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# DYE COUPLING IN HYPOTHALAMIC SLICES: DEPENDENCE ON *IN VIVO* HYDRATION STATE AND OSMOLALITY OF INCUBATION MEDIUM[1]

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**Abstract**

Electrotonic coupling is one mechanism which may coordinate the electrophysiological activity of a population of neurons. By measuring the incidence of dye coupling, we have investigated whether conditions that stimulate hormone secretion by hypothalamic magnocellular neuroendocrine cells affect coupling between these neurons. Neurons in the magnocellular regions of the paraventricular nucleus (PVN), in slices prepared from normally hydrated or chronically dehydrated male rats, were intracellularly injected with the fluorescent dye Lucifer Yellow CH. The dye coupling index (DCI), the ratio of the number of dye-coupled neurons to the total number of filled cells, was determined for each treatment group. The DCI for slices from dehydrated animals incubated in 310 milliosmoles/kg of medium (0.121) was significantly lower than that for slices for hydrated animals incubated in medium of the same osmolality (0.333). This decrease was reversed when slices from dehydrates were incubated in medium having an osmolality of 340 milliosmoles/kg (DCI = 0.307). There was also evidence for an interaction between slices incubated in the same chamber: the DCI in slices from dehydrated animals was significantly higher (0.475) when slices from normally hydrated rats were also present in the incubation chamber. Based on these data and on cited evidence, we suggest that the osmolality of the extracellular fluid and the local concentration of sex steroid hormones may influence dye coupling in the PVN.

Most magnocellular neuroendocrine cells (MNCs) of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus project to the posterior lobe of the pituitary and secrete either vasopressin or oxytocin. In the rat, these neurons normally fire in a slow, irregular manner. However, during conditions of increased hormone demand, the neurons adopt one of two characteristic firing patterns. In lactating animals, oxytocinergic neurons have been characterized as continuously firing with a superimposed, high frequency burst of action potentials preceding each milk ejection (Wakerley and Lincoln, 1973). Vasopressinergic neurons have been characterized as phasically firing after the plasma osmolality was increased (Brimble and Dyball, 1977) or hypovolemia was induced (Poulain et al., 1977). Coordination of neurons of each MNC type appears to be important to produce the rapid changes of hormone concentration in the plasma which occur during increased hormone demand. This coordination could be achieved by several mechanisms: synaptic input from intra- or extranuclear sources, local changes of extracellular ionic concentrations, local electrical field (ephaptic) effects, and electrotonic coupling between MNCs (see Dudek et al., 1983).

Electrotonic coupling of mammalian central neurons is extremely difficult to show directly: except between cultured neurons, the only such demonstration has been in the hippocampal slice (MacVicar and Dudek, 1981). However, two phenomena have been considered as indirect, physiological/anatomical demonstrations of electrontrotonic coupling (Bennett and Goodenough, 1978; Dudek et al., 1983): dye coupling, that is, the intercellular movement of a low molecular weight, intracellularly injected dye, and the presence of gap junctions between neurons. In the PVN and SON, dye-coupled magnocellular neurons have been observed after injection of the fluorescent dye Lucifer Yellow CH (LY) (Stewart, 1978) into cells in the hypothalamic slice preparation (Andrew et al., 1981; Mason, 1983), and gap junctions, although rarely seen in ultrathin sections, have been shown in freeze-fractured material (Andrew et al., 1981). These observations indicated that electrotonic coupling may occur between hypothalamic presumed MNCs under basal conditions but gave no indication of the physiological mechanisms which influence coupling in the two nuclei.

This paper describes the results obtained in an investigation of the relationship between the incidence of coupling in the PVN and hormone demand, using chronic dehydration as the *in vivo* stimulus to increase demand. We have observed that the incidence of dye coupling between MNCs in hypothalamic slices is dependent on the previous *in vivo* hydration state and on the osmolality of the incubation medium. We have also seen that slices from normally hydrated rats unexpectedly influenced the amount of coupling in slices from dehydrated rats when these two types of slices were incubated together. Prelim-

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inary reports of the data have been published (Cobbett and Hatton, 1983; Hatton and Cobbett, 1983).

## Materials and Methods

Male rats (Holtzman strain), aged 35 to 95 days, were housed in group cages in a 12-hr light/12-hr dark cycle. All experiments were done during the light phase of the cycle. Dry laboratory food was available *ad libitum*. For the 8 days immediately before decapitation, rats were given either water (control) or 2% NaCl solution (dehydrate) to drink *ad libitum*; until this time all animals had water.

Coronal hypothalamic slices containing the PVN were prepared and incubated in a recording chamber as described previously (Hatton et al., 1980). Briefly, the brain was removed after decapitation and a tissue block containing the hypothalamus was prepared. This block was cut into 500-μm-thick slices with a tissue chopper. Those slices containing PVN were transferred to the recording chamber where they were maintained at a medium-oxygenated atmosphere interface at 34°C. A "static" chamber was used in all experiments: that is, the medium in the chamber was unchanged throughout each experiment. Slices were always placed in the chamber (containing ~2 ml of medium) with their rostral surface up. In some experiments, slices from control and dehydrate rats were incubated separately; in others, slices from these two treatment groups were incubated together.

Two different media were used, both adapted from that of Yamamoto (1972). The first, designated 310 medium, had an osmolality of 310 milliosmoles/kg. The second, designated 340 medium and made by increasing the concentration of all solutes in the 310 medium by the same percentage, had an osmolality of 340 milliosmoles/kg. Since glucose (~10 milliosmoles/kg in these solutions) is a minimally active osmotic solute on brain tissue, these media had effective osmolalities similar to that of plasma from normally hydrated (310 medium) and from dehydrated (340 medium) rats (Jones and Pickering, 1969).

Slices were incubated for at least 3 hr before electrophysiological recordings were made. Recordings and injections were made for up to 9 hr after slice preparation. Conventional intracellular recording techniques were used. Microelectrodes were filled with 5% LY (Sigma Chemical Co.) in 0.25 M lithium acetate and had resistances of 50 to 400 megohms when filled with this solution. Electrodes were positioned visually on the slice surface in the magnocellular areas of the PVN. Dye was injected using pulsed or continuous negative current (<1.0 nA) for periods between 90 sec and 23 min during which the amplitudes of the resting and action potentials were monitored. To prevent spurious staining of cells and incorrect observation of dye coupling, several precautions were taken: (*a*) neurons were penetrated only by passing brief positive current pulses through the recording electrode (negative pulses or overcompensation of the negative capacitance feedback of the preamplifier were never used, as these procedures would have ejected into the extracellular space dye which might have been taken up by damaged neurons close to the electrode tip); (*b*) impalement of a neuron was always terminated if the amplitude of action potentials decreased to 40 mV because continued dye "injection" after further deterioration of the impalement and/or viability of the impaled neuron might rather have caused dye ejection into the extracellular space; and (*c*) only one injection was made on each side of a slice even if it lasted less than 90 sec and, as such, was omitted from this study.

Slices were fixed in paraformaldehyde (4% in 0.1 M sodium cacodylate, pH 7.25, for 2 hr at 5°C). After being rinsed and stored for up to 5 days in 0.05 M Tris-buffered 0.15 M NaCl solution, slices were completely dehydrated in ethanol, and were cleared and mounted on glass slides in methyl salicylate. Cleared slices were examined using epifluorescence microscopy.

## Results

Resting potentials recorded from neurons in this study were −40 to −75 mV. The amplitude of the overshooting action potentials recorded immediately after penetration of a neuron, and for up to 23 min afterwards, was 50 to 90 mV (see Fig. 1*A* for an example); input resistances were in the range of 50 to 300 megohms. Recordings were made from slowly and irregularly firing cells, from fast firing (≥4 Hz) and phasically firing neurons, and also from neurons which were normally silent but which could be made to fire by intracellular injection of depolarizing current. Since these properties are similar to those recorded previously from magnocellular PVN neurons using electrodes which did not contain LY (Andrew et al., 1981), it appeared that LY affected the quality of recordings made in this study minimally, if at all.

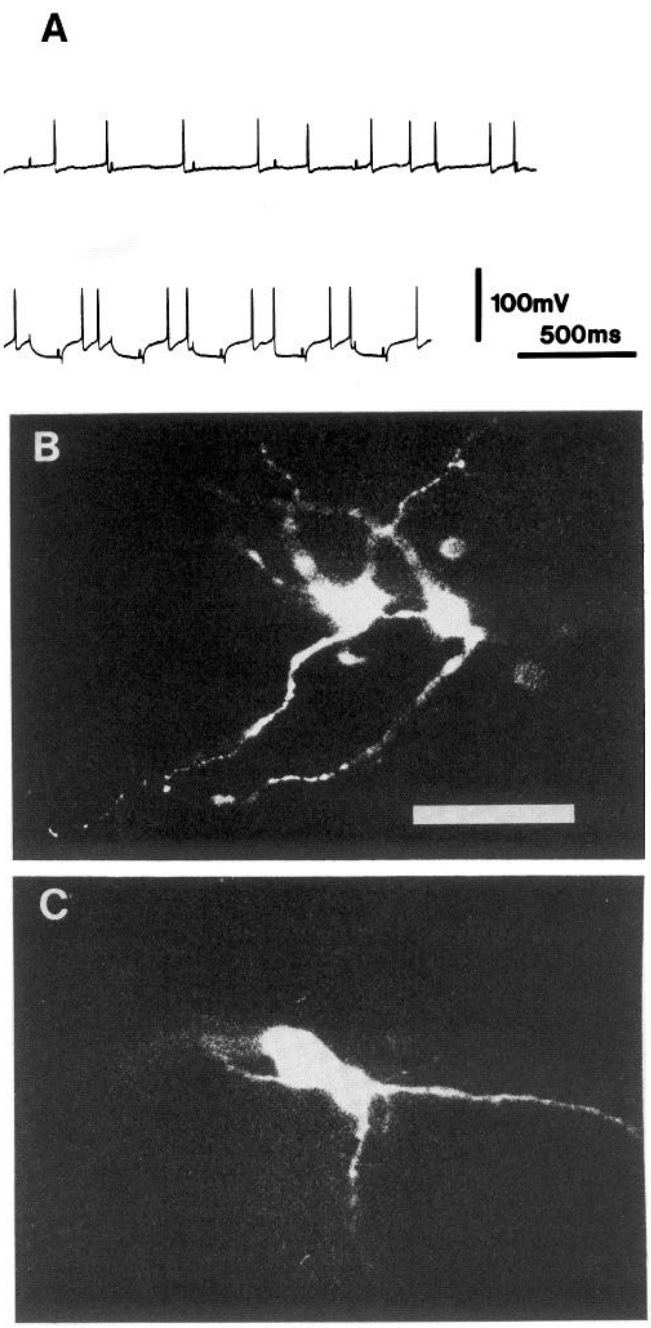


*Figure 1.* *A*, Spontaneous action potentials recorded 30 sec after impalement of a neuron in the paraventricular nucleus of a hypothalamic slice (*upper trace*). Evoked action potentials recorded from the same neuron 11 min after impalement (*lower trace*) were evoked by intracellularly injected current pulses. (The small depolarizations in each *trace* are 10-mV, 5-msec calibration pulses.) During this impalement the resting potential increased LY was injected into the neuron. *B*, Subsequent examination of the slice revealed two dye-filled neurons. The somata of these two magnocellular neurons are clearly separate, indicating that dye transfer had taken place through dendrosomatic or dendrodendritic junctions. *C*, The somata of two other magnocellular neurons, which were filled after a single injection, were closely apposed. In this case, dye transfer was probably through somatosomatic junctions. The *bar* in *B* represents 100 μm for *B* and *C*.

Examination of the dye-filled cells revealed that all were within the borders of the PVN and were magnocellular. That is, the neurons were in the magnocellular areas of the PVN and had a somatic diameter of 15 to 35 μm (Armstrong et al., 1980). All injections produced one or more dye-filled neurons, and dye-filled glial cells were never observed. Most injections produced a single dye-filled neuron. When more than one cell was filled with dye after a single injection, two neurons were seen in all cases except two: one injection filled three neurons, and the other filled four neurons. There was no obvious relationship between injection duration or current intensity and the incidence of dye coupling; for instance, the incidence of coupling did not increase with injection duration.

If the somata of the coupled neurons were apparently closely apposed when observed in a whole slice, coupling was designated as somasomatic. The apparent close apposition between the somata of the coupled neurons in these cases was also seen in 5-μm sections prepared from these slices and examined using epifluorescence microscopy. Coupling was designated as nonsomasomatic if the somata of the coupled neurons were clearly separate: further separation into dendrosomatic and dendrodendritic subgroups was not made. Examples of dye-coupled magnocellular neurons are shown in Figure 1, *B* and *C*.

A total of 116 intracellular injections of neurons with LY were made in 84 slices. Treatment groups, numbers of injections for each treatment group in each incubation condition, and the numbers of single and coupled neurons which were found to be dye filled following these injections are shown in Table I. The dye coupling index (DCI) was calculated as the ratio of the total number of coupled dye-filled neurons to the total number of dye-filled neurons. This index provides a more complete analysis of coupling than one based only upon instances of coupling, since the latter conveys no information on the number of dye-filled cells when coupling occurs. The percentage of somasomatically coupled magnocellular neurons for each group was calculated as the ratio of the number of somatosomatically coupled neurons to the total number of coupled cells. The percentage of non-somasomatically coupled magnocellular neurons was calculated in a similar manner.

*Incidence and mode of dye coupling in slices incubated separately.* The incidence and mode of dye coupling between magnocellular neurons were dependent on the *in vivo* hydration state and on the osmolality of the incubation medium (Fig. 2).

Dehydration *in vivo* and subsequent incubation of slices from these dehydrates in 310 medium produced a significantly smaller DCI (0.121) compared to that of controls in 310 medium (0.333; $\chi^2 = 4.6$, $p < 0.05$). When *in vivo* dehydration was "prolonged" by incubation of dehydrate slices in 340 medium, the DCI (0.307) was similar to that of control slices, but not significantly different from that of dehydrate slices in 310 medium ($\chi^2 = 3.1$, $p < 0.1$). However, the composite DCI for slices that were in *in vitro* osmotic environments similar to those in which the tissue had been *in vivo* (i.e., data from controls in 310 medium and dehydrates in 340 medium added together; DCI = 0.323) was significantly different from that for slices for which the *in vivo* and *in vitro* conditions were dissimilar (i.e., dehydrates in 310 medium; $\chi^2 = 4.0$, $p < 0.05$).

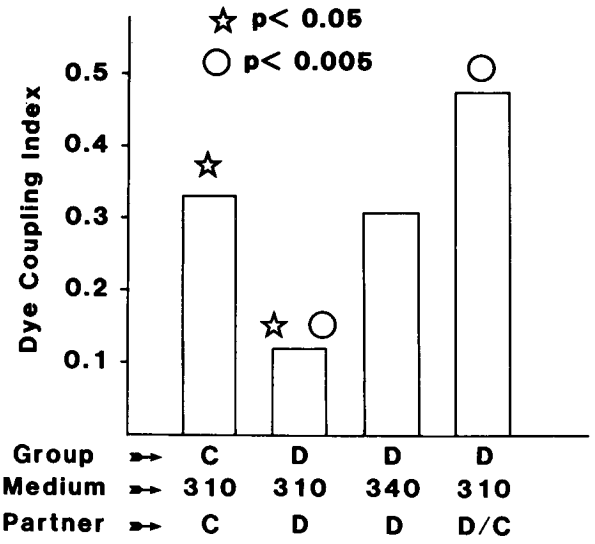


*Figure 2.* The DCI for injected neurons in the four treatment groups. Neurons were injected in slices prepared from control (*C*) or dehydrated (*D*) animals (*Group*) and incubated in medium (*Medium*) having an osmolality of 310 milliosmoles/kg (*310*) or 340 milliosmoles/kg (*340*). Slices from controls were incubated only with slices (*Partner*) from controls, but slices from dehydrates were incubated either only with slices from other dehydrates or with slices from dehydrates and controls (*D/C*).

Analysis of the mode of coupling between neurons, i.e., somasomatic or nonsomasomatic, revealed that the coupling was predominantly somasomatic in slices from control animals incubated in 310 medium (71%) and in slices from dehydrates in 340 medium (75%). This was not the case in slices from dehydrates incubated in 310 medium: equal amounts of each type of coupling were seen. Since the population of coupled neurons in each group was small, these differences were not statistically different.

*Incidence and mode of dye coupling in slices incubated together.* Slices from dehydrate and control animals were incubated together in a separate set of experiments as described under "Materials and Methods." The DCI for dehydrate slices was higher (0.475) when control slices were placed in the same chamber than when slices from control animals were absent (0.121; Fig. 2); this increase was statistically significant ($\chi^2 = 10.5$, $p < 0.005$). However, there was no effect on the DCI of control slices in the same conditions. Concurrent with the DCI change in the dehydrate slices, there was reversion to the predominance of somasomatic coupling (77%) as in control slices.

TABLE I

*LY injections of magnocellular neurons in the in vitro PVN*

The table shows for each treatment group the *in vivo* hydration state of the animals (C, control; D, dehydrate), the osmolality of the incubation medium (310, 310 milliosmoles/kg; 340, 340 milliosmoles/kg), and the hydration state of other animals from which other slices in the chamber were prepared (C, control; D, dehydrate; D/C, dehydrate and control). For each treatment group the number of slices used, the number of injections made, the number of single and coupled cells, and the DCI are given.

| Hydration state | C | D | D | D |
|---|---|---|---|---|
| Medium | 310 | 310 | 340 | 310 |
| Incubation partner | C | D | D | D/C |
| Slices | 25 | 23 | 19 | 16 |
| Injections | 35 | 31 | 22 | 30 |
| Single cells | 28 | 29 | 18 | 21 |
| Coupled cells | 14 | 4 | 8 | 19 |
| DCI | 0.333 | 0.121 | 0.308 | 0.475 |

## Discussion

The large number of LY injections made in this study has allowed a more detailed analysis of the incidence and mode of coupling between magnocellular neurons of the hypothalamus *in vitro* than was possible previously (Andrew et al., 1981; Mason, 1983). In another study, all except one of more than 50 dye-filled and, in some instances, dye coupled magnocellular

neurons (in slices from males and females) were characterized immunocytochemically as neuroendocrine (Cobbett et al., 1982; Smithson et al., 1984). We therefore consider the magnocellular neurons observed in this study also to be neuroendocrine; that is, the dye-filled neurons are MNCs.

Of particularly importance was the finding that DCIs of some animal treatment groups were significantly different statistically. This is, we believe, the first study in which a physiological stimulus applied *in vivo* and/or *in vitro*, in this case dehydration, has been demonstrated to affect dye coupling between neurons. Recently, it has been reported that a neuron and a glial cell may be simultaneously impaled with a single electrode, and it was suggested that dye coupling could result from the simultaneous injection of two cells (Alger et al., 1983). Our data show that the amount of coupling between MNCs was dependent upon the treatment group from which slices were prepared and on the origin of other slices in the incubation chamber. In addition, we never observed a dye-filled glial cell after injection of a neuron. These data, taken together with the precautions taken in this study to avoid inadvertent staining of cells, and the fact that we never observed two or more dye-filled neurons that were not in "contact" when a single cell was injected, argue strongly against the notion that the dye coupling that we observed is an artifact.

The overall effect of dehydration was seen by comparison of control slices in 310 medium and slices from dehydrates in 340 medium. This showed that the incidence and percentage of somasomatic coupling were not changed, a somewhat surprising result since ultrastructural changes during dehydration have been described previously for the PVN (Gregory et al., 1980) and, to a much greater extent, for the SON (Tweedle and Hatton, 1976, 1977). In those studies, the amount of somasomatic, direct membrane apposition between MNCs was significantly increased during dehydration, presumably by retraction of interneuronal glial processes. The ultrastructural data suggested that the amount of somatic membrane available for the formation of gap junctions would therefore be increased during dehydration and, thus, an increased incidence of coupling would be expected. It is possible that ultrastructural changes occurring *in vivo* were reversed *in vitro* despite the high osmolality of the incubation medium. We do not believe this to be the case, however, since the effects in *in vivo* dehydration on the ultrastructure of the neural lobe of the pituitary can also be produced *in vitro* when the osmolality of the incubation medium is high (340 milliosmoles/kg; Perlmutter et al., 1984). The discrepancy between the increased amount of membrane apposed and the unaltered amount of dye coupling during chronic dehydration was, therefore, unexpected.

We suggest that the overall effects of dehydration on dye coupling may be divided conceptually into two parts: (*a*) the direct effect of an increased osmolality of the extracellular fluid on the MNCs and other cells in the nucleus, and (*b*) the indirect effects of altered hormonal input from extrahypothalamic sources to the nucleus. An example of a direct osmotic effect on the magnocellular hypothalamic nuclei was the demonstration that neurons in the SON rapidly and reversibly respond to osmotic stimulation *in vitro* (Mason, 1980). Indirect effects which might be expected to have a greater latency and be more prolonged have not been suggested previously in *in vitro* studies. The direct effects of dehydration were observed in this study by comparison of dehydrate slices in 310 medium and in 340 medium. Dehydration per se appeared to influence the DCI and the percentage of somasomatically coupled MNCs. The observed changes are consistent with the increased somasomatic apposition, produced by dehydration, previously noted in the PVN (Gregory et al., 1980) and SON (Tweedle and Hatton, 1976, 1977).

The indirect effects of *in vivo* dehydration were recorded by comparing slices incubated in 310 medium from control and dehydrate animals. The incidence of coupling in dehydrates was significantly lower and, interestingly, the predominance of one mode of coupling which was seen in controls was abolished. It would appear that control slices, and dehydrate slices to a lesser extent, contain a substance, or "coupling factor," that facilitates coupling. This substance, we suggest, is one or more of the androgenic hormones. This hypothesis is supported by the presence of androgen- and estrogen-concentrating cells in the magnocellular PVN (Sar and Stumpf, 1975; Stumpf, Sar and Keifer, 1975; Rhodes et al., 1981). Furthermore, vasopressin reduces testosterone production by testicular cells *in vitro* (Adashi and Hseuh, 1981), and, in rats, testicular function, measured as the weight of the seminal vesicles, is reduced by chronic dehydration (our unpublished observations). There is also evidence for sex steroid hormones affecting the incidence of coupling, at least in non-neural tissues (e.g., rat myometrium; Garfield et al., 1978). These data suggest that a gonadal-MNC interaction may be present and that one role of androgens in the PVN is to control the incidence of coupling between neurons. Experiments to test this hypothesis are currently in progress. Estrogens may also affect coupling between MNCs in the PVN, but we have not recorded changes in slices from females which may be attributed to these hormones (unpublished observations; controls: DCI = 0.182; dehydrates: DCI = 0.229).

The interaction between slices from different treatment groups was an unexpected result and is, we believe, the first evidence for interaction between brain slices incubated together. It is possible that the presence of slices from control animals affected, for instance, the osmolality of the medium and thus the incidence of coupling in slices from dehydrates. However, this, like other similar explanations that we have considered, is unlikely to be correct since we did not record a similar interaction between slices from females (unpublished observations; dehydrates only: DCI = 0.229; dehydrates with controls: DCI = 0.216). Instead, these data may support our hypothesis for an androgenic control of coupling. We believe that the interaction may only have been recorded because we used a "static" incubation chamber (see "Materials and Methods"). In other types of chambers where slices are continuously perifused, the "coupling factor" would not have accumulated in the incubation medium and, thus, this interaction would not have occurred.

In this study, arbitrary separation of coupling into somasomatic and non-somasomatic groups has been made. It is not clear whether these differences really exist, since we did not determine the presence of gap junctions or even the close, direct apposition of membrane between coupled cells by electron microscopic examination of the tissue. Furthermore, it is possible that neurons apparently coupled by connections between their somata were also coupled via dendritic junctions. Whether there is a functional significance to the different modes of coupling between MNCs is uncertain. It may be that somasomatic coupling is predominant between vasopressin-secreting MNCs, and non-somasomatic coupling might then be expected to be associated with oxytocin-containing neurons. Although dye-coupled MNCs have been shown to contain the same hormone-associated neurophysin (Cobbett et al., 1982), there is no clear evidence for an association between coupling mode and hormone content. However, the results suggest that mode of coupling may be influenced by osmolality of the extracellular space and by local concentrations of steroid hormone, since we observed trends for different modes of coupling between treatment groups.

Although dye coupling is an indirect demonstration of electrotonic coupling, there is evidence that dye coupling actually underestimates the number of neurons that are electrotonically

coupled. Dye coupling probably only represents instances where electrotonic coupling is strong (Dudek et al., 1983; Murphy et al., 1983). Whether the changes in the incidence and mode of dye coupling between hypothalamic MNCs seen in this study reflect changes in the coupling strength between MNCs already electrotonically coupled or in the total number of coupled neurons is not known. Nor is a study to determine such changes presently feasible, since it would require many instances of simultaneous intracellular recordings to be made from coupled pairs over a period of several hours.

In conclusion, we have found that the incidence and mode of dye coupling between MNCs in the *in vitro* PVN is influenced by *in vivo* hydration state, by the osmolality of the *in vitro* medium, and by the origin of other slices present in the recording chamber. The data suggest that electrotonic coupling *in vivo* in the PVN is also modulated by the osmolality of the extracellular space and by hormonal influences.

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