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Heavy Water Lengthens the Period of Free-Running Rhythms in Lesioned Hamsters Bearing SCN Grafts

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LESAUTER, J. AND R. SILVER. *Heavy water lengthens the period of free-running rhythms in lesioned hamsters bearing SCN grafts.* PHYSIOL BEHAV **54**(3) 599–604, 1993.—Heavy water (D_2O) lengthens the period of free-running circadian rhythms in most organisms. We compared the effect of D_2O on freerunning locomotor activity rhythms in intact and SCN-lesioned (SCN-X) hamsters that had recovered circadian rhythmicity following implantation of SCN grafts. The animals were housed individually in cages equipped with running wheels, and locomotor activity was monitored using a computer-based data acquisition system. At the end of the behavioral tests, animals were anesthetized and perfused. Brain sections were immunostained for vasoactive intestinal peptide (VIP) and vasopressin (VP) to evaluate the extent of the lesion and the presence of a functional graft. The D_2O similarly lengthened the period of free-running activity without affecting amount of activity in both intact and in SCN-X grafted animals. The results indicate that D_2O acts directly on the SCN to lengthen the free-running period, and suggest that coupling between pacemakers within the grafted SCN is as efficient as in the intact SCN.

Heavy water Deuterium oxide Hamsters SCN lesions SCN grafts Circadian rhythms

IT is well established that the suprachiasmatic nuclei (SCN) of the hypothalamus are important for the expression of circadian rhythms in mammals (28,34). Destruction of the SCN abolishes circadian rhythms in behavioral and physiological responses. The SCN have a circadian rhythm of metabolic and electrical activity. Finally, circadian locomotor rhythms are restored by implantation of fetal hypothalamic grafts containing the SCN (1,13,22,35,37), and the restored period is that of the donor rather than that of the host (32).

While circadian rhythms can be sustained following lesions that spare as little as 25% of the paired SCN (12), such lesions may decrease normal coupling between paired SCN. Pickard and Zucker (30) found that stability of the circadian system in response to heavy water (D_2O) is reduced in animals bearing SCN lesions. The effect of D_2O on circadian pacemakers has been widely studied. D_2O has a consistent effect across species in lengthening the period (the interval between activity onsets on successive days) of free-running circadian rhythms. For example, D_2O lengthens the period of activity rhythms of the rat (33), the hamster (10,16), the deer mouse *Peromyscus* (39), and the intertidal isopod *Excirolana* (15); the ERG rhythm of the crayfish (29); the sleep movement rhythm of the bean *Phaseolus* (7); and the phototactic rhythm of *Euglena* (6). The period of circadian activity increases linearly with the concentration of D_2O (10,14,39).

In the present experiment, the effect of D_2O was tested on SCN-lesioned (SCN-X) adult hamsters that had recovered circadian locomotor rhythmicity following grafting of fetal donor SCN tissue. In this model, connections between the host brain and the SCN graft are very limited. Only partial reinnervation of the SCN by host 5-HT fibers was seen following grafting of fetal cell suspensions containing mesencephalic raphe neurons (11). There was no apparent localization of reinnervating fibers to any particular part of the nucleus, thus differing from normal 5-HT innervation of the SCN. This was in marked contrast to 5-HT hyperinnervation observed in neighboring areas such as supraoptic nucleus, a region that normally receives few 5-HT fibers. Similarly, only sparse label was seen in a few functional SCN grafts following retrograde transport of fluorescent microspheres injected into host PVN, PVT, or septum (5). In SCN grafts that restore rhythmicity, NPY fibers cross the host–graft border, and in some but not all instances, they reach the area of the SCN within the graft (18,24,32); whether these fibers establish functional connections remains to be determined.

Afferent connections to the SCN from retinal ganglion cells are also markedly reduced or absent. Though a few instances of entrainment to bright light have been reported [unpublished data of Aguilar et al. and Bittman et al., cited in (24)], SCN-lesioned animals bearing fetal hypothalamic grafts generally do not entrain to a photic signal of 700 lux (5,25,35). This may be attributable to the fact that retinal afferents to the grafted SCN occur only when the attachment site of the graft lies in the floor of the third ventricle, above the optic chiasm. Furthermore, even in instances where retinofugal fibers have been demonstrated, animals are unable to entrain (25), suggesting that retinofugal fibers may not reach appropriate targets within the SCN.

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Neural connections from the graft to the host are reduced in SCN-lesioned, grafted animals. When SCN grafts containing vasopressin are implanted into vasopressin-deficient Brattleboro rats (42), sparse vasopressin fibers are seen entering the host brain. When fetal tissue grafts rich in VIP cells, such as bed nucleus of the stria terminalis, medial amygdaloid nucleus, and SCN, were implanted in the lateral ventricle of Brattleboro rats (2), both stria terminalis and amygdaloid nucleus grafts densely innervated the lateral septum, whereas the SCN graft made very few connections. In SCN grafts that restore locomotor activity rhythms, a few VIP fibers have been seen crossing the graft–host border, particularly where the ependymal cells of the ventricle wall are absent (25); however, in other functional grafts, VIP fibers from SCN clusters remain clearly limited to the graft (22,25). Most VIP, CCK, and NP fibers project extensively within the graft itself rather than into the host brain. The presence of neural efferents from the functional graft into the host brain is not sufficient to establish that such connections are necessary for restoration of locomotor rhythms.

The hypothesis underlying the present experiment is that pharmacological agents that act directly on the pacemaker cells of the SCN should produce similar effects on the period and/or phase of circadian rhythms in intact and in SCN-X grafted animals. Agents that act on extra-SCN sites may have no effect in SCN-X grafted subjects (8). A second issue addressed here concerns the restorative effects of SCN grafts on free-running circadian rhythms. If coupling among pacemakers within the grafted SCN resembles that of the intact SCN, then the change in period in response to D_2O treatment should not be more variable in recovered SCN-X grafted animals than in intact animals. If, on the other hand, the stability of the circadian system is disturbed in SCN-X grafted animals, one might predict an increase in variability in response to D_2O, as has been reported in SCN-X hamsters (30).

METHOD

Subjects

Adult male LVG hamsters (*Mesocricetus auratus*) were housed individually in translucent propylene cages (48 × 27 × 20 cm) equipped with running wheels (17 cm diameter) for 4–6 weeks prior to the start of the experiment. The room was kept at about 23°C and maintained in constant darkness (DD); a dim red light (<1 lux; Kodak filter No. 2, 7.5 W bulb) allowed for animal maintenance. The room was equipped with a white noise generator (91 dB spl) to mask environmental sounds. Animals were provided with running wheels in their cages and had ad lib access to food. Tap water, or 10% D_2O in tap water, was also available ad lib.

Procedure

Experimental animals were SCN-lesioned hamsters implanted with fetal SCN grafts ($n = 8$), and controls were intact hamsters ($n = 7$). The activity of intact hamsters was monitored for 2–8 weeks prior to the start of the experiment. After the lesion, the activity of experimental animals was monitored for 6 weeks prior to implantation of anterior hypothalamic tissue containing the SCN.

To make the lesion, and for the subsequent graft implantation, the hamsters were anesthetized with pentobarbitol (100 mg/kg) and placed in a stereotaxic instrument. Bilateral electrolytic lesions of the SCN were made using a Grass LM-5 lesion maker using stainless steel #00 insect pin electrodes, insulated except at the tip (0.25 mm) with epoxylite. Current was passed for 20 s, at 0.55 mA. The SCN lesions were placed 0.8 mm anterior to bregma, 0.1 mm lateral to the midline, and 7.9 mm below the dura.

After 6 weeks, pregnant female hamsters (gestation day 15, with day 1 representing the first day after mating) were sacrificed with an overdose of pentobarbitol. The pups were quickly removed, their brains were dissected out and placed on a sterile petri dish. Under aseptic conditions, the fetal tissue was microdissected from the ventral surface of the hypothalamus with coronal cuts just rostral and caudal to the optic chiasm, and lateral cuts equidistant from the midline. Embryonic segments from two donors were pooled into a drop of sterile saline in a sterile petrie dish. The grafts were implanted via a modified 18-ga needle that was lowered 7.6 mm below dura, using the opening in the skull made during the lesion. After recovery from anesthesia, the animals were returned to their wheels.

The D_2O administration began 6–10 weeks after graft implantation, when animals had recovered sustained free-running, wheel-running rhythms. In administering D_2O, the investigator was blind as to which hamsters were intact or SCN-X grafted.

Histology and Immunocytochemistry

At the end of the sequence of behavioral tests, histological and immunocytochemical tests were performed to verify the completeness of the lesion and the presence of a functional graft in the SCN-grafted animals (see Fig. 1). The hamsters were deeply anesthetized (Nembutal 75 mg/kg) and perfused intracardially with a brief wash of 0.9% saline followed by 300–400 ml of 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.3. Brains were postfixed for 12–18 h at 4°C, and coronal sections (50 μm) were cut on a vibratome and subsequently processed as free-floating sections for immunocytochemistry. Polyclonal antisera to VIP 1:10,000, and NP 1:10,000 (Incstar) were used with a modified avidin–biotin–HRP procedure (Vectastain Elite Kit, Vector Laboratories). The NP antibody recognizes VP within the SCN. HRP was demonstrated using diaminobenzidine (DAB) as the chromogen. Every fourth section was processed for Nissl staining using cresyl violet (ChromoGesselshaft) to determine the borders of the grafts, and the presence of necrotic tissue at lesion site. An SCN lesion was judged as complete if no VP or VIP fiber was present in host tissue at the site of the host SCN. The presence of the SCN in the graft was defined by the characteristic nonoverlapping distribution of VP and VIP fiber plexi (9).

Data Collection and Analysis

Locomotor activity was monitored using a computer-based data acquisition system (DataQuest, Datasciences, St. Paul, MN). The period was calculated using the TAU program (Mini-Mitter, Sunriver, OR), which uses the chi-square periodogram method (36) to give the period of activity onset. The period was calculated on three 7-day intervals, before, during, and after the end of D_2O treatment.

RESULTS

Histological Results

The histological analysis of SCN-X grafted animals is shown in Table 1. Five of eight animals had complete lesions with no detectable VIP or VP fibers or cells remaining in the area of the host SCN [Fig. 1(B), #15-31]. In the other three animals, immunostaining was seen in the region of the host SCN. In one of the animals with spared host SCN (#55-247), the lesion was 99% complete with two to three VIP and VP fibers and few VIP cells detectable in the host animal. In a second such hamster, the

TABLE 1

SUMMARY OF TAU BEFORE, DURING AND AFTER D_2O TREATMENT

Group	Animal	Before	During	After	Lesion
Intact	#3-30	24.05	24.45	23.94	—
	#9-03	24.05	24.32	24.07	—
	#20-26	23.96	24.39	24.05	—
	#23-04	24.06	24.26	23.98	—
	#24-05	23.97	24.27	23.88	—
	#28-25	24.13	24.45	24.07	—
	#33-23	24.55	24.73	24.35	—
Mean		24.11	24.41	24.05	
SE		0.08	0.06	0.06	
Grafted	#7-32	23.60	24.89	24.20	complete
	#8-33	23.53	24.45	24.30	complete
	#15-31	24.13	24.87	23.98	complete
	#16-47	24.24	24.65	24.20	complete
	#17-48	24.05	24.98	24.95	complete (large)
	#55-247	23.80	24.94	24.11	99%
	#18-57	23.90	24.62	23.95	90%
	#14-40	24.03	24.54	24.26	75%
Mean		23.91	24.743	24.244	
SE		0.09	0.07	0.11	

lesion was 90% complete (#18-57). In the third animal, 75% of the SCN was destroyed (#14-40).

All experimental animals had robust SCN grafts, determined by the presence of clusters of fibers and cells immunostained for VP and VIP [Fig. 1(B)]. In all the SCN-X grafted animals, grafts were located in the third ventricle, and portions of the graft appeared to merge with the host brain. A few fibers could be detected crossing the graft–host border for short distances (100–200 μm).

Behavioral Results

The average amount of deuterated water intake was 18.57 ml/day for the intact animals, and 24.60 ml/day for the grafted animals.

As summarized in Table 1, D_2O increased the period of the free-running rhythm in every individual animal in this study, and following termination of D_2O treatment, the period shortened in every animal, $F(2, 26) = 42.39$, $p = 0.0001$. In intact animals, the mean of the free-running period was 24.11 prior to D_2O treatment, increased to 24.41 during D_2O treatment, and decreased to 24.05 after D_2O withdrawal. A representative intact animal (#28-25) is shown in Fig. 2(A). A similar period-lengthening effect was seen in SCN-X grafted animals, with free-running periods of 23.91, 24.74, and 24.24 before, during, and after treatment. A representative animal (#17-48) from this group is shown in Fig. 2(B). The magnitude of the D_2O effect on period was different in the intact and grafted groups, with a change from baseline of 0.30 + 0.04 h in the former and 0.83 + 11 in the latter, $t(13) = 4.51$, $p > 0.001$. There was no difference in variability of change in period in intact vs. SCN-X grafted hamsters.

The latency of the response to D_2O presentation and removal was similar for both intact and SCN-X grafted animals. Lengthening of period occured 3–4 days after D_2O presentation, and it took 3–4 days for the period to restabilize at predeuteration levels after D_2O removal.

There did not seem to be an association between the size of the host lesion and the magnitude of the change in period (Table 1). In the animal with damage to only 75% of the SCN, the D_2O-induced change in period was 0.51, while the animal with damage to 99% of the SCN had a change of 1.14, somewhat above the group mean. Among the animals with complete lesions, there was no correlation between lesion size and the change in period produced by D_2O ($r = 0.69$, $p = 0.19$). Administration of D_2O produced a change in the period of 0.93 in the animal with the largest hypothalamic lesion, with total destruction of the SCN of the host.

A significant decrease in overall amount of activity was seen in SCN-X grafted animals compared to intact controls, $F(1, 13) = 21.58$, $p = 0.0005$. Nevertheless, there was no significant effect of D_2O intake on amount of activity, $F(2, 26) = 1.80$, $p = 0.18$, Fig. 3).

DISCUSSION

D_2O increased the period of the free-running rhythm in all SCN-X grafted and intact animals, and had no effect on amount of activity in either group. The latency to respond to D_2O administration was similar in both groups. Since the grafted SCN has reduced functional afferents and efferents from and to the host brain (see the Introduction), these results indicate that D_2O acts directly on the SCN pacemaker cells within the graft. We have previously shown that triazolam phase shifts intact but not SCN-X grafted hamsters that recovered locomotor rhythmicity (8), indicating that triazolam does not act directly on the grafted SCN. On the other hand, we have shown that lithium lengthens the free-running period in a similar way in intact and SCN-X grafted animals (26), indicating that lithium, like D_2O, acts directly on the SCN to modulate circadian rhythms.

It is clear, nevertheless, that D_2O has widespread effects (21), acting on both SCN and extra-SCN sites. For example, D_2O inhibits angiotensin II-induced drinking in sheep (19). D_2O has also been shown to act on rats' vascular smooth muscle (27),

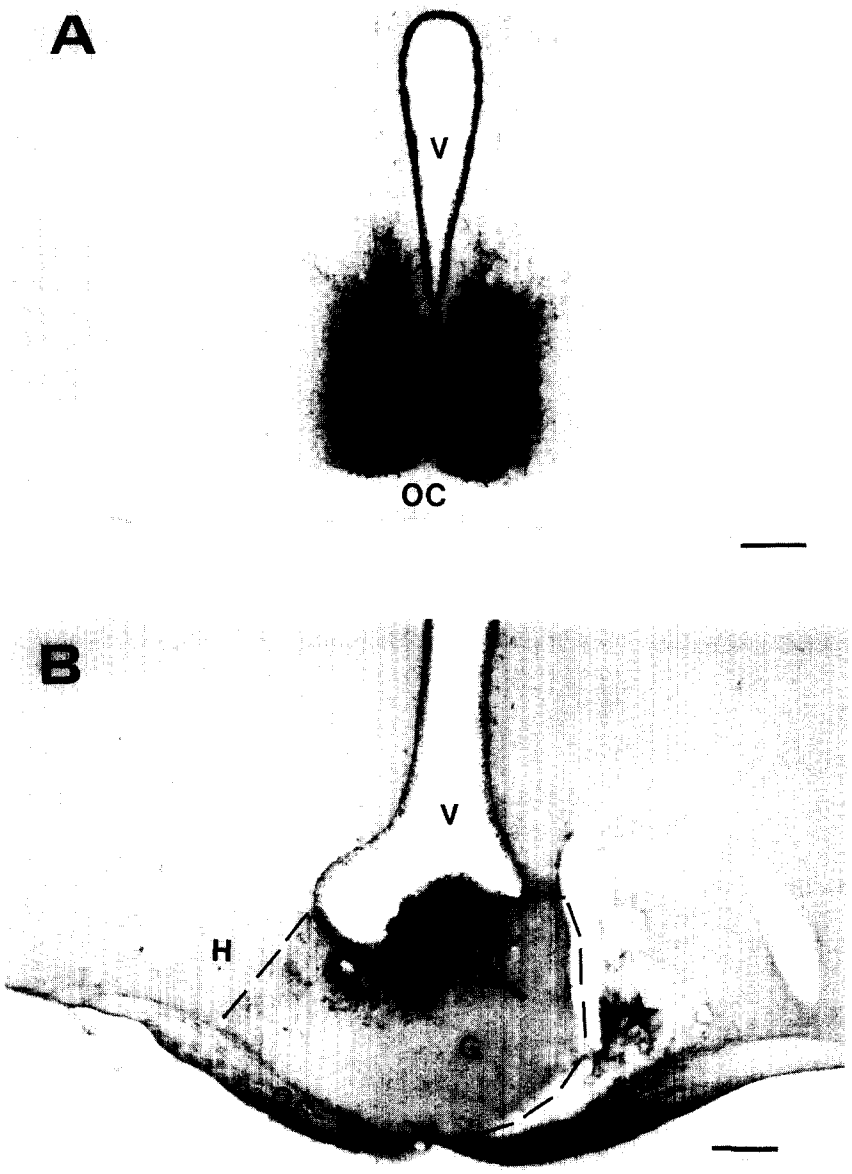

FIG. 1. Photomicrographs of coronal brain sections. (A) SCN of an intact animal immunostained for VIP. (B) Lesion site and graft of a complete SCN-X animal (#15-31) immunostained for VIP. No VIP fibers are detectable within the host brain. A cluster of VIP fibers (arrow) can be seen within the graft, indicating the presence of SCN in the graft. The star indicates necrotic tissue at lesion site. The borders of the grafts are indicated by dashed lines. OC, optic chiasm; V, third ventricle; H, host brain; G, graft. Bars, 200 μm.

on guinea pig myocytes (31), on the barnacle's muscle fibers (20), on animal cell membranes (23), and on cell division (17).

While it has multiple effects on chemical and physical reactions (41), the mechanism of action of D_2O is thought to be due mainly to the exchange of hydrogen by deuterium at specific sites. The deuterium bond is more stable than the hydrogen bond, and requires more activation energy (4), thereby slowing down biological and biochemical reactions (20).

The latency of D_2O effect on period after its presentation, or after its removal, was three to four cycles in both intact and in SCN-X grafted animals. This indicates that D_2O accesses both the intact and the grafted SCN with the same latency. The 3–4-day delay may be due to progressive increases in D_2O concentration over this period. D_2O might access the SCN immediately, but the replacement of hydrogen bonds by deuterium bonds, and vice versa at the end of treatment, might occur slowly. However, this process is thought to occur over several hours, not days (3). Another possibility is that D_2O exerts its biochemical effects quickly, and the behavioral expression of the clock's new period takes a few days (40).

The magnitude of the change in period was greater in SCN-X grafted animals than in intact animals. It is not likely that this is due to a lesion effect in experimental animals, as Pickard and Zucker (30) showed that the response to deuteration was

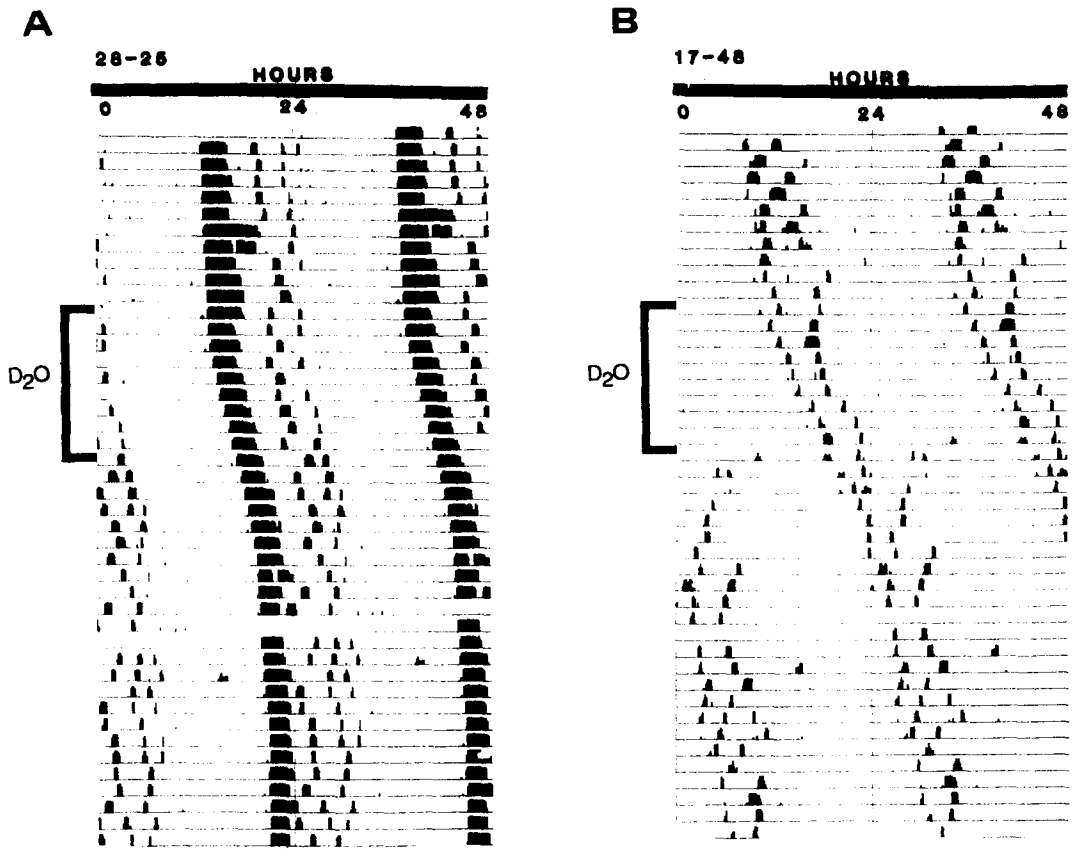

FIG. 2. (A) Double-plotted locomotor activity rhythm of a representative intact animal (#28-25). A lengthening in period is seen starting 3–4 days after the begining of D_2O treatment. The period was 24.13, 24.45, and 24.07 h before, during, and after D_2O treatment. (B) Double-plotted locomotor activity rhythm of representative SCN-X grafted animal (#17-48). In this animal, the effect of D_2O seems immediate, although in most grafted animals, a change in period was seen after 3–4 days. The period was 23.90, 24.62, and 23.95 h before, during, and after D_2O treatment.

independent of baseline period, or of the extent of damage to the SCN. It is likely that differences between groups can be attributed to the greater consumption of D_20 by the SCN-X grafted animals (2.46 ml/day SCN-X grafted vs. 1.86 ml/day intact). While we cannot account for the increased intake of the SCN-X hamsters, a linear relationship between D_20 intake and magnitude of the induced period change is well established (10,14,39).

The presence of remnant SCN tissue in the lesioned host did not influence any parameter of the response to D_20 (Table 1). Additionally, there was no apparent influence of the size of SCN grafts within the third ventricle on the response to D_2O. This is consistent with previous reports (30) that neither coupling of the bilaterally symmetrical SCN nor lesions of up to 50% of the SCN alters the period-lengthening effects of D_2O, and suggests substantial redundancy in the neural system that responds to this agent.

The effects observed here on period of the free-running rhythms, on latency to respond to D_2O administration, and on amount of activity are consistent with previous results on intact (16) and partially lesioned hamsters (30). On the other hand, Daan and Pittendrigh (10) found a longer latency to return to pre-D_2O period after removal of 20% D_2O in the deer mouse. This could be a dose effect, or species difference.

It is noteworthy that in the study of Pickard and Zucker (30), the response to D_2O was more variable in animals sustaining SCN lesions than in intacts. They concluded that a subpopulation of the normal complement of SCN neurons is sufficient to permit full responsiveness of the circadian system to D_2O and that the full complement of SCN neurons enhances the stability of the system. While it is true that the intact animals are more precise in their activity onset than are the SCN-X grafted animals

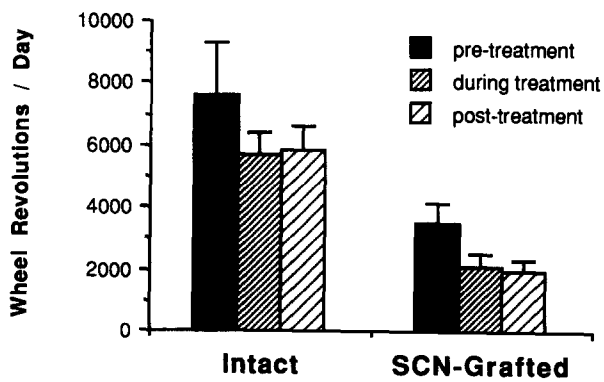

FIG. 3. The average daily amount of wheel running of intact and SCN-X grafted animals before, during, and after D_2O treatment. The SCN-X grafted animals had much lower activity level than intact animals, but there was no effect of D_20 intake on amount of activity.

(25,35), and that the lesioned grafted animals have a reduced activity level, probably due to the hypothalamic lesion (38), there was no apparent increase in variability in response to D_2O in the latter animals. This provides further evidence of the power of the restorative effects of the SCN grafting procedure on the organization of the circadian system.

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