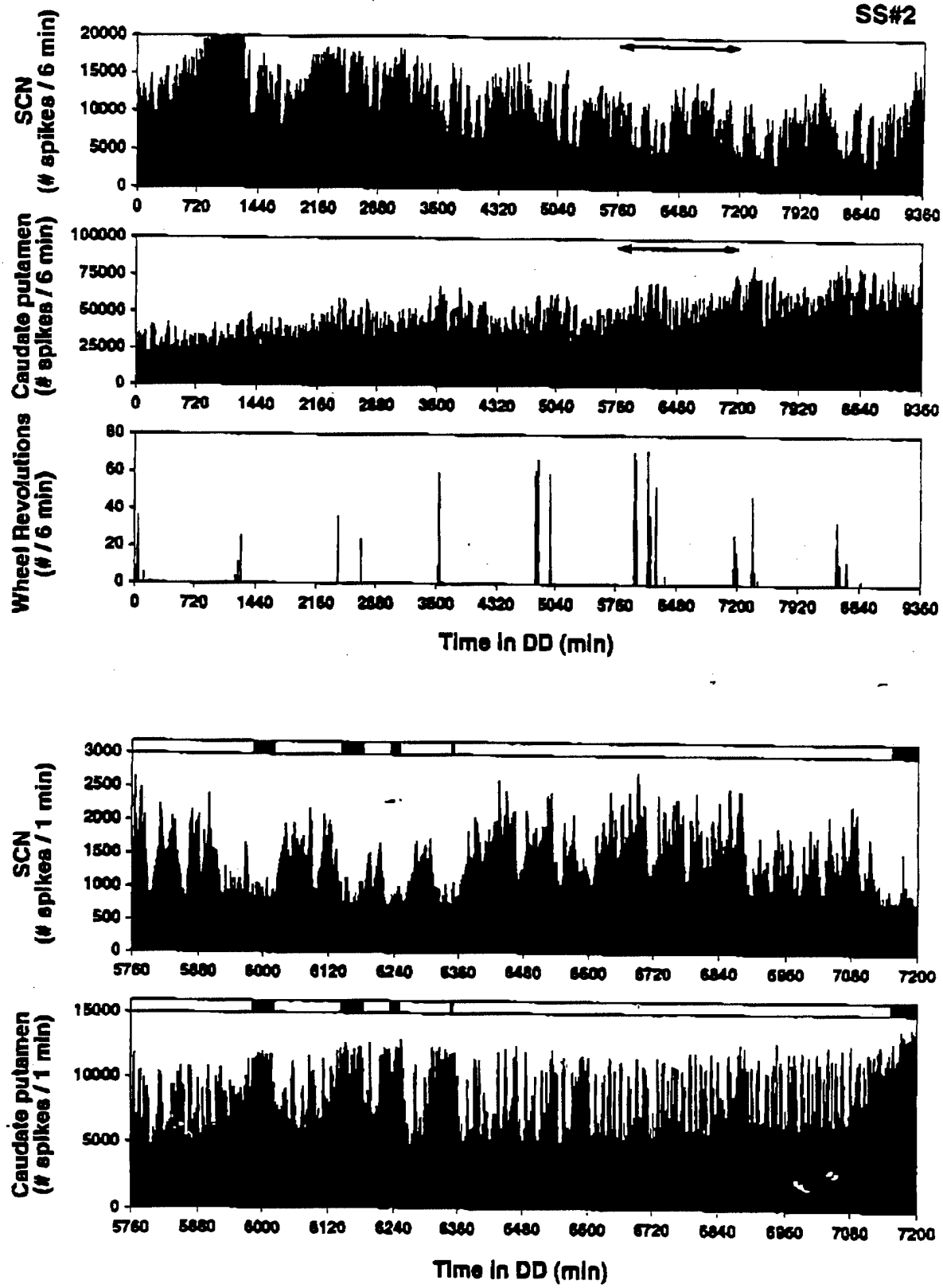

Fig. 8. Neural activity records from an awake, behaving *tau* mutant hamster. Data are plotted as in Figure 7. The period of the rhythm in the SCN is approximately 20 hours and it is 180° out of phase with both the locomotor activity and the electrical activity in the caudate putamen. The phase relationships are maintained at the level of fine temporal structure (lower two panels).

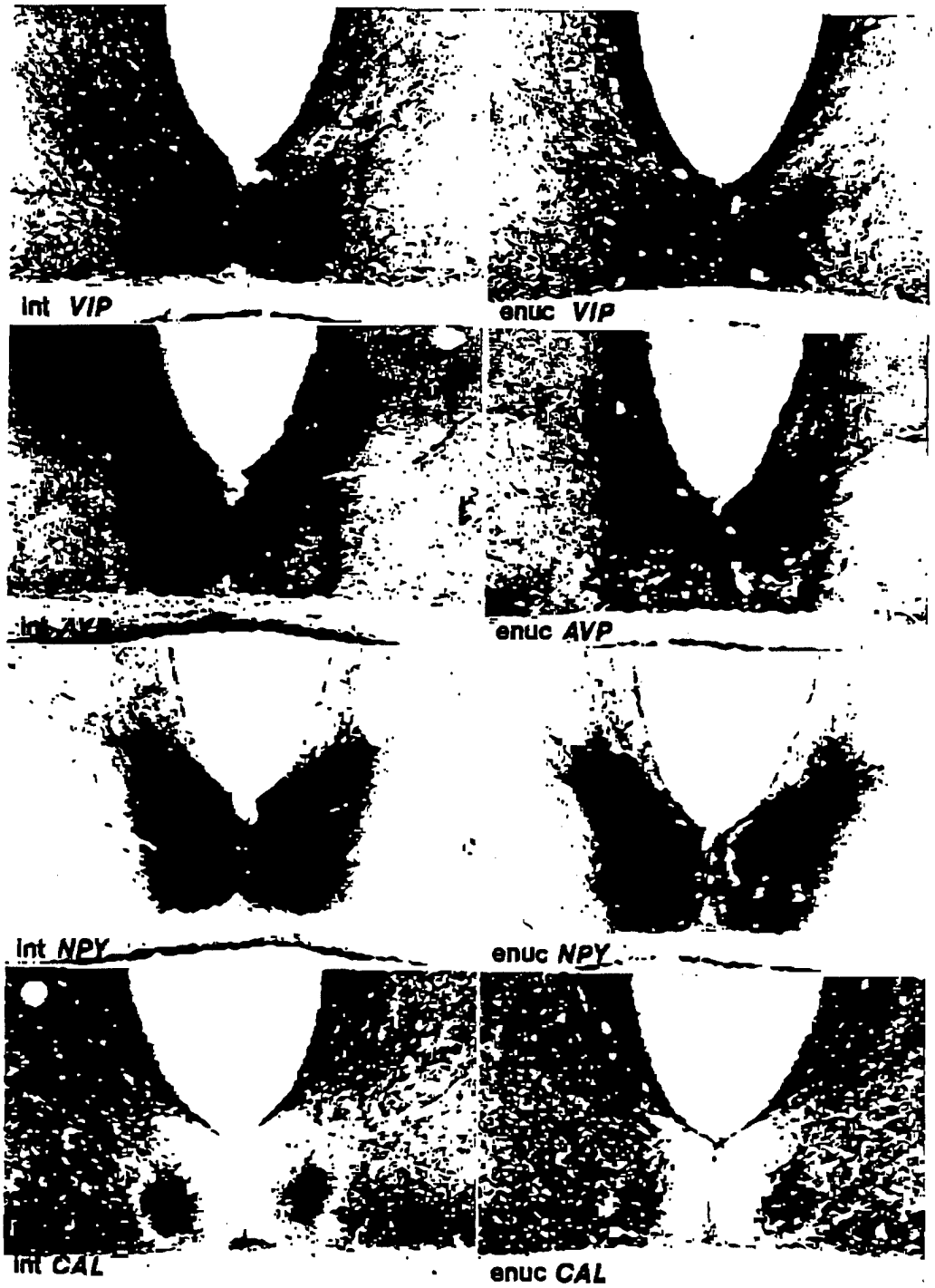

Fig.9. Serial coronal sections through the SCN of enucleated (right panel) and intact controls (left panels) stained for VIP, AVP, NPY, and Calbindin. There are no clear differences between enucleated animals and intact controls kept in DD, either in localization or content of VIP, AVP, NPY or Calbindin, demonstrating that enucleation does not cause major non-specific damage to the SCN.

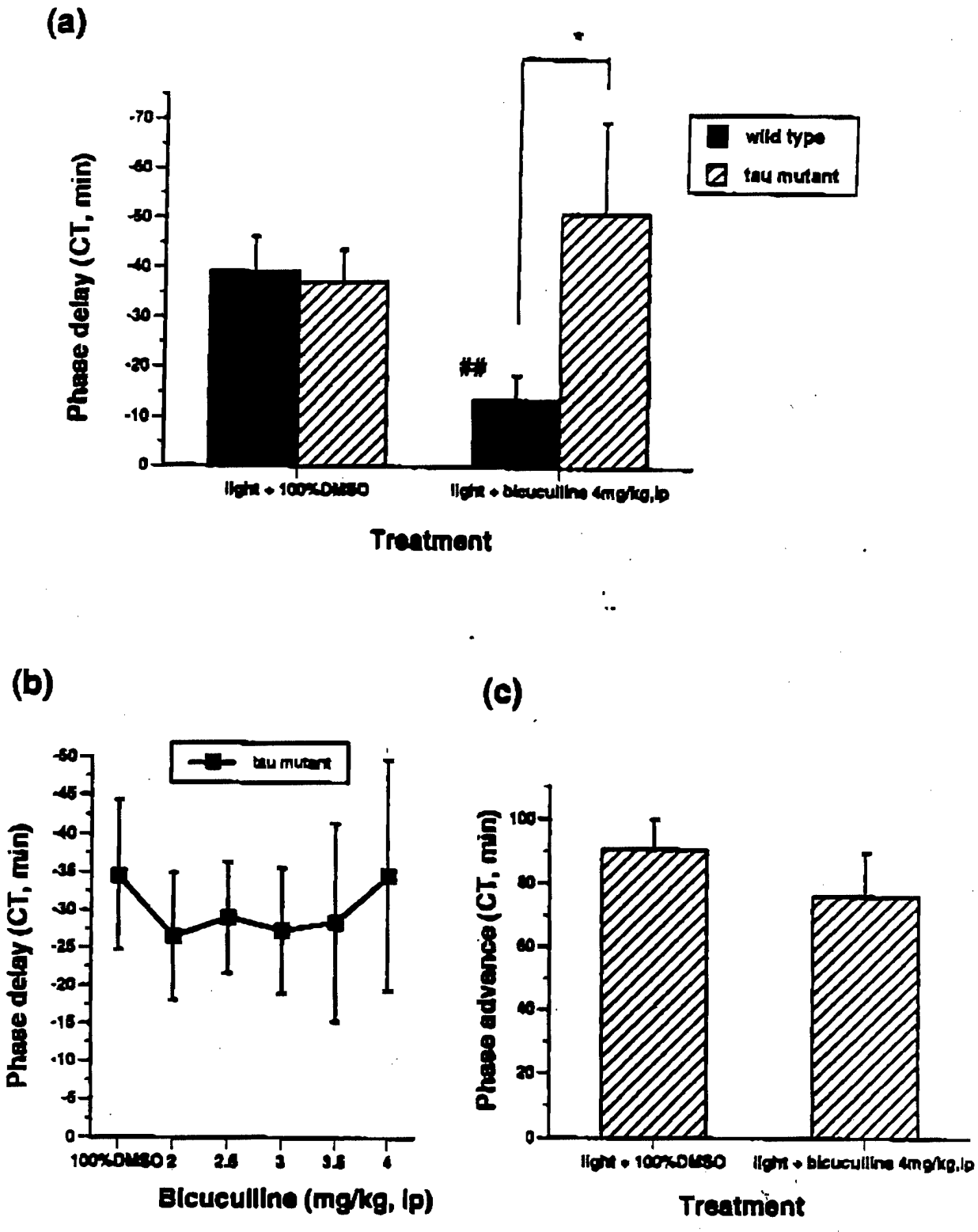

Fig. 10. The effects of bicuculline on light-induced phase shifts in wild-type and *tau* mutant hamsters. (a) Vehicle does not block phase delay in either group, bicuculline blocks phase delay in wild-type but not in *tau* mutant animals. (*: $p<0.05$; ##: $p<0.01$; n=6-9 for each column) (b) Lack of dose/response relationship in *tau* mutant hamsters (n=5-7 for each point; dose/response curve for wild-types published in ref# 11); (c) Lack of effect of bicuculline on phase advancing light pulse given at CT 18 to *tau* mutant hamsters (n=6 for each column; the same lack of effect is seen in wild-types, see ref# 11).

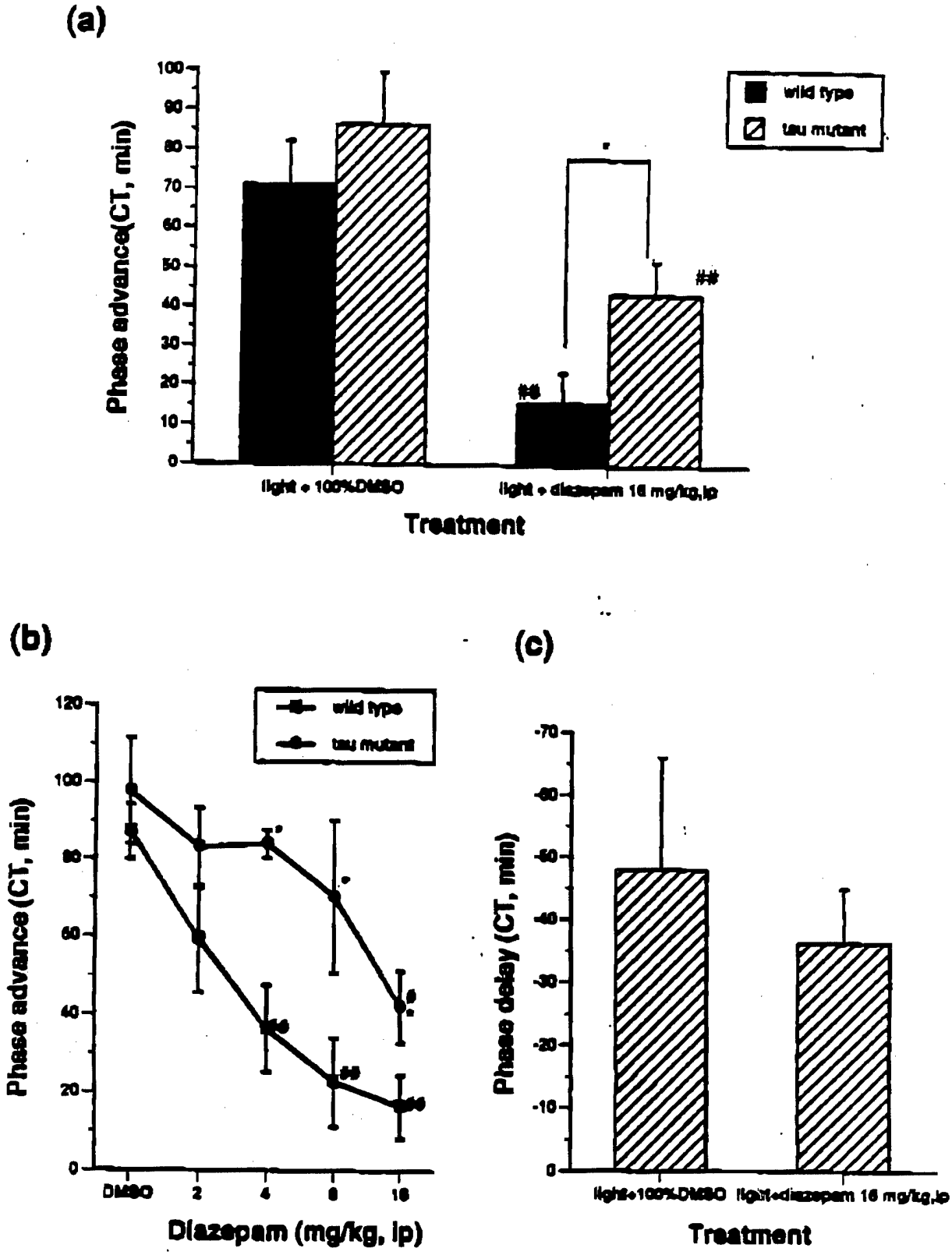

Fig. 11. The effects of diazepam on light-induced phase shifts in wild-type and *tau* mutant hamsters. (a) Vehicle does not block phase advances in either group, diazepam does not block phase advances to the same extent in *tau* mutants as it does in wild-types (#: $p<0.05$, ##:$p<0.01$; n=8 for each column). (b) Dose response curves for the blocking effect of diazepam in wild-type and *tau* mutant animals (n=4-7 for each point; #:$p<0.05$; ##:$p<0.01$; *:$p<0.05$). Note that the effects on *tau* mutants are significantly smaller at the three higher doses. (c) Diazepam does not have a significant blocking effect on light-induced delays at CT 13.5 (also true for wild-types, see ref.# 12).

neurotransmitter during the day[14]. Our preliminary data indicate that the *tau* mutation dramatically alters the hamster's response to both bicuculline and diazepam.

In marked contrast to its effects in wild type hamsters, bicuculline fails to block the phase delaying effects of single light pulses administered in the early subjective night; furthermore the effect of diazepam on phase advances produced by single light pulses administered in the late subjective night is greatly attenuated in *tau* mutant as compared with wild type animals (Figs. 10 and 11). These data suggest that the sensitivity of the circadian oscillators to the modulatory effects of GABA has been altered by the *tau* mutation, an effect which is not predicted by anything that we currently understand about the mechanism underlying the effects of any circadian period mutation. Any such effect raises a great many questions, e.g.: might a change in GABA sensitivity account for the entire effect of the mutation? If not (as seems likely), then does the effect on GABA sensitivity occur at the system level--among and between neurons within the SCN, or those outside it which regulate its function--or at the cellular level, in the channels and receptors of the SCN cells that are expressing circadian oscillations, or at both levels?

In future work, we will attempt to begin to unravel this complex situation by extending our analysis from the behavioral to the neurophysiological level. In awake, behaving animals we will record (for the first time) the neural response of the SCN to single light signals presented either in early or late subjective night. We will assess this response in both wild type and *tau* mutant animals and then determine how it is affected by bicuculline and diazepam.

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