Paraventricular neurones in the rat hypothalamic slice: Lucifer Yellow injection and immunocytochemical identification[1]

B. E. J. Kayser, M. Mühlethaler and J. J. Dreifuss

Department of Physiology, Centre Médical Universitaire, 1 rue Michel Servet, CH-1211 Geneva 4 (Switzerland), 15 July 1981

Summary. Intracellular records were obtained from paraventricular neurones injected with Lucifer Yellow in slices from the rat hypothalamus. Slices containing fluorescent neurones were then serially cut and alternating sections were stained immunocytochemically for vasopressin or oxytocin. Double-labelled cells were found which fluoresced and which had reacted with either vasopressin or oxytocin antiserum.

Reaves and Hayward[2,3] combined 3 techniques to characterize neurones of the preoptic nucleus of the goldfish hypothalamus which, in the higher vertebrates, differentiates to form the hypothalamic, supraoptic and paraventricular nuclei. Briefly, they obtained intracellular records of a neurone projecting to the posterior pituitary lobe, injected Lucifer Yellow into the cell and later characterized immunocytochemically the neurohormone it produced. It would be of major interest to relate similarly electrophysiological data, cell shape and peptide content of magnocellular endocrine neurones in the mammalian hypothalamus. We report here preliminary results on the immunocytochemical identification of intracellularly injected paraventricular neurones in a hypothalamic slice preparation.

Materials and methods. Male Sprague-Dawley rats, approximately 200 g in b. wt, were decapitated and their brains quickly removed. Coronal slices (400–500 µm thick) were cut with a tissue chopper from a block of brain tissue containing the hypothalamus. The slices including the paraventricular nuclei were transferred to a recording chamber and laid on a nylon grid at the interface between the perifusion medium (kept at 36–37 °C) and a humidified, oxygenated gas mixture[4]. Intracellular recordings from neurones located in the area of the lateral and medial magnocellular paraventricular subnuclei[5] were obtained, 3–5 h after the death of the animal, with glass micropipettes filled with a 4% solution of Lucifer Yellow and which had a tip resistance of 200–300 MΩ. Lucifer Yellow was generously supplied by Dr W. W. Stewart. NIAMDD, Bethesda, MD. The dye was ejected electrophoretically for 1–5 min with a DC hyperpolarizing current of 0.5–5.0 nA.

Following injection of several cells, each slice was fixed either in a solution containing 4% paraformaldehyde in a 0.2 M phosphate buffer or in Bouin's fixative (saturated aquous picric acid; formaldehyde; glacial acetic acid at 150:50:10). Slices were fixed overnight and then left in a phosphate buffered sucrose solution (30%) for approximately 20 min. Serial sections (9 µm in thickness) were cut on a cryostat, collected on chromalum-albumin covered slides and air-dried for 10 min. Following a 10 min wash in phosphate buffered saline (PBS), the sections were counterstained with a 0.05% solution of Evans-Blue in PBS. The sections were then observed under a Leitz-Ploemopak fluorescence microscope and photographs were taken of the sections containing Lucifer Yellow-injected cells.

The sections were thereafter processed for immunocytochemistry according to the method of Sternberger[6]. Serum

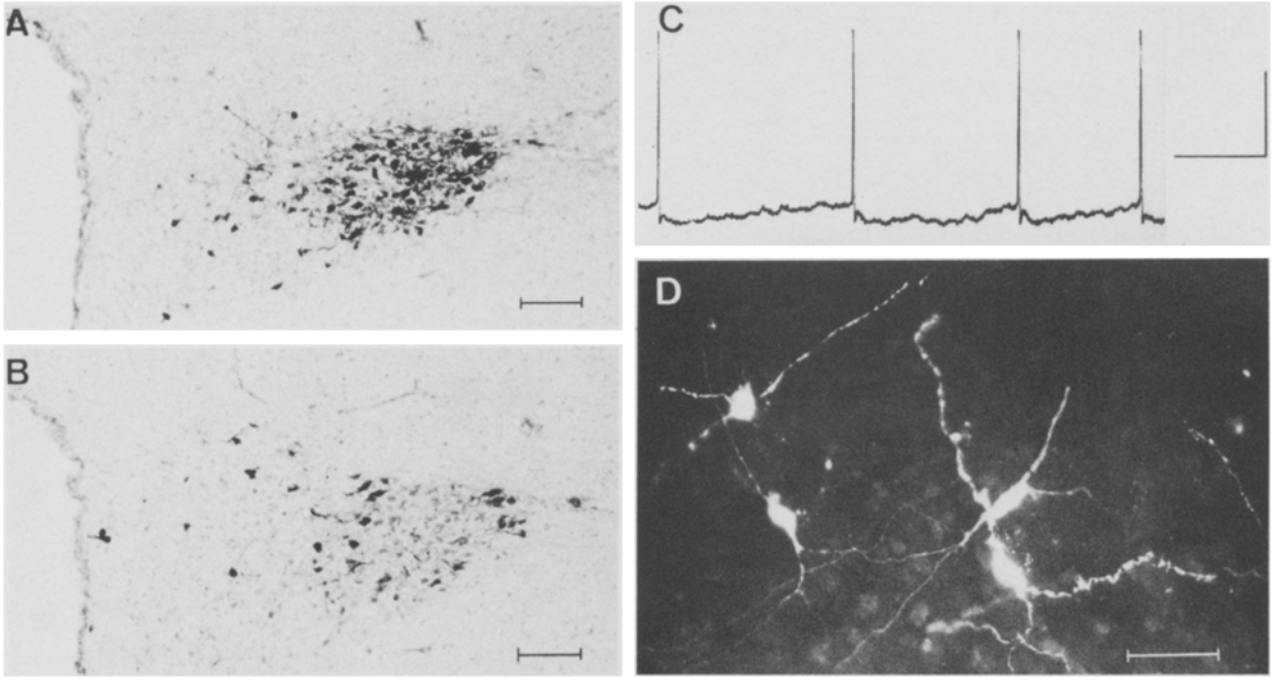

Figure 1. A and B Micrographs of 2 adjacent, 9 µm thick, coronal sections obtained from a slice fixed in 4% paraformaldehyde, and immunocytochemically stained for vasopressin (A) and oxytocin (B). Note the differential distribution of vasopressinergic and oxytocinergic neurones. Calibration: 100 µm. C Action potentials generated by a paraventricular neurone in a 450 µm thick slice. Vertical bar: 25 mV; horizontal bar: 100 msec. D 4 Lucifer Yellow-injected neurones in a 60 µm thick section cut from a paraformaldehyde fixed slice. Calibration: 50 µm.

containing antibodies against arginine-vasopressin or against oxytocin was kindly provided by Dr F. Vandesande, Gent. Since hypothalamic magnocellular neurones synthesize either oxytocin or vasopressin[7], we used a liquid-phase absorption procedure so that cells containing the heterologuous antigen would not be stained or would only be faintly stained. We therefore added 12.5 IU of heterologuous antigen and 100 µg of Bacitracin/ml to the diluted (1:1000) antiserum.

For immunocytochemistry all incubations were done at room temperature and sera were applied drop-wise according to the following protocol:

- incubation for 15 min with normal goat serum (1:20) in PBS containing 0.4% (v/v) Triton X 100;
- 5 min rinse in PBS;
- incubation for 48 h with rabbit serum (1:1000) containing antibodies directed against vasopressin or against oxytocin in PBS containing 0.4% Triton X 100;
- 5 min rinse in PBS;
- incubation for 30 min with goat anti-rabbit serum (1:40) in PBS;
- 5 min rinse in PBS;
- incubation for 30 min with peroxidase-antiperoxidase complex (1:50) in PBS;
- quick wash in PBS, followed by a 5 min wash in TRIS/HC1 buffer 0.05 M, pH 7.6;
- incubation for 8 min in TRIS-HC1 buffer containing 75 mg of diamino-benzidine (DAB) in 100 ml. Immediately prior to use 30 µl of a 30% solution of H_2O_2 was added;
- 5 min wash in distilled water.

Sections were then counterstained with Toluidine Blue[8], dehydrated through a series of alcohols and xylol, mounted and observed under the microscope. Photographs were taken of the regions containing the Lucifer Yellow-injected cells.

Results and discussion. Figure 1 shows adjacent coronal sections of the paraventricular hypothalamus stained for vasopressin or oxytocin in a slice. Vasopressinergic neurones were located predominantly in the lateral magnocellular subnucleus whereas oxytocinergic cell bodies were scattered at the edge of the subnucleus and more medially amongst unlabelled parvocellular cells; no cells reacted with both antisera.

In 7 slices, 42 neurones located in the area of the paraventricular nucleus yielded stable recordings (fig. 1C) and could be injected with Lucifer Yellow. In 11 of these cells, the diameter of the cell body was smaller than 15 µm and these cells were deemed to be parvocellular. The 31 remeining neurones were larger (fig. 1D). The slices were then serially sectioned and treated with the antisera. The number of dye-injected and immunocytochemically identified neurones was small due to technical difficulties, such as loss of sections and non-uniform DAB-staining. Our initial fear that the DAB-deposit might mask the Lucifer Yellow-induced fluorescence led us to counterstain and to photograph the areas containing injected cells both before and after the immunocytochemical procedure. Actually, especially in slices fixed in Bouin's fixative, the cell nucleus did not react with DAB and remained brightly fluorescent. Double-labelled neurones were therefore characterized by having a cytoplasm colored dark brown and a yellow-fluorescing nucleus.

In 2 slices where the whole procedure was successfully completed, 4 of the large Lucifer Yellow-injected cells contained DAB reaction product; 3 were vasopressinergic and 1 was oxytocinergic. Two cells which reacted with vasopressin antiserum are shown in figure 2. Specificity was ascertained by lack of staining of the same cells with the 2nd antibody in the adjacent section (fig. 2E).

Lucifer Yellow is a fluorescent 4-aminonaphtalimide dye[9,10] which can be used to stain living cells during

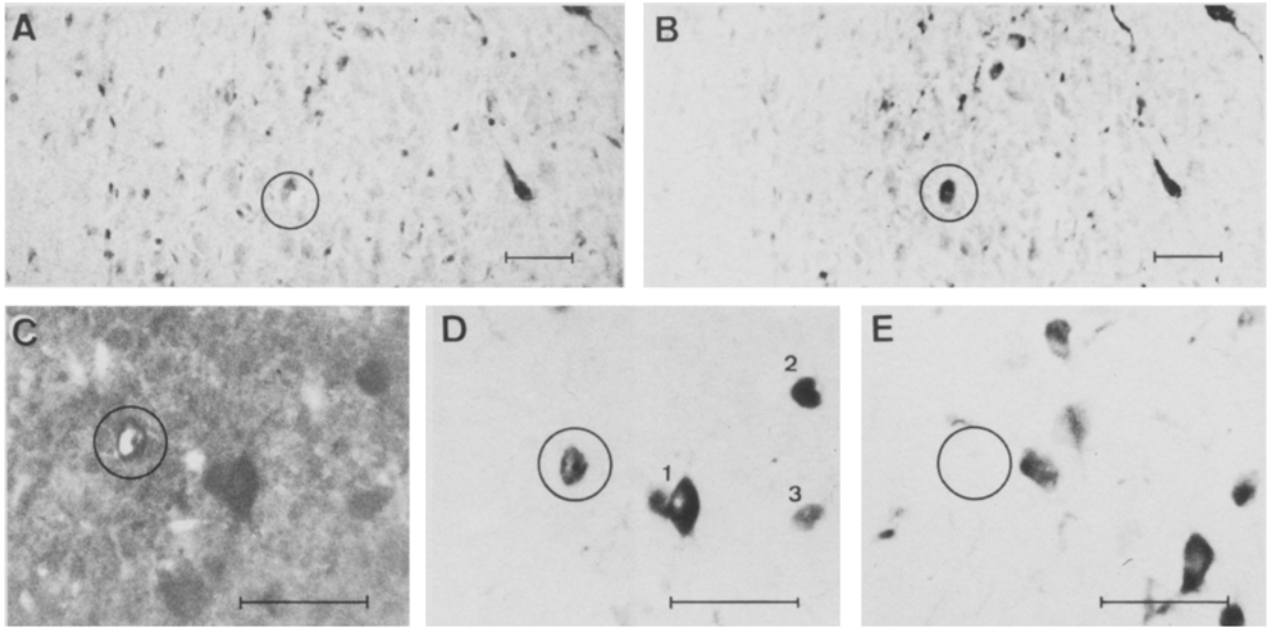

Figure 2. Immunocytochemical staining (PAP-method) of 2 vasopressinergic neurones. A and B are from a slice fixed in Bouin's fixative, C–E from another slice fixed in paraformaldehyde. Photographs were taken with UV and visible light illumination (A, C) and visible light alone (B, D, E), respectively. Lucifer Yellow-injected cells are outlined by a circle. Cell A has a white nucleus (with fluoresces in yellow on the original color photograph) which is surrounded by a dark (brown) cytoplasm. Note the presence of other vasopressin-reactive cells in B. In C, note clear (fluorescent) nucleus of an immunoreactive cell (circled). D shows the same cell, as well as 3 additional neurones (1–3) which contain DAB reaction product. E shows the absence of staining of the injected neurone on the immediately adjacent section stained for oxytocin. Calibrations: 50 µm.

electrophysiological recording[11,12]. In the neuroendocrine hypothalamus, for example, cultured neurones from the area of the supraoptic nucleus have been injected in order to study their dendritic and axonal processes[13]. Andrew et al.[14] showed that dye-transfer amongst magnocellular hypothalamic neurones occurred frequently in a slice preparation, an observation which we failed to confirm. Other studies, aimed at describing the morphology and the electrophysiological characteristics of neurones located in the arcuate-ventromedial region of hypothalamic slices, have used a different fluorescent dye, i.e. Procion Yellow[14,15].

Many workers have recently used the hypothalamic slice preparation to study the bioelectrical and pharmacological properties of endocrine neurones located in the supraoptic[17–19] and paraventricular nuclei[4,14,20–22], including studies by intracellular recording[14,19,21]. We therefore investigated whether it might be possible to apply the methods pioneered by Reaves and Hayward[2,3] to the rat hypothalamus in vitro and thereby to characterize its magnocellular neurones by the use of a combination of electrophysiology, dye injection and immunocytochemistry. The results thus far obtained are encouraging, since they showed the persistence of peptide hormone immunoreactivity in hypothalamic slices kept in vitro for several hours. The existence of a differential sensitivity of presumptive oxytocinergic neurones ('milk-ejection cells')[23] and vasopressinergic neurones (i.e. cells not accelerated at milk ejection) to transmitter substances has been suggested[24–26]. The method described here should allow a direct test of this conjecture.

1 This study was supported in part by grant 3.469.79 from the Swiss National Science Foundation.

2 T.A. Reaves, Jr, and J.N. Hayward, Cell Tissue Res. *202,* 17 (1979).
3 T.A. Reaves, Jr, and J.N. Hayward, Proc. natl Acad. Sci. USA *76,* 6009 (1979).
4 M. Mühlethaler, B.H. Gähwiler and J.J. Dreifuss, Brain Res. *197,* 264 (1980).
5 W.E. Armstrong, S. Warach, G.I. Hatton and T.H. McNeill, Neuroscience *5,* 1931 (1980).
6 L.A. Sternberger: Immunocytochemistry, 2nd edn. John Wiley, New York 1979.
7 F. Vandesande and K. Dierickx, Cell Tissue Res. *164,* 153 (1975).
8 M.V. Sofroniew, A. Weindl, I. Schinko and R. Wetzstein, Cell Tissue Res. *196,* 367 (1979).
9 W.W. Stewart, Cell *14,* 741 (1978).
10 W.W. Stewart, Nature, Lond. *292,* 17 (1981).
11 B.H. Gähwiler, J. Neurobiol. *12,* 187 (1981).
12 B.H. Gähwiler, Proc., R. Soc., B *211,* 287 (1981).
13 J.J. Dreifuss and B.H. Gähwiler, J. Physiol., Paris, in press (1982).
14 R.D. Andrew, B.A. MacVicar, F.E. Dudek and G.I. Hatton, Science *211,* 1187 (1981).
15 M.J. Kelly, U. Kuhnt and W. Wuttke, Exp. Brain Res. *34,* 107 (1979).
16 M.J. Kelly, U. Kuhnt and W. Wuttke, Exp. Brain Res. *40,* 440 (1980).
17 E.W. Haller, M.J. Brimble and J.B. Wakerley, Exp. Brain Res. *33,* 131 (1978).
18 E.W. Haller and J.B. Wakerley, J. Physiol., Lond. *302,* 347 (1980).
19 W.T. Mason, Nature, Lond. *287,* 154 (1980).
20 G.I. Hatton, W.E. Armstrong and W.A. Gregory, Brain Res. Bull. *3,* 497 (1978).
21 F.E. Dudek, G.I. Hatton and B.A. MacVicar, J. Physiol., Lond. *301,* 101 (1980).
22 Q.J. Pittman, J.D. Hatton and F.E. Bloom, Proc. natl Acad. Sci. USA *77,* 5527 (1980).
23 J.J. Dreifuss, E. Tribollet and M. Mühlethaler, Biol. Reprod. *24,* 51 (1981).
24 T. Akaishi and N. Negoro, Endocr. jap. *26,* 725 (1979).
25 G. Clarke, D.W. Lincoln and P. Wood. J. Physiol., Lond. *303,* 59P (1980).
26 G. Clarke, R. Noble and J.B. Wakerley, J. Physiol., Lond., in press (1981).

Cytomegalovirus-like particles in the red-backed vole submandibular gland

M. Odajima

Department of Oral Anatomy, Hokkaido University School of Dentistry, North 13 West 7, Kita-ku, Sapporo, 060 (Japan), 26 January 1981

Summary. Cytomegalovirus-like particles were observed in the acinar cells and in the intralobular ducts of the submandibular gland of the red-backed vole. The particles were target-shaped, had a glycoprotein-positive, electron-dense central core, and the core was surrounded by a protein-rich layer and glycoprotein-positive outer layer.

During a study of ultracytochemistry[1], the author observed a virus infection in the submandibular glands of the red-backed vole (*Clethrionomys rufocanus bedfordiae,* Thomas). The ultrastructural morphology of cells infected with cytomegalovirus, a Herpes group virus, has been previously reported[2–6]; the target-shaped virus particles observed in the present study were very similar to those of the cytomegalovirus in their morphological features. This report describes the ultracytochemistry of the mature form of the cytomegalovirus-like particles.

Materials and methods. 25 red-backed voles (9 adult male and 16 female voles) were used in this study. Trapped in Sapporo, Hokkaido, all animals appeared healthy and free of desease. They were killed shortly after capture. The submandibular glands were fixed in buffered 10% formalin for 6 h and processed for paraffin sectioning. Sections (5–6 μm) were cut serially and stained with hematoxylin-eosin, Millon reaction for tyrosine, the p-DMAB method for tryptophan, 0.05% toluidine blue (McIlvaine, pH 2.5, 4.1 and 7.0) for acid and neutral mucopolysaccharides, or the PAS reaction for neutral mucopolysaccharides. For the ultrastructural investigations, small tissue blocks were dissected from the submandibular glands of 18 voles, and fixed after continuous shaking in a cold 5% formalin fixative (pH 7.4, phosphate buffer) for 3 h or in a cold 50% Karnovsky fixative (the same buffer) for 3 h. The blocks were rinsed and shaken in the same buffer for 6–12 h, after which half of the blocks were dehydrated with ethanol and embedded in Epon. The rest of the blocks were post-fixed in a cold 1% OsO_4 solution (pH 7.4, phosphate buffer), dehydrated, and embedded in Epon. Ultrathin sections were cut by a LKB 8800 ultramicrotome and observed under a Hitachi HU-11DS electron microscope. To determine the presence of glycoprotein, the PA-TSC-SP staining method[7] was used for the ultrathin sections. The presence of protein was determined by pronase digestion[8] (Kaken