A non-photic gateway to the circadian clock of hamsters

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Abstract. This paper considers the neural mechanisms underlying a particular kind of non-photic phase shifting, that produced by novelty-induced wheel running in the hamster. The projection from the intergeniculate leaflet (IGL) to the suprachiasmatic nucleus (SCN) appears to be an important part of the mechanism mediating such phase shifts. A number of experiments support this view. First, expression of immediate-early genes in the IGL is induced by non-photic phase-shifting stimuli. Second, Fos-like immunoreactivity in the IGL co-localizes with neuropeptide Y (NPY) immunoreactivity. Third, direct application of NPY to the SCN produces phase shifts which do not depend on the hamsters becoming active following the injections. Fourth, blocking the normal actions of NPY at the SCN blocks or greatly attenuates the phase shifting that is normally produced by novelty-induced wheel running. Progress on the physiological basis of phase shifts associated with activity, or a correlate, depends on understanding the behavioural aspects of this phenomenon. The activity–shift response curve is especially useful.

1995 Circadian clocks and their adjustment. Wiley, Chichester (Ciba Foundation Symposium 183) p 154–174

There are many different phase-shifting stimuli and entraining situations that can be classified as non-photic; for example, social zeitgebers, pharmacological treatments and entrainment by periodic feeding. Most have received little study. We have no idea whether or not they depend on the same neural mechanisms. It is definitely premature to attempt to review the mechanisms of non-photic entrainment in general. Instead, I shall concentrate on a particular type of non-photic phase-shifting, that produced by confinement of hamsters (*Mesocricetus auratus*) to novel running wheels for a period of a few hours in the middle of their subjective day. The procedure is simple (Mrosovsky & Salmon 1987, Reebs & Mrosovsky 1989a). For most of a test, a hamster is kept in its home cage which usually contains its own wheel for monitoring circadian activity rhythms. At the desired time, the animal is placed in a nearby clean running wheel from which there is no exit (Fig. 1). Its main options are to run or to rest. In our laboratory conditions most hamsters choose to run. After a standard period,

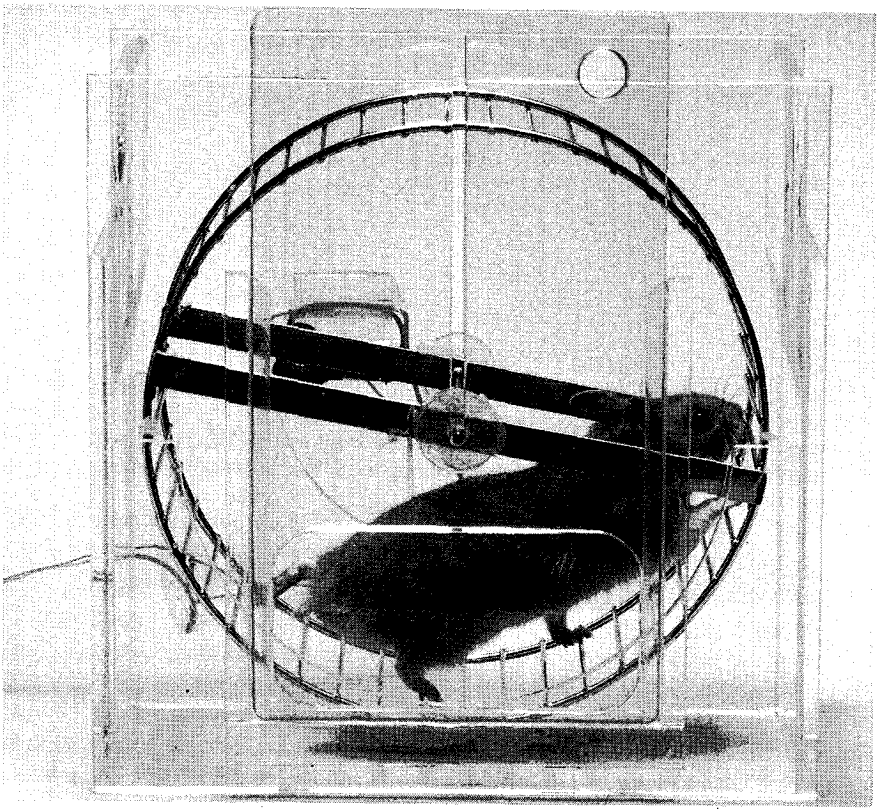

FIG. 1. A simple apparatus for producing non-photic phase shifts in hamsters. The procedure is also simple: the hamster is removed from its home cage and put in the novel wheel for a specified time, usually three hours in our experiments. The animal is confined to the wheel by the plastic sides. The number of wheel revolutions produces a measure of activity.

three hours usually, the animal is returned to its home cage. Novelty-induced running is not so esoteric a starting point as it may appear and has a number of considerations in its favour.

(a) It is a manipulation capable of producing large phase shifts, as large in fact as those produced by light in hamsters (Fig. 2).

(b) Novelty-induced wheel running shares some features with other non-photic phase-shifting stimuli. It is probably just one way of tapping into a phase-shifting system that requires arousal or activity to become engaged. For example, the phase response curve (PRC) for triazolam injections is similar in shape to that for novelty-induced running, and it has been pointed out that triazolam often makes hamsters hyperactive (Mrosovsky & Salmon 1987). It has now been shown that phase-shifting effects of triazolam are greatly attenuated by restricting the

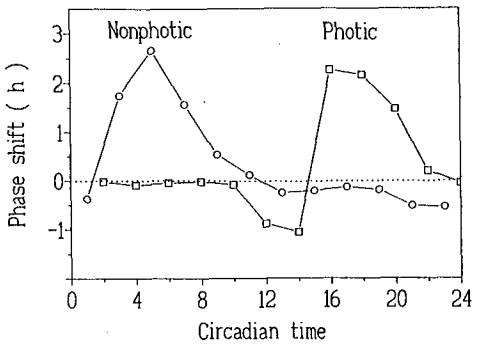

FIG. 2. Non-photic (○) and photic (□) phase response curves (PRCs) for hamsters kept in the dark. The non-photic PRC is for three-hour pulses of novelty-induced wheel running (replotted from Mrosovsky et al 1992). The photic PRC is for one-hour pulses of saturating light (replotted from Takahashi et al 1984). Points show mean values for two-hour bins.

hamster's activity after injection (Van Reeth & Turek 1989, Mrosovsky & Salmon 1990). Phase response curves for pulses of social interaction, for cage changing and for neuropeptide Y (NPY) injections, as well as those for application of a number of substances *in vitro* to suprachiasmatic nucleus (SCN) slices (Gillette 1991, Prosser et al 1993), have some similarities to those for activity pulses or triazolam. Some authors classify PRCs into two main families, one with shapes similar to the classic PRC for light pulses and the other with shapes similar to those for dark pulses and various non-photic stimuli (Smith et al 1992). The latter family has also been designated Y-type because 'in all probability, the phasically active peptide in hamster brain is neuropeptide Y' (Morin 1991). This might turn out to be correct, but grouping PRCs into families without explanation of the considerable differences within a family could be superficial. PRCs within a family can have peaks differing by as much as five hours (Smith et al 1992). Also, the delay portions of the curves within a family often do not match up well; for example, NPY produces phase delays when given in the early subjective night (Albers & Ferris 1984) but triazolam produces delays mainly when given in the late subjective night. Nevertheless, despite many differences in detail, there could be some underlying features common to PRCs with advance portions somewhere in the subjective day.

(c) When studies on activity pulses (Mrosovsky et al 1989, Janik & Mrosovsky 1993) are taken together with those on triazolam (Turek 1988), they constitute the beginnings of a database.

(d) Finally, there is some recent information about the physiological substrates for novelty-induced wheel running.

For these reasons, it may be profitable at this stage to try to understand one particular kind of non-photic phase shifting, and to go on from that base to ask

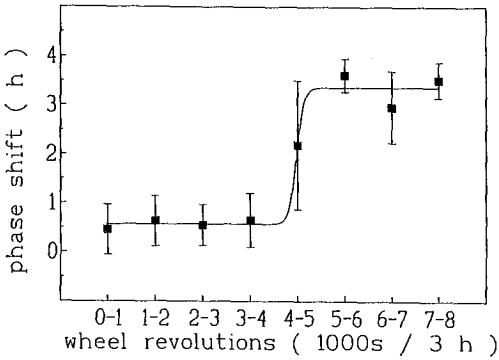

FIG. 3. Phase shifts (mean ± SD) in three–five-month-old male hamsters as a function of the number of revolutions made in a novel wheel during a three-hour pulse starting at zeitgeber time (ZT) 4. Data are replotted from Janik & Mrosovsky (1993), with a sigmoid curve fitted by GraphPad InPlot, with the lower and upper asymptotes fixed at 0.56 and 3.37 h respectively ($R^2 = 0.99$). An Aschoff (1965) type II procedure was used; the maximal phase shifts with this procedure were similar to those obtained with the Aschoff type I procedure (see Mrosovsky et al 1992). It should not be assumed that the threshold value, lying between 4000 and 5000, will be exactly the same in other strains or ages or sexes of hamsters, or with different test conditions, wheel sizes, etc. The construction of similar curves for particular situations is advocated to assist in the interpretation of data on non-photic effects on circadian rhythms.

whether other non-photic inputs to the clock depend on the same or different mechanisms.

Activity-response curves and their implications

Before the results of physiological manipulations can be interpreted with confidence, it is essential to appreciate that brief bouts of activity are relatively ineffective non-photic phase shifters (Honrado & Mrosovsky 1989, Reebs & Mrosovsky 1989b). Although it is not known what the critical feature of confinement to a novel wheel is, passing a threshold number of wheel revolutions *predicts*, but does not necessarily *cause*, the occurrence of a large phase shift (Fig. 3). In the particular conditions of our tests, hamsters making fewer than 4000 revolutions in the middle of the subjective day are unlikely to show a phase shift, whereas those making more than 5000 have a high probability of shifting (Fig. 3).

It has been realized that when phase shifting is produced by a drug, it is necessary to know whether the drug is simply a fancy way of producing activity or is having a more direct effect on the circadian system. As mentioned above, tests with hamsters whose movements are restricted indicate that phase shifts produced by triazolam depend on activity, or a correlate. In contrast, those produced by chlordiazepoxide (Biello & Mrosovsky 1993) and melatonin (Redman & Roberts 1991) do not seem to be mediated in this way. In other

cases direct tests have not been done, but at least the possibility of behavioural mediation of phase-shifting effects has been recognized as a possibility (Tsujimaru et al 1992, Edgar et al 1993).

The converse, that loss of non-photic phase shifting following a manipulation may depend on decreased activity or arousal in response to non-photic stimuli, does not seem to have been generally appreciated. For example, after chemical lesions of the intergeniculate leaflet (IGL), triazolam no longer produces phase shifting (Johnson et al 1988), but no evidence has been provided to show that this benzodiazepine induces the same amount of activity in the lesioned and non-lesioned hamsters. After electrolytic lesions of this area, hamsters become less active and do not run as much when confined to a novel wheel (Janik & Mrosovsky 1994, Wickland & Turek 1992). Johnson et al (1988) wrote: 'the requirement of an intact LGN [lateral geniculate nucleus] for triazolam to shift circadian phase suggests that the LGN may be a site through which stimuli gain access to the circadian clock'. An alternative is that stimuli reach the circadian clock by some other route and the IGL lesions simply prevent hamsters from becoming sufficiently active or aroused for adequate stimulation of those pathways.

Although experiments with lesions have not yet established that triazolam-induced phase shifts depend on information to the SCN being routed through the IGL, ironically the conclusions that have been drawn from such experiments are probably correct. Supportive evidence comes from several other types of experiment.

The involvement of c-*fos*

Non-photic phase shifting can induce expression of *fos*-related genes in the IGL (Janik & Mrosovsky 1992; Fig. 4). This experiment was guided by a knowledge of the behavioural predictors of large phase shifts (Fig. 3). Animals that ran more than 5000 revolutions in a novel wheel were selected and compared with those left in their home cages. No expression of *fos* was found in the SCN, and this accords with the results of others working with other non-photic stimuli (Mead et al 1992), though in some of those experiments one cannot be sure that the manipulations would have resulted in phase shifts in the conditions used for the study of c-*fos* (Cutrera et al 1993). Induction of immediate-early genes in the IGL, but not in the SCN itself, suggests that information about non-photic events is conveyed from the IGL to the SCN in some way that does not involve induction of *fos*. One obvious candidate is the NPY projection from the IGL to the SCN (Mikkelsen 1990, Card & Moore 1991, Mikkelsen & O'Hare 1991, Moore 1992), especially since the PRC for NPY injections into the SCN (Albers & Ferris 1984) has some rough similarities to those for novelty-induced activity and triazolam. However, before getting carried away by this line of reasoning, it is necessary to consider the possible involvement of increased activity in phase shifts produced by NPY.

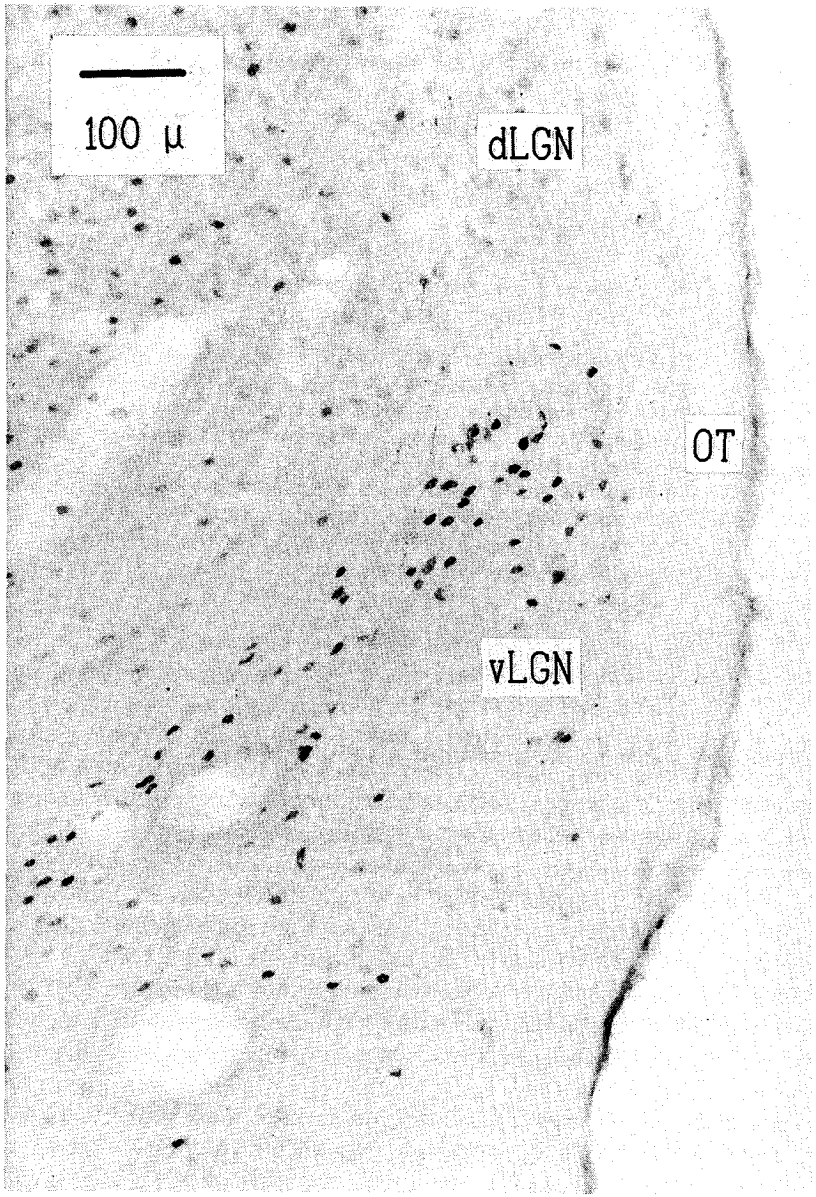

FIG. 4. Fos-immunoreactivity in the IGL of a hamster following a three-hour pulse of novelty-induced wheel running. Antibody provided by Drs D. Hancock and G. Evan; photomicrograph by Dr J. D. Mikkelsen.

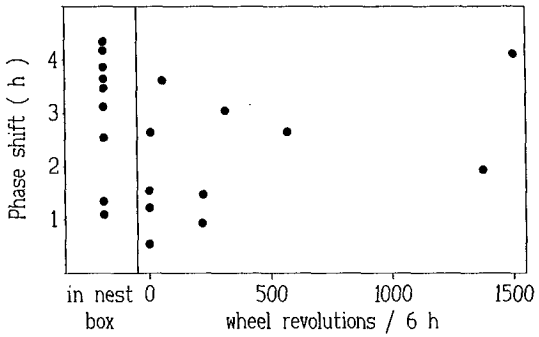

FIG. 5. Phase shifts of hamsters given SCN injections of 400 ng of NPY at ZT 4 in an Aschoff type II test procedure. Data from Biello et al (1994) are plotted as a function of the number of wheel revolutions made in the six hours following the injections. The left panel shows shifts from hamsters confined to their nest boxes for this six-hour period. Phase shifts of confined hamsters were not significantly different from those in hamsters with access (but not confined) to wheels. The correlation between the number of wheel revolutions and the extent of phase shifts was not significant ($r=0.46$, $P=0.15$ two-tailed test, slope not significantly different from 0). Note also that the number of wheel revolutions are below the values associated with phase shifts in Fig. 3. However, in the graph and all calculations, one point is excluded, that from a test in which an animal ran 10 983 revolutions after receiving NPY; the associated shift of 3.05 h may well have been mediated by activity or a correlate. Inclusion of this animal would have reduced the correlation between activity and phase shifts to even less significant values.

NPY-induced phase shifts and motor activity

In Albers & Ferris's paper (1984), the actograms illustrating shifts in response to NPY show prominent bouts of wheel running immediately after the injections. Their work was done before the chronobiological effects of activity/arousal were discovered (Mrosovsky 1988, Mrosovsky & Salmon 1987) and they did not study activity systematically. If phase shifts produced by NPY depend on the animal becoming active, NPY is not a good candidate for *mediating* shifts produced by novelty-induced wheel running; administration of NPY would simply be another way, a biochemical way, of initiating or *mimicking* such shifts.

To investigate this we measured wheel running in hamsters given NPY injections to the SCN at about zeitgeber time (ZT) 4. There was no correlation between the amount of running and the extent of the phase shift. Also, in every case except one, the animals made fewer than 4000 revolutions in the six hours following the injections (cf., Fig. 3). Other hamsters were confined to small nest boxes for six hours after receiving NPY, but this did not prevent NPY from producing significant phase advances (Fig. 5). These results show that NPY can produce phase advances in the absence of appreciable amounts of wheel running. These necessary preliminaries fuelled our enthusiasm for NPY as a candidate for conveying non-photic input to the SCN.

Co-localization of Fos and NPY

In our studies of immediate-early gene expression in the IGL, D. Janik noticed that cells with Fos-like immunoreactivity seemed to occupy a field roughly similar in extent to the NPY field described by Morin et al (1992). To look into this more closely, we double-labelled sections with antibodies to Fos and antibodies to NPY. Brains of hamsters that had run more than 5000 revolutions during three hours of confinement to a novel wheel were sent with brains of control hamsters to J. D. Mikkelsen in Copenhagen for blind assessment. Using a different antibody and greater dilutions than in Janik & Mrosovsky's (1992) initial work, he confirmed that immediate-early genes are turned on in the IGL by this manipulation. It also became apparent that about 70% of the NPY-immunopositive cells also expressed c-*fos*.

Antibodies to NPY

If activation of NPY-containing cells in the IGL and subsequent release of NPY at terminals in the SCN mediates non-photic phase shifting, it should be possible to block such shifts by interfering with the ability of NPY to bind to receptors. Unfortunately, there are no readily available well-studied NPY antagonists, so we tried another approach, injecting antiserum to NPY into the SCN before placing animals in a novel wheel. These experiments were done by S. M. Biello using a well-characterized antiserum generously provided by J. M. Polak (Allen et al 1983). In view of the dose–response requirements for non-photic shifts (Fig. 3), it was essential that injection of the antiserum should not interfere with the hamsters' responses to the novel wheel. Fortunately, the treated animals generally ran as much as those given normal rabbit serum. Of course, some hamsters, even untreated ones, decline the opportunity to run when placed in a novel wheel; such sluggards are not expected to show phase shifts (Fig. 3). However, from data on animals that made more than 5000 revolutions both on their tests with NPY antiserum and on those with normal serum, it was clear that the phase-shifting effect of confinement to a novel wheel can be greatly attenuated without there being reductions of wheel running during the pulse (e.g., Fig. 6; Biello et al 1994).

Does exercise increase NPY release?

So far, we know that immediate-early genes are expressed in NPY-containing cells in the IGL after a well-characterized non-photic phase-shifting stimulus; we know that these NPY neurons send axons to the SCN; and we know that interfering with the action of NPY at the terminals of these axons by introducing antiserum to NPY greatly attenuates the phase shifts. To complete this story, it would be nice to demonstrate an increased release of NPY in the SCN in

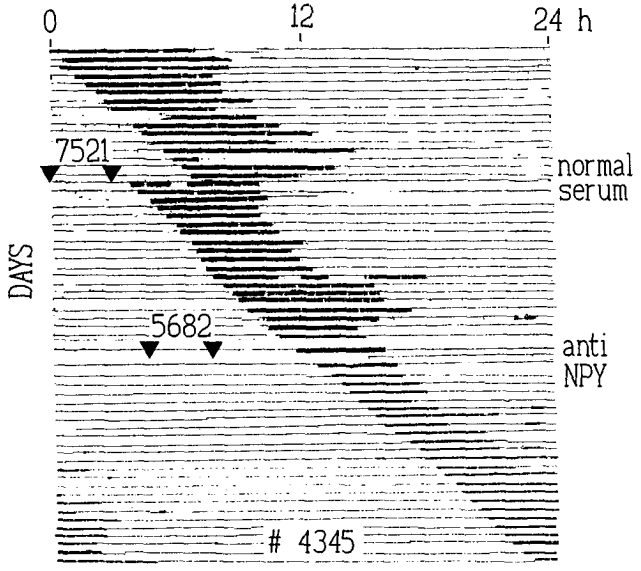

FIG. 6. Actogram from Esterline Angus records of a hamster in continuous darkness given injections to the SCN shortly before being placed in a novel wheel for three hours. First injection, normal rabbit serum; second injection, antiserum to NPY. Numbers by triangles show the number of wheel revolutions made in the three-hour pulses. Pen deflections show wheel running at other times. (Data from Beillo et al 1994.)

response to a non-photic stimulus. Initial attempts to detect this immunocytochemically have not been promising, and we have turned to other methods. It is interesting to note that NPY increases in a number of hypothalamic areas in rats in a state of chronic negative energy balance produced by a combination of intense wheel running and limited rations (Lewis et al 1993). Whether a single bout of running increases NPY concentration or NPY turnover in the SCN has not been investigated.

Limitations and problems

Gaps

A major gap in our understanding of the physiology of non-photic effects is how information reaches the IGL. Another obvious limitation is that the experiments cited on NPY have been done with only one species, and mostly in the advance portion of the PRC. A topic almost untouched at the physiological level is the interaction between photic and non-photic phase-shifting events. Some striking interactions have been demonstrated at the behavioural level (Mrosovsky 1991, Ralph & Mrosovsky 1992).

Direct input or coupling?

Another question is whether the NPY pathway to the SCN mediates simple phase-shifting effects or represents a coupling pathway to the light-entrainable oscillator in the SCN from a non-photically entrainable *oscillator* elsewhere. Because all the wheel-running activity, not just a component, can be entrained non-photically in hamsters (Mrosovsky 1988, Rusak et al 1988, Reebs & Mrosovsky 1989a, Mrosovsky et al 1989), one must infer that the light-entrainable oscillator is affected by non-photic stimuli. There could be direct influences on the light-entrainable oscillator(s) in the SCN, or a coupling influence from another oscillator (Mrosovsky 1988, Rusak et al 1988).

If circadian rhythms could be entrained by non-photic means in animals with SCN lesions, this would support the idea of there being some non-photic oscillator outside the SCN. It has been reported (Mistlberger 1992) that hamsters with SCN lesions do entrain to a two-hour stay each day in a different cage, but because of the low levels of activity and the lack of stability in period and in phase angle during the days when the pulses were given, the data are not compelling. If an animal is activated by being in a new cage, it is possible that it will rest more in the few hours after it gets back to its home cage. Non-random distribution of activity over a 24 h period might result in this way from masking effects. Without clear free runs after withdrawal of the putative zeitgeber, rhythms of activity during its presence become harder to interpret. Finally, persistence of non-photically entrained rhythms was weaker or absent in the hamsters with the larger lesions; this suggested that there are some non-photically entrainable oscillators in or near the SCN (Mistlberger 1992).

Canbeyli et al (1991) found that SCN-lesioned hamsters whose rhythmicity had been restored by transplants of fetal SCN failed to show a phase shift in response to triazolam. They suggested that afferents outside the SCN mediate the shifting effects of triazolam. Although the results are consistent with this interpretation, the loss of phase shifting in response to triazolam is not instructive because the amounts of activity induced by the drug were not adequately quantified. Even if the amounts of activity after the triazolam injections had reached levels associated with shifting in intact animals, a failure to find shifting in the transplanted animals may reveal more about the ability of transplanted tissue to fulfil normal functions than about the normal site of action of triazolam.

Dark pulses

A potential problem with the view that non-photic phase shifts associated with activity require the NPY projections to the SCN comes from experiments with dark pulses. Because preventing wheel running greatly attenuates the phase-shifting effect of dark pulses, it has been concluded that phase shifts produced

by dark pulses are mediated by activity or some correlated variable (Reebs et al 1989, Van Reeth & Turek 1989). If this is so, lesions of the LGN should attenuate phase shifts after dark pulses. However, Harrington & Rusak (1986) reported that this occurs only when six-hour dark pulses start at circadian time (CT) 5–7, and not at other times. This half answer is difficult to interpret. The advance portion of Harrington & Rusak's dark pulse PRC for control hamsters peaked at CT 11–13, whereas previously Boulos & Rusak (1982) found the peak for six-hour dark pulses some six hours earlier in the same species. It is possible that some dark pulse PRCs are mediated by activity, but that others are not, or not to the same extent. Some experimenters use procedures likely to promote activity, such as 'jostling the cages to awaken all animals' at the start of the pulse (Ellis et al 1982). Another complication is the differences in PRCs for different durations of dark pulses (see Boulos & Rusak 1982, Ellis et al 1982). After a long dark pulse starting towards the end of the subjective day, the lights will tend to come on in an advance portion of the photic PRC. The background intensity of light is another poorly researched variable. Mark Twain's comments about the ability of researchers to cast much darkness on a subject might be doubly apt for this case.

Summary

This paper makes two main points. The first is that work on the physiological basis of non-photic effects on circadian rhythms must be informed by an understanding of the behaviour that is associated with phase shifting (e.g., Fig. 3). This methodological point is probably more important than the second and more substantive point: that the NPY projection from the IGL to the SCN is the mechanism through which certain non-photic events affect the circadian clock in hamsters.

With respect to the latter point, the suggestion is that NPY is a non-photic *gateway* to the SCN, not that it is the *pathway*. By what route behavioural and neural information arrives at that gateway remains a blank. A major difficulty in mapping the complete path from the non-photic inputs to the SCN is that there is no clear starting point. With photic entrainment in mammals, the eye is the obvious starting point. With non-photic shifting events, such as confinement to a novel wheel, it is not known whether it is the induced activity, the associated arousal, or some other variable that is critical for phase shifting. Perhaps with non-photic entrainment it will be possible to start at the other end of the system, at the clock itself, and follow the thread back to the periphery, and so discover more about pathways and inputs. The recent findings with NPY and c-*fos* put the end of that thread into our hands.

Acknowledgements

I thank Daniel Janik, Stephany Biello and Jens Mikkelsen for collaboration, ideas and comments. Peggy Salmon and Kelly Foster assisted with the experiments. J. M. Polak gave us NPY antiserum. The Medical Research Council of Canada provided support.

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DISCUSSION

Miller: It is implicit in your model that the gene encoding NPY must be a target gene for Fos, which in turn suggests that there's an AP1 binding site on the NPY gene.

Mrosovsky: I'm not sure there has to be an AP1 binding site on the NPY gene. There could be several intermediate steps.

Mikkelsen: It's likely that Fos turns on the NPY gene, even though a direct contact between c-*fos* expression and NPY mRNA expression has not been demonstrated. In other systems, such as the polymorph layer of the dentate gyrus, NPY mRNA expression is increased after seizures (Gall et al 1990). We have recently shown that *fos* is induced in NPY-immunoreactive cells (M. Woldbye & J. D. Mikkelsen, unpublished observations) after seizures and take this as evidence for the presence of an AP1 site(s) on the NPY gene.

Moore: Could I respond to your comments about our paper on IGL lesions (Johnson et al 1988). At the time we did that work it was not recognized that activity was a component of the response and we didn't look at activity; but, after all, the fact that we didn't look at it doesn't mean that the activity was not induced in the lesioned animals. Another piece of information that we published in that paper indicated that the IGL is a component of the non-photic pathway: R. F. Johnson made one set of lesions with an excitatory amino acid, and that agent initially stimulated the IGL prior to destroying it. This was done with the animals under anaesthesia and, in that situation, obviously without any locomotor activity, there were subsequent changes in the phase of the animals' wheel running that were dependent on the time that the lesion was made. This clearly implicates the IGL–geniculohypothalamic tract pathway in non-photic entrainment.

The IGL, like the SCN, surprisingly has relatively few inputs from other brain areas. The predominant input, other than that from the retina, is from the retrochiasmatic area, which is a crossroads for all kinds of inputs coming through the supraoptic commisures, including ones from the brainstem and from the

forebrain. The retrochiasmatic area input is likely to be the most important input other than the visual input.

Mrosovsky: I should have emphasized that Johnson et al's paper (1988) was an early paper on IGL lesions; and the point you made about the phase shifts induced under anaesthesia is compelling. I just wanted to stress that one has to take activity into account in interpreting the results of such manipulations.

Turek: The type of activity may not be particularly important, though. We see major phase shifts in animals that do not have a running wheel, who have never seen a wheel. We give them triazolam and they are active afterwards, and though they are not running for 4000–5000 wheel revolutions they still show phase shifts. If you give a running wheel to animals that have never had one before, they show even larger phase shifts than an animal that has had a running wheel all the time and is given a novel one. We certainly agree with Dr Mrosovsky that the amount of activity is important but it does not have to be in a wheel. If you stimulate only a little activity you will not stimulate a phase shift.

Mrosovsky: We confine the hamsters to the running wheel because we then get a good measure of their activity. Basically, they can either sleep or run, but not much else. But we also have evidence that activity other than wheel running can have some effects (Reebs et al 1989, Honrado & Mrosovsky 1989).

Menaker: Have you looked at the interaction of non-photic stimulation with light?

Mrosovsky: The interaction between non-photic and photic effects will, I predict, become an important research area. Such interactions may be relevant to some of the anatomical complexity in the circadian system. Why isn't the retinohypothalamic tract sufficient to get light information into the SCN and to induce a phase shift? Why do we need all this other stuff? The answer could be that the circadian system, in addition to handling photic phase shifting, is doing several other things, one of which is arranging for interactions between photic and non-photic inputs. Hamsters put into a novel wheel just before being given a sub-saturating light pulse at CT 18 show attenuated phase shifts. That is an example of an interaction between photic and non-photic inputs—one which, incidentally, has possible implications for interpreting the blocking of photic effects with MK801, a substance which has been reported to activate rats, at least. Another type of interaction occurs when a non-photic pulse given at about CT 5 is followed by a photic pulse; the advance normally produced by the non-photic stimulus is virtually abolished. This is not a simple subtractive effect, because in hamsters the phase delay in response to a photic stimulus is about 1.5 h maximum, whereas the non-photically induced advance is around three hours, so a photic stimulus coming after a non-photic stimulus can eliminate at least 1.5 h of the advance (Mrosovsky 1991). There are complicated synergistic actions that may allow us to give 'double whammies' (photic and non-photic) to the system to achieve greater phase shifts.

Arendt: Is there any scope for considering changes in body temperature as a common pathway for a whole variety of agents?

Mrosovsky: There's certainly scope for that, but there is scope for a whole lot of other things that happen when the animal exercises—interleukins, corticosteroids and many other things change. I don't view a fishing expedition as a pleasant way to proceed unless there is hope of catching a big fish. We have done some work on temperature. We can't mimic the effects of running in a novel wheel by passive warming of the animal, but that may not be critical, because there is an argument about whether temperature goes up passively with exercise or rises to meet an elevated set point. Wickland & Turek's (1991) work also suggests that temperature is not a factor.

Turek: We can induce a rise in temperature which is similar to that observed in response to wheel running, but we don't see phase shifts in response to the temperature rise. We can restrain animals after treatment with triazolam and still see a rise in temperature, but no phase shifts are observed (Wickland & Turek 1991).

Menaker: Is it correct that enforced running doesn't produce phase shifts?

Mrosovsky: This is a highly relevant point, relevant also to Dr Block's attempts to induce phase shifts in *Bulla* by putting them in a jacuzzi (p 64). Mistlberger (1991) got only marginal entraining effects with rats forced to run on a treadmill.

Meijer: Forced wheel running does not induce phase shifts in hamsters either (M. J. De Vries & J. H. Meijer, unpublished results).

Menaker: That's an important result, because it eliminates a whole class of possible inputs.

Mrosovsky: If we put the sluggardly hamsters, the ones that don't spontaneously run in our novel wheels, into a cold environment (5–10 °C) they will run, but we do not correspondingly increase the percentage of animals that show a phase shift. In other words, some of these sluggardly animals (as defined by their disinterest in running in our normal warm test conditions) don't shift even after considerable running in the cold wheels. That, we think, is instructive. It's not necessarily the running *per se* which stimulates the shift. The reason for running seems to be important.

Miller: This discussion reminds me of conversations I have had with my colleague Dr Dale Edgar, who has speculated for some time about that bugbear for psychologists, the notion of volition in locomotion. Although this is anathema to many of us, perhaps there is an element here that you would not see in forced locomotion or in cold-induced locomotion. Are there other techniques by which you could increase this volitional running and convert your sluggards into runners? Operant-conditioned locomotion would be one possibility and another would be to reduce the body weight by 20%, which should cause a general increase in locomotion.

Mrosovsky: There are many ways in which one can get a hamster to run, but to isolate the volitional aspect one would need a situation in which the hamster wants to run but does not.

Roenneberg: I'm confused about the need for running. Could it be that it is not easy to make the sluggards nervous, even if you make them run? Is running essential or not?

Mrosovsky: The methodological point is that in normal warm conditions the running is predictive of a phase shift; this allows us to pick animals that we know will respond with shifts for our work with NPY antiserum. But is it the running *per se* which is responsible for the shift? Dr Meijer's work with enforced running and our experiment with running at reduced temperatures tells us that running is not *sufficient*. Once we can say that, we can go on to ask whether it is *necessary* at all, but we don't have the answer to that question yet. The motivational context in which the running occurs is important.

Daan: What happens when you block the running wheel?

Mrosovsky: We have done some experiments with blocked wheels (Honrado & Mrosovsky 1989, Reebs et al 1989). Phase shifting is reduced but by how much depends on whether or not the hamsters have access to their home cages. We don't really need to block the wheel, because we have the sluggards who won't run.

Roenneberg: But they might be a different type of animal. There may be sluggards who wouldn't shift even if you forced them to run, but also runners who would indeed shift if they are nervous in spite of not being allowed to run.

Czeisler: Or are perhaps just thinking about running!

Mrosovsky: Agreed; we should take individuals that we know from previous work will run and put them in a blocked wheel.

Armstrong: I wonder how far these findings will extend to other species. We have all seen articles in newspapers recommending jogging for jet lag on the basis of work in hamsters.

Curt Richter (1967) gave just about every physiological, endocrinological and neurological insult to wild and laboratory rats. For example, he induced prolonged sleep deprivation by forcing rats to swim in a tank for between 19 and 42 hours (see Fig. 22 in Richter 1967). Although these types of treatment often destroyed or interfered with the manifestation of behaviour, the effect was temporary, lasting for only a few days, and the onset of running always reappeared at the expected time, indicating that although locomotor behaviour had been interfered with by the stressor, the biological clock was still keeping time. Nevertheless, if you look carefully at Richter's published data, you can in fact find phase shifts of about an hour. Now, you might interpret this to support what Dr Mrosovsky has been saying, but I would argue that the phase shifts are small given the magnitude of the stress applied to the animal.

In Dr Menaker's laboratory in Oregon, I investigated immobilization stress in C57BL mice and saw exactly the same phenomenon as that reported by Richter for rats. Our mice ($n = 6$) were immobilized for 30 min at the same time every day. In one mouse, although wheel-running behaviour was interfered with for a few days at the time when the onset of the activity rhythm coincided with

the 30 min immobilization, the rhythm reappeared at exactly the time predicted from the pre-immobilization running pattern. In other cases, there was no effect on running at activity onset, but some disruptions at the end of activity (see Fig. 5 in Armstrong & Redman 1985). With Long-Evans hooded rats, we've tried 30 min of immobilization, novelty stress and daily handling (Barrington et al 1993). In some rats you do get effects (entrainment, period changes and phase shifts) but I would underline the fact that the most sensitive time is at subjective dawn, the end of the active period, and not at subjective dusk.

Turek: Was 30 min the maximum time of immobilization?

Armstrong: Yes.

Turek: You need to immobilize hamsters for three hours to get a phase shift.

Armstrong: In terms of corticosteroid release, 20 min is fine in rats. Indeed, after several days of daily stress at the same time, corticosterone will rise in anticipation of the stressor.

Turek: Adrenalectomized hamsters will show phase shifts in response to an activity-inducing stimulus, so there is no reason to invoke the involvement of corticosteroids in this species.

Armstrong: I'm not saying they are necessarily involved in a causal way, but simply a marker of stress. Our rats were certainly stressed daily as indicated by plasma corticosterone concentrations.

Basically, I'm just wondering if there are marked differences between rodents, and the extent to which we can generalize from hamsters to other mammals, including humans. When I started thinking about this I went back to the origin of the golden hamster. As you may know, all the hamsters, *Mesocricetus auratus,* we have in Cambridge, England, in Toronto and in Virginia, USA came from one male and three females many years ago, as the following quotation from Harrison & Bates (1991, p 265–266) shows:

> 'According to Reynolds (1954), this species was first brought back alive to Britain by J. H. Skene, the former Consul General at Aleppo, in 1880. It apparently bred in captivity for 30 years before dying out. In 1930, a female and 12 young were found by I. Aharoni near Aleppo; they were taken to the Hebrew University at Jerusalem. Arahoni (1932) stated that from one male and three females of this stock up to 150 specimens were raised in one year. Apparently, the vast numbers of hamsters now found as captive stock around the world are all derived from the pregnant female found by Arahoni.'

What this says about the genetics I won't go into here. I happened to be talking to an Israeli ecophysiologist, Professor Abraham Haim, and asked what had happened to all the hamsters, whether they had all died out or had been eaten! He told me that as far as he was aware they exist still, but are subterranean and hardly ever come up. A male will come to the surface about once a day, will sniff around and will go back down again unless there's a female in oestrus,

when it will start running around. These are animals which would actually see very little light, in which case non-photic stimuli would be important, especially interaction with conspecifics underground. What I can't make sense of is the elegant work on photoperiodism in this species.

Mrosovsky: Non-photic effects have been demonstrated in scorpions (Hohmann et al 1990), sparrows (Reebs 1989) and mice (Edgar & Dement 1991). It would be convenient if they could be demonstrated in the rat because we know so much about rat physiology. Forcing rats to swim is on the aversive end of the volition–compulsion spectrum. Also, there may be important differences between species in what constitutes an appropriately arousing stimulus for phase shifting. Rats and hamsters differ in terms of what they like to do in a wheel. When you first give a rat the chance to run in a wheel, it runs rather little. It takes two or three weeks before it reaches a reasonable level of running (Cornish & Mrosovsky 1965). When you give a hamster the chance to run in a wheel, the first time it hits the wheel it engages in a prolonged marathonian bout of running that can last for hours. The rat is simply a less 'wheely' animal and we may have to look for other ways of producing non-photic shifts in rats.

Block: The 23 h enforced swimming is problematic because you are integrating over the advance and delay region of the PRC. A shorter bout of swimming might give a good phase shift.

Waterhouse: We've spoken at considerable length about the input to this system. The output, as I understand it, is activity. Is there evidence that other outputs from the clock are changed? Dr Arendt and Dr Lewy have found circumstances under which the activity seems to be adjusted in humans but other rhythms are not.

Mrosovsky: The hamster's phase response to light is shifted by our non-photic manipulation (Mrosovsky 1989). We tried, in collaboration with Dr Lewy, to check whether the melatonin rhythm was affected but we didn't have enough animals to be sure. If non-photic manipulation of circadian rhythms is to be valuable to people, it is important to learn whether or not many different rhythms are phase shifted.

Turek: With triazolam, we have shifted the circadian pre-ovulatory luteinizing hormone surge, a hormonal rhythm which is not dependent on activity, and we also shifted the body temperature rhythm, though I'll admit that the temperature rhythm may be masked or linked to the activity rhythm. So, I think it is clear that an activity-inducing stimulus can affect at least one hormonal and one metabolic rhythm, but I agree, it would be nice to have data on a few more metabolic and/or hormonal rhythms.

Mikkelsen: If we consider the non-photic and photic inputs to the SCN to be anatomically distinct, we also need to discuss whether and where those two inputs meet. So far it looks as if there are two parallel projections. Photic and non-photic stimuli induce *fos* expression in different cells in the IGL. Also, in the SCN the NPY input and the glutamatergic input may innervate two different populations of cells. It has not yet been shown that they go to the same

population of neurons, and there's actually some indirect evidence that that's not the case. The distribution of *fos* expression in the SCN induced by light is different from the distribution of a protein involved in phosphorylation (Mikkelsen & Gustafson 1993), even though both are present in the ventrolateral part of the SCN. This may indicate that there are two populations of tightly coupled neurons in the SCN, one regulated by glutamate (photic) and coupled to c-*fos* expression, the other regulated by neuropeptide Y and/or serotonin (non-photic) and coupled to cyclic AMP.

Miller: There is evidence for co-innervation of vasoactive intestinal peptide (VIP) neurons by the geniculohypothalamic tract, the retinohypothalamic tract and the 5-HT projection from the dorsal and medial raphe. The same recipient cell could have two different signal transduction pathways for different zeitgebers. There's no reason why there necessarily have to be different populations of cells.

Mikkelsen: We have double-stained for Fos and VIP in hamsters and rats exposed to a light pulse at CT 14 and CT 18, and have not found more than about 10% of the VIP neurons containing Fos.

Lewy: Dr Mrosovsky, are responses to dark pulses mediated by increases in activity, and, if so, in what species?

Mrosovsky: I know of two relevant experiments on hamsters, both showing that restricting movement greatly attenuates the phase-shifting effects of dark pulses (Reebs et al 1989, Van Reeth & Turek 1989). Nevertheless, there may be different types of PRC produced by dark pulses. Some investigators (Ellis et al 1982) note in their methods section that they jostle the hamsters' cages in order to wake them up before giving them the dark pulse, to make sure the animals are aware of the dark pulse, whereas other investigators may not do that. Also, the PRCs produced by dark pulses are very variable. Harrington & Rusak (1986) obtained a peak advance at close to CT 12 whereas previously Boulos & Rusak (1982) got a peak 5–6 h earlier. The other work that I should mention is that by Dr Takahashi on the effects of dark pulses on cells in culture; unfortunately, those cells were not running around in their culture dish! There are too many discrepancies in the data from experiments with dark pulses to answer your question with confidence.

Roenneberg: Giving a dark pulse to *Gonyaulax* during the day produces a nice PRC. We discovered only recently that if you put the cells into darkness they cease to aggregate, but when you release them into light, they begin to aggregate again with an enormous density. This raises the question, what does a dark pulse really do? Perhaps in animals active at night activity is induced during a dark pulse, but in organisms active during the day activity might be reduced during the pulse and may come back with a huge surge afterwards. This brings us to the question about the importance of the onset and the end of pulses; which of them is providing the information, or are both doing something additively? In any case, *Gonyaulax*, which is far away from the

hamster, seems to react with increased activity following dark pulses but not following light pulses in background dim light.

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