Immunocytochemical Demonstration of Peptidergic Neurons in the Central Nervous System of the Pond Snail *Lymnaea stagnalis* with Antisera Raised to Biologically Active Peptides of Vertebrates

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Summary. Perikarya and nerve fibers were immunocytochemically identified in the central nervous system of the pond snail *Lymnaea stagnalis* by means of the unlabelled antibody enzyme method with antisera to 15 biologically active peptides of vertebrates: vasopressin, vasotocin, oxytocin, α -melanocyte stimulating hormone (α -MSH), met-enkephalin, somatostatin, glucagon, insulin, glucose-dependent insulinotropic peptide (GIP), vaso-active intestinal polypeptide (VIP), gastrin, secretin, pancreatic polypeptide (PP), Substance P, calcitonin. No immunostaining was obtained with antisera to β -endorphin, cholecystikinin (CCK), neurophysin I and II. Particular neurons could be identified with two antisera (anti-vasopressin/vasotocin, anti- α -MSH/met-enkephalin, anti-substance P/PP, anti-PP/gastrin). Apparently this indicates that populations of cells identified with a given antiserum may consist of more than one cell type.

Only a few of the new peptidergic cells appeared to be identical with classical neurosecretory cells. Thus the growth hormone producing Light Green Cells stained with anti-somatostatin and the axon terminals of the ovulation hormone producing Caudo-Dorsal Cells with anti-met-enkephalin. Whether this indicates structural identity of the growth hormone with somatostatin and of the ovulation hormone with met-enkephalin remains to be investigated.

Just like the classical neurosecretory cells a number of the new peptidergic cells (anti-glucagon, -insulin, -met-enkephalin, -somatostatin, and -PP positive cells) send their axons to the peripheries of commissures, connectives or nerves. Thus these cells can be considered as probably neuroendocrine. The classical neurosecretory cells release their products into the haemolymph from these sites. Other new peptidergic cells (e.g., anti-vasopressin, -vasotocin, -oxytocin

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The authors wish to thank Prof. Dr. J. Lever for reading the manuscript, Mr. G.W.H. van den Berg for drawing the figures, and Mrs. Dagmar Reichelt for technical assistance

and -GIP positive cells) have axons that terminate, probably synaptically, on other neurons, indicating that they are "more conventional" neurons, their products being neurotransmitters/neuromodulators. It can also not be excluded that some cells of a population containing a given peptide are neuroendocrine and others make contact with other neurons.

Key words: Immunocytochemistry – Biologically active peptides – *Lymnaea stagnalis* – Neurohormone – Neurotransmitter

From immunocytochemical (ICC) studies and radioimmunoassays (RIA) it is clear that in vertebrates numerous biologically active peptides (BAP) are not only synthesized in (neuro)endocrine cells (e.g., in the hypothalamus, the hypophysis and the gastro-enteropancreatic system), but also in conventional neurons, viz. in the central and peripheral nervous system (e.g., Polak and Bloom 1978; Scharrer 1978; Hökfelt et al. 1980). These findings suggest a fundamental relationship among BAP producing cells. A theoretical basis for this suggestion may be found in the APUD cell concept (Pearse and Takor Takor 1976), which suggests that all BAP producing cells have a neuroectodermal origin, and in the common precursor theory, which supposes that various BAP producing cells synthesize the same prohormone as primary translation product, from which each cell type forms its own specific end product (Van der Donk et al. 1978; Lips et al. 1978). Morphological and functional studies have shown that a particular BAP may act either as (neuro)hormone or as neurotransmitter/neuromodulator, depending on the site where it is released (e.g., De Wied and Gispen 1977; Polak and Bloom 1978; Emson 1979; Buijs and Swaab 1980).

The fact that BAP producing cells have various characteristics in common speaks for a close evolutionary relationship of these cells (e.g., Scharrer 1978). Recently this hypothesis has been sustained by ICC and RIA studies on invertebrates (insects, molluscs, ascidians) demonstrating that vertebrate BAP or closely related substances are present in these animals, in particular in elements of the nervous system (e.g., Grimm-Jørgensen 1975; Straus et al. 1975; Fritsch et al. 1978, 1979, 1980; Grimm-Jørgensen et al. 1978, 1979; Strambi et al. 1978, 1979; Boer et al. 1979; Duvé and Thorpe 1979, 1980a, b; Van Noorden et al. 1980). It has furthermore been shown that invertebrate BAP occur in vertebrates (Schaller et al. 1977; Boer et al. 1980).

The aim of the present research is to further document the hypothesis of the general occurrence of BAP by investigating with ICC the possible presence of vertebrate BAP in the central nervous system (CNS) of the pond snail *Lymnaea stagnalis*. It has previously been shown that in the CNS of this animal 2 electrotonically coupled giant neurons contain an ACTH-like substance (Boer et al. 1979). With the alcian blue-alcian yellow (AB/AY) staining method for neurosecretion 10 types of peptidergic cells have been identified in this animal (Wendelaar Bonga 1970). Ultrastructural observations and results obtained with the toluidine blue method for small peptides (Solcia et al. 1968) have suggested that many more peptidergic cells are present in the CNS of *L. stagnalis*.

Materials and Methods

Laboratory bred adult specimens of *L. stagnalis* (shell height 25–30 mm) were used. The CNS were either fixed overnight at room temperature in a mixture of glutaraldehyde, picric acid, and acetic acid (GPA, see Boer et al. 1979) or quenched in liquid nitrogen-cooled Freon 22 (Dupont), freeze-dried in a tissue freeze-drier (Edwards), and fixed for 3 h at 60 °C in p-benzoquinone vapour (for details, see Pearse and Polak 1975). The CNS were embedded in paraffin and 6–10 µm sections were cut. Inhibition of pseudo-peroxidase activity was carried out with absolute methanol (30 min) and 0.0125 % H₂O₂ in PBS, pH 7.45 (30 min). After this treatment the sections were immunocytochemically stained with the unlabelled antibody enzyme (peroxidase-anti-peroxydase, PAP) method (Sternberger 1974). For the demonstration of insulin the enzyme labelled indirect method was used.

The following antisera were used (dilutions in parentheses): anti-vasopressin (1 : 500, 1 : 3000); anti-vasotocin (1:500, 1:3000); anti-oxytocin (1:500, 1:3000); anti- α -melanocyte stimulating hormone (α -MSH) (1:500, 1:3000); anti- β -endorphin (1:6000); anti-met-enkephalin (C-terminal) (1:1600); anti-glucagon (C-terminal) (1:5000); anti-insulin (1:800); anti-glucose dependent insulinotropic peptide (GIP) (mid portion of the molecule) (1:12000); anti-vasoactive intestinal polypeptide (VIP) (C-terminal) (1:2000); anti-gastrin (C-terminal) (1:2000); anti-cholecystokinin (CCK) (mid portion of the molecule) (1:5000); anti-secretin (1:6000); anti-bovine-pancreatic polypeptide (PP) (1:12000); anti-somatostatin (1:6000); anti-calcitonin (1:1200); anti-Substance P (C-terminal) (1:16000); anti-neurophysin I (1:500); anti-neurophysin II (1:500). When two dilutions were used the incubation time with the antiserum was 1 h (low dilution) or 48 h (high dilution). When one dilution was used the incubation time was 48 h. The sections were then incubated with swine-anti-rabbit γ -globulin (Nordic 1:60) and with PAP (Sternberger-Meyer 1:200). The peroxidase was made visible with a solution of 0.05 % 3,3'-diaminobenzidine in 0.05 M Tris-HCl, pH 7.6, containing 0.01 % H₂O₂. The number of CNS used to test each antiserum was 4–20 (average number 6).

The controls for specificity included the use of (1) non-immune serum, and (2) the antiserum absorbed with the homologous antigen. In the case of anti-vasopressin, anti-vasotocin, anti-oxytocin and anti- α -MSH solid phase absorption was applied (Swaab et al. 1975). All other antisera were absorbed by incubating the diluted antiserum overnight with 10 nMol peptide/ml.

Results

Anatomy

The anatomy of the central nervous system of *L. stagnalis* has been described in detail by, e.g., Hekstra and Lever (1960), Wendelaar Bonga (1970) and Winlow and Benjamin (1976). The CNS surrounds the oesophagus and consists of 11 ganglia (Fig. 1): the paired buccal, cerebral, pleural, parietal and pedal ganglia and the unpaired visceral ganglion. To each cerebral ganglion is attached a small additional ganglion, the lateral lobe. On the surface of each cerebral ganglion a medio- and a latero-dorsal body are located.

Controls

With non-immune serum no reaction was obtained. In all cases, except for anti-VIP (see below), neither cells nor fibers were stained with antisera absorbed with their homologous antigen.

Immunocytochemical Observations

Positive immunostained perikarya and fiber tracts were observed with 15 antisera (Figs. 1–15). The extensiveness of the peptidergic systems varies greatly. In some

Figs. 1–15. Diagrams of central nervous system of *L. stagnalis* with neuron perikarya and fibers identified by different antisera. **Fig. 1:** anti-vasopressin. B buccal ganglion; C cerebral ganglion; LL lateral lobe; Pa parietal ganglion; Pe pedal ganglion; Pl pleural ganglion; V visceral ganglion; a bucco-cerebral connective; b subcerebral commissure; c cerebral commissure; d dorsal pedal commissure; e ventral pedal commissure. 1–23 nerves: 1 radular, 2 gastric, 3 pharyngeal, 4 tentacular, 5 optic, 6 nuchal, 7 superior frontal lip, 8 median lip, 9 penial, 10 static, 11 left pallial, 12 cutaneous pallial, 13 anal, 14 intestinal, 15 genital, 16 right internal pallial, 17 right external pallial, 18 superior pedal, 19 superior cervical, 20 median pedal, 21 inferior cervical, 22 columellar, 23 inferior pedal nerve. **Fig. 2:** anti-vasotocin; 3: -oxytocin; 4: - α -MSH; 5: -met-enkephalin; 6: -somatostatin; 7: -gastrin; 8: substance P; 9: -PP

cases axons of individual neurons or of groups of cells identified with a particular antiserum, can be followed over relatively long distances. In other cases, however, fibers of several cells and cell groups intermingle, forming extensive pathways in the CNS (Figs. 1, 7); in these instances it is not possible to follow fibers of individual cells or of particular cell groups.

Fig. 10: anti-secretin; 11: -VIP; 12: -GIP; 13: -glucagon; 14: -insulin; 15: -calcitonin

Fixation

The results obtained with the two fixatives used (GPA, p-benzoquinone) were not completely identical. With some antisera the largest number of stained cells was found after GPA fixation, with others after p-benzoquinone fixation. The descriptions of the distribution of positive cells and of fiber tracts are based on the fixation that gave the most extensive results.

Anti-Vasopressin (Fig. 1) (GPA)

Positive perikarya were observed in the buccal, cerebral, and pedal ganglia. Their axons form pathways extending into all central ganglia. Furthermore positive fibers

run from this "network" into a number of nerves: gastric, pharyngeal, tentacular, right internal pallial, intestinal, and pedal (median, inferior, superior).

The following perikarya and groups of perikarya were identified. Scattered in each buccal ganglion 3–5 cells ($\varnothing$ perikarya 15–25 μm) were found. A small number of positive fibers terminate, probably synaptically, on other, unstained, neurons (Fig. 16). In each cerebral ganglion two groups of cells are located. The first consists of 30–50 cells in the left and of 10–15 cells in the right ganglion ($\varnothing$ 10–30 μm). They are located in the ventro-rostral part of the ganglia. The other groups (10–15 cells, $\varnothing$ 15–30 μm) are located in the medio-dorsal part of each ganglion. In each pedal ganglion a group of 15–20 positive cells ($\varnothing$ 20–30 μm) lies near the origin of the inferior pedal nerve.

Anti-Vasotocin (Fig. 2) (GPA)

With this antiserum also a "network" of fibers extending into all central ganglia was observed. Fiber tracts were found in the following nerves: pharyngeal, tentacular, median lip, left pallial, right internal pallial, and the visceral ganglion nerves.

Positive perikarya were identified in all but the pleural ganglia. In each buccal ganglion 2–4 cells ($\varnothing$ 15–50 μm) lie near the origin of the bucco-cerebral connective. In each cerebral ganglion two groups of positive cells are present, one (2–4 cells, $\varnothing$ 25–40 μm) lies just dorsal to the lateral lobe, the other group (10–15 cells, $\varnothing$ 10–40 μm) near the origin of the cerebro-pedal connective. In each ganglion the anti-vasopressin positive cell group stained also with anti-vasotocin. In addition to this group 3–8 scattered cells staining with anti-vasotocin, but not with anti-vasopressin, occur in each ganglion. In the caudo-lateral part of the left parietal ganglion a group of 5–8 positive cells ($\varnothing$ 10–50 μm) is located. In the right parietal ganglion 3 cell groups were found. One group (7–10 cells, $\varnothing$ 20–40 μm) lies caudo-ventral to the parieto-visceral connective, the second (10–15 cells, $\varnothing$ 30–40 μm) is situated medio-rostral to the parieto-visceral connective, and the third (15–20 cells, $\varnothing$ 15–50 μm) lies near the parieto-pleural connective; 2–4 further positive cells are located in the medio-lateral part of this ganglion. A small number of fibers from these cells seem to end synaptically on 2–4 neurons located in the lateral part of the ganglion. In the visceral ganglion a group of 20–40 cells ($\varnothing$ 15–50 μm) lies in the caudal part of the ganglion, near the origin of the anal, intestinal, and genital nerves.

Anti-Oxytocin (Fig. 3) (p-Benzquinone)

Extensive pathways of positive fibers are present within the CNS and positive fiber tracts occur in all nerves connected with the CNS.

Positive perikarya were observed in all ganglia, except in the left pleural and the left parietal ganglion. In each buccal ganglion 2 positive cells ($\varnothing$ 20 μm) are located near the origin of the gastric nerve and another 2 ($\varnothing$ 20 μm) near that of the bucco-cerebral connective. In this ganglion particular fibers end on unstained neurons. Scattered in each cerebral ganglion 8–15 cells ($\varnothing$ 30–60 μm) are present, and anti-oxytocin positive fibers were also seen to terminate on unstained cells (Fig. 17). In the ventral part of each pedal ganglion, near the origin of the columellar and

Fig. 16. Anti-vasopressin positive fiber (*arrow*) ending on neuron in buccal ganglion. Fix. GPA $\times 530$

Fig. 17. Anti-oxytocin positive fibers (*arrows*) ending on neuron in cerebral ganglion. GPA $\times 430$

Fig. 18. Anti-met-enkephalin positive axon terminals (*arrows*) in periphery of cerebral commissure (C), CT connective tissue. Fix. p-benzoquinone $\times 940$

Fig. 19. Anti-glucagon positive fibers (*arrows*) in periphery of genital nerve (GN), G granular cell. Fix. p-benzoquinone $\times 710$

inferior cervical nerves, a group of 10–20 cells ($\varnothing$ 10–20 μ m) is present. In the caudal area of the right pedal ganglion 1 further positive cell ($\varnothing$ 40 μ m) was observed. Scattered in the right pleural ganglion 5–10 cells ($\varnothing$ 15–25 μ m) are present. In the rostro-dorsal part of the right parietal ganglion a group of 10–15 cells ($\varnothing$ 60–80 μ m) is located. A second group (15–20 cells, $\varnothing$ 15–30 μ m) lies near the origin of the internal pallial nerve and a third (15–20 cells, $\varnothing$ 15–20 μ m) near the parieto-pleural connective. In the ventro-caudal part of the visceral ganglion a group of 20–30 cells ($\varnothing$ 30–50 μ m) is present. Several fibers were found to terminate on perikarya or on axons of neurons situated in an area ventral to the origin of the right visceroparietal connective.

Anti- α -MSH (Fig. 4) (GPA)

Positive perikarya were observed in the cerebral and pedal ganglia only. In each cerebral ganglion 2 cell groups are present. The first (8–10 cells, $\varnothing$ 12–20 μ m) is located in the medio-dorsal part of the ganglion, just ventral to the growth hormone producing Light Green Cells (Wendelaar Bonga 1970; Geraerts 1976). The other group lies just dorsal to the lateral lobe. In the right ganglion this group consists of 8–15 cells ($\varnothing$ 15–20 μ m), in the left of 6–10 cells ($\varnothing$ 15–50 μ m). Positive fibers were observed in the median lip nerve and in the superior frontal lip nerve. In the pedal ganglia two groups of positive cells were found. One group (10–15 cells, $\varnothing$ 25–35 μ m) lies in the ventro-medial part of these ganglia near the origin of the inferior pedal nerve. The fibers of some of these cells run into this pedal nerve. The other group (15–20 cells, $\varnothing$ 20–40 μ m) lies in the lateral part of the ganglion near the inferior pedal commissure and the superior pedal nerve. Some cells of this group have fibers that project into this nerve. The cells of both groups can also be identified with anti-met-enkephalin (see below). In addition to the groups a few positive perikarya occur scattered in the medio-lateral part of the pedal ganglia.

In the buccal ganglion only positive fibers were observed. They enter the ganglion via the gastric nerve. Some fibers terminate on a neuron situated near the origin of the gastric nerve. Others run to the contra-lateral pharyngeal nerve via the buccal commissure and the contra-lateral buccal ganglion. Moreover 2 or 3 fibers run to the bucco-cerebral connective.

Anti-Met-Enkephalin (Fig. 5) (p-Benzoquinone)

Positive cells were observed in the cerebral and pedal ganglia. In each cerebral ganglion 2 groups of cells are present. The first (10–15 cells, $\varnothing$ 10–15 μ m) lies in the medio-lateral part of the ganglion, the second (6–10 cells, $\varnothing$ 10–15 μ m) just dorsal to the lateral lobe. In addition, 2 positive cells ($\varnothing$ 15–20 μ m) were observed in the medio-ventral area and 1 ($\varnothing$ 35 μ m) near the origin of the median lip nerve. In addition, numerous positive axon endings were seen along the entire periphery of the cerebral commissure (Fig. 18). In each pedal ganglion a group of 35–40 cells ($\varnothing$ 40–50 μ m) is located in the lateral part. Some of these cells also stain with anti- α -MSH. A second group (8–10 cells, $\varnothing$ 20–40 μ m) lies in the ventro-medial area of the ganglion. These cells also stain with anti- α -MSH. Anti-met-enkephalin positive fibers were not observed.

Figs. 20, 21. Consecutive sections of cerebral ganglion stained with anti-gastrin (20) and anti-PP (21), respectively. Note cells stained with either anti-gastrin (A), anti-PP (B) or with both antisera (C). Fix. p-benzoquinone Fig. 20 $\times 460$, Fig. 21 $\times 410$

Anti-Somatostatin (Fig. 6) (p-Benzoquinone)

In each cerebral ganglion the two groups of Light Green Cells (LGC) were faintly stained. The axons run to the median lip nerve. In the lateral lobe the giant Canopy Cell ($\varnothing$ 100 μ m) was stained. The axon of this cell runs via the cerebral commissure to the contra-lateral median lip nerve (cf. Van Minnen et al. 1979).

Anti-Gastrin (Fig. 7) (p-Benzoquinone)

Positive cells were observed in all ganglia. The fibers of these cells form an extensive network from which axon tracts run into the commissures (exception: inferior pedal commissure), connectives, and into most of the nerves (exception: radular and nuchal nerves).

In each buccal ganglion 4 positive cells are present, 2 ($\varnothing$ 30 and 40 μ m) in the ventral and 2 ($\varnothing$ 30 and 80 μ m) in the dorsal area. The fibers of these cells run to the neuropile where they ramify. In each cerebral ganglion numerous positive cells occur. A group of 10–15 cells ($\varnothing$ 25–40 μ m) lies in the medio-dorsal part. Just dorsal to the lateral lobe a group of 20–25 perikarya ($\varnothing$ 15–30 μ m) is located. Ventral to the lateral lobe 5–10 cells ($\varnothing$ 20–30 μ m) are situated. In addition to these groups about 40–50 positive cells ($\varnothing$ 10–50 μ m) occur scattered throughout the ganglion. A number of these cells (10–15) also stain with anti-PP (Figs. 20, 21). In each pedal ganglion a group of 30–50 positive cells ($\varnothing$ 20–30 μ m) was observed in the dorsal part, near the origin the superior pedal nerve. Ventral to the median pedal nerve lies a group of 30–40 cells ($\varnothing$ 15–25 μ m). A third group (30–40 cells, $\varnothing$ 20–35 μ m) is located in the ventro-medial part of the ganglion. In addition to these groups 30–60 positive cells ($\varnothing$ 10–50 μ m) occur scattered in the ganglion. In each pleural ganglion 2–4 cells ($\varnothing$ 70–90 μ m) were observed. In the right parietal ganglion 2 groups of positive cells are present. The first (15–20 cells, $\varnothing$ 20–30 μ m) lies near the origin of the right internal pallial nerve, the second (5–7 cells, $\varnothing$ 15–20 μ m) in the median part of the ganglion. Furthermore 2–4 cells ($\varnothing$ 80–100 μ m) lie in the medio-lateral part. In the left parietal ganglion a group of 7–10 cells ($\varnothing$ 15–20 μ m) is present near the origin of the left pallial nerve. Just dorsal to this group 3–5 positive cells ($\varnothing$ 90–100 μ m) were observed. Finally, in the visceral ganglion 2 groups of cells are present. The first (5–8 cells, $\varnothing$ 30–40 μ m) lies near the left viscero-parietal connective, the second (3–5 cells, $\varnothing$ 20–30 μ m) in the medio-ventral part of the ganglion. In addition 5–10 cells ($\varnothing$ 20–30 μ m) occur scattered in the ganglion.

Anti-Substance P (Fig. 8) (p-Benzoquinone)

Positive cells were observed in the cerebral and pedal ganglia. In each cerebral ganglion 2 groups of cells are located. One (6–10 cells, $\varnothing$ 15–20 μ m) lies near the cerebro-pedal connective. Lateral to this group one large cell ($\varnothing$ 60 μ m) was found, which was also positively stained by anti-PP. The second group (3–5 cells, $\varnothing$ 30–40 μ m) is situated just dorsal to the lateral lobe. Positive fibers were observed in the median lip nerve, the tentacular nerve and the cerebro-buccal connectives. It is not clear from which perikarya these fibers originate. In the dorsal part of each pedal ganglion a group of positive cells is located near the superior cervical nerve. In the right ganglion this group consists of 15–20 cells ($\varnothing$ 20–25 μ m), in the left of 6–10

cells ($\varnothing$ 20–30 μm). In addition 2 cells ($\varnothing$ 40–50 μm) lie in the rostral part of each ganglion. The fibers of these cells run to the central neuropile where they ramify into small branches, which intermingle with other positive fibers. From here a small number of fibers run to the contra-lateral pedal ganglion.

Anti-PP (Fig. 9) (p-Benzoquinone)

Positive perikarya were found in all but the left pleural and the buccal ganglia. The fibers of these cells form an extensive network in the central ganglia. Fiber tracts were observed in the following nerves: the tentacular, median pedal, superior cervical, inferior pedal, right internal pallial, and left pallial nerve, and in all nerves of the visceral ganglion. Some fibers seem to terminate in the periphery of the left pallial nerve.

In each cerebral ganglion 2 groups of positive cells are located. The first (4–5 cells, $\varnothing$ 30–40 μm) lies in the latero-dorsal part of the ganglion, the other (30–40 cells, $\varnothing$ 15–60 μm) near the cerebro-pleural connective. A number of these latter cells are also anti-gastrin positive (Figs. 20, 21). Moreover one large cell ($\varnothing$ 60 μm) of this group is also anti-Substance P positive (see above). In the lateral lobe 3–5 positive cells (10 μm) are present. The fibers of these cells run to the cerebral ganglion via the dorsal lateral lobe connective. In each pedal ganglion a group of 15–20 cells ($\varnothing$ 10–20 μm) is located near the origin of the columellar nerve. Furthermore, a group of 3–5 cells ($\varnothing$ 20–30 μm) was observed near the pedo-pleural connective. In the medio-lateral part of the right pleural ganglion a group of 8–10 cells ($\varnothing$ 10–25 μm) is located. In the right parietal ganglion two groups of 30–40 positive cells ($\varnothing$ 15–80 μm) are situated in the medio-rostral and medio-lateral parts, respectively. In the left parietal ganglion a group of 4–6 cells ($\varnothing$ 25–30 μm) lies just caudal to the parieto-visceral connective. In the visceral ganglion 2 groups of cells are present. The first (40–50 cells, $\varnothing$ 30–50 μm) lies near the origins of the anal and intestinal nerves, the other (4–6 cells, $\varnothing$ 30–40 μm) is located anterior to the left viscero-parietal connective.

Anti-Secretin (Fig. 10) (p-Benzoquinone)

Positive perikarya were found in the cerebral ganglion and in the lateral lobe. Positive fibers were not observed. In each cerebral ganglion 2 positive cells ($\varnothing$ 15–20 μm) are located near the origin of the optic nerve and one cell ($\varnothing$ 15 μm) in the ventro-lateral part of the ganglion. In each lateral lobe 1 positive cell ($\varnothing$ 40 μm) was found.

Anti-VIP (Fig. 11) (p-Benzoquinone)

Two positive anti-VIP cells ($\varnothing$ 30 μm) were observed, one in the medio-dorsal area of each cerebral ganglion. The fibers of these cells run to the neuropile where they ramify. Positive fibers were also observed in the pedal ganglia and in both pedal commissures. However, the staining of these pedal fibers was probably non-specific, as they were still found, in contrast to the cerebral cells and fibers, in preparations stained with anti-VIP absorbed with its homologous antigen.

Anti-GIP (Fig. 12) (p-Benzoquinone)

With this antiserum no positive perikarya were found. Positive fibers, on the other hand, were observed in the cerebral, parietal and visceral ganglia, in a number of nerves (tentacular nerve, both right pallial nerves, anal and intestinal nerve) in the parieto-visceral connectives, and in the cerebral commissure. There are indications that fibers terminate in the ganglia on perikarya and on axons of unstained neurons.

Anti-Glucagon (Fig. 13) (p-Benzoquinone)

Cells containing a glucagon-like substance were observed in the lateral lobes and in the cerebral, right parietal and visceral ganglia. In the lateral lobe 7–10 positive cells ($\varnothing$ 15–20 μ m) are present. The axons of these cells run to the cerebral ganglion via the dorsal lateral lobe connective. Some of the cells have a second projection running to the follicle gland, a vesicular epithelial structure in the lateral lobe (Lever et al. 1959). In each cerebral ganglion 1 positive cell is present, near the origin of the median lip nerve. Its fiber ramifies in the neuropile into very thin branches. In the medial area of the right parietal ganglion 2 positive cells ($\varnothing$ 25–35 μ m) were observed. The fibers of these cells ramify in the neuropile of the ganglion. In the visceral ganglion 3 cells ($\varnothing$ 20–25 μ m) were observed just dorsal to the left visceroparietal connective. In addition to these perikarya and their fibers, fiber tracts were observed in the median lip nerve, in the tentacular nerve and in the perineurium of the central ganglia. Furthermore a tract extending into the cerebro-pedal connective and in the median pedal nerve, and another running through the cerebral, pleural, parietal and visceral ganglia, were observed. The latter tract is connected with fibers running into the pallial nerves and in the nerves of the visceral ganglion. Fibers seem to terminate in the peripheries of the median pedal nerve and of the visceral nerves (Fig. 19).

Anti-Insulin (Fig. 14) (p-Benzoquinone)

In all 4 CNS studied with anti-insulin, positive cells were observed in the cerebral and pedal ganglia and positive fibers in the tentacular nerve, the median lip nerve, the median pedal nerve, and in the cerebral and dorsal pedal commissures. In the medio-ventral part of each cerebral ganglion 3 cells ($\varnothing$ 20–25 μ m) are located. The fibers of these cells ramify near the perikarya; some of the branches run into the cerebral commissure. In the medio-caudal part of each pedal ganglion 6–8 cells ($\varnothing$ 30–40 μ m) are situated. In one of the preparations, further perikarya and fibers were observed in the right parietal and in the visceral ganglion and in nerves of these ganglia. In the right parietal ganglion 4 cells ($\varnothing$ 30–50 μ m) were found near the parieto-visceral connective; 7 other positive cells ($\varnothing$ 30–40 μ m) and positive fibers were observed in the right internal pallial nerve. In the visceral ganglion 6 cells ($\varnothing$ 25–35 μ m) were seen near the right visceroparietal connective. Positive fibers were found in the anal and in the intestinal nerve. In the first nerve 4 perikarya ($\varnothing$ 40–45 μ m) were also identified.

Anti-Calcitonin (Fig. 15) (p-Benzoquinone)

Positive cells were observed in the cerebral and pedal ganglia. Positive fibers run in the tentacular nerve, the optic nerve, the median lip nerve, in all nerves of the parietal and visceral ganglia (except for the cutaneous pallial nerve), and in the cerebro-buccal, the cerebro-pedal, and the pedo-pleural connectives, and the cerebral commissure.

In each cerebral ganglion a group of 7–10 cells ($\varnothing$ 10–30 μ m) is located near the cerebro-pleural connective. A second group, of 3 cells ($\varnothing$ 20–30 μ m) in the left and of 5–8 cells ($\varnothing$ 15–30 μ m) in the right ganglion, lies just dorsal to the lateral lobe. In addition 4 groups of 4–10 cells each ($\varnothing$ 10–20 μ m) were observed in the dorsal part of the right cerebral ganglion. Two cell groups of 4–8 cells ($\varnothing$ 10–15 μ m) are located in the dorso-lateral part of each pedal ganglion. Furthermore a small number of cells ($\varnothing$ 5–15 μ m) occur scattered in these ganglia.

Other Antisera

With the following antisera neither perikarya nor fibers were stained: anti-CCK, anti- β -endorphin, anti-neurophysin I and II.

Discussion

Several authors have argued that it is not possible to identify a peptide with ICC beyond any doubt (e.g., Swaab et al. 1977). Additional experimental evidence must be presented to prove the identity. This conclusion is sustained by preliminary results with RIA and iso-electrofocussing on extracts of the CNS of the pond snail. Whereas in RIA's the presence of substances that are chemically related to mammalian vasopressin, oxytocin, α -MSH and ACTH was suggested, iso-electrofocussing experiments on the snail's "oxytocin" showed that its structure is not identical to that of the mammalian hormone (H.M.L. Van Pelt-Heerschap and G.J. Boer, unpubl. results). The present observations seem to underline the statement that results obtained with ICC should be interpreted cautiously. Particular neurons in the CNS of *L. stagnalis* could be identified with two antisera (anti-vasopressin/vasotocin, anti- α -MSH/met-enkephalin, anti-Substance P/PP, or anti-PP/gastrin). The fact that in each of these cases, in addition to the cells that stained with two antisera, cells or fibers were observed that stained only with either one of the pair, indicates that 3 cell types are involved. It would not seem impossible that cells exist that can be identified with even more than 2 antisera (Pevet et al. 1980). Preliminary observations have indicated that the anti-vasopressin/vasotocin positive cells of the pedal ganglia can also be stained with an antiserum to the molluscan cardio-excitatory FMRF-amide. Furthermore there were some indications that a group of small anti-PP/Substance P positive cells in the pedal ganglia is also positive with anti-gastrin. The conclusion that a population of cells that stains with an antiserum may consist of more than one cell type is supported by the observation that in some cases the fixative appeared to influence the number of cells that is stained with a particular antiserum. The problem should be studied further with adequate specificity tests. Nevertheless the present data suggest that in

the CNS of *L. stagnalis* there are many cells that contain peptides that have antigenic determinants in common with vertebrate BAP. This supports the hypothesis that BAP, or substances with structures closely related to them, have a wide distribution in the animal kingdom (e.g., Scharrer 1978; Boer et al. 1980).

In the periphery of the cerebral commissure numerous anti-met-enkephalin positive axon terminals were found. Since the vast majority of terminals in this area are derived from the ovulation-hormone producing Caudo-Dorsal Cells (CDC) of the cerebral ganglia (Wendelaar Bonga 1971; Geraerts and Bohlken 1976), it can be assumed that the stained terminals are CDC terminals indeed. It is not clear why the CDC perikarya were not positive. As the amount of neurosecretory material in the CDC perikarya is usually rather small (Roubos 1973), possibly the peptide was present, but in too low concentration (Hökfelt et al. 1980). Another possibility would be that the peptide is transformed during axonal transport (Pickering and Jones 1971; Gainer et al. 1977).

In comparison with the results obtained with AB/AY and phloxin staining for neurosecretion (Wendelaar Bonga 1970), it appears that only a few of the classical neurosecretory cells can also be identified with any of the antisera used in the present study. The growth hormone producing cerebral LGC (Geraerts 1976) were stained with anti-somatostatin. It seems, however, unlikely that the snail's growth hormone is chemically identical with somatostatin, since extracts of the median lip nerve (the neurohaemal area of the LGC) stimulate a.o. the activity of the enzyme ornithine-decarboxylase (Dogterom and Robles 1980), whereas somatostatin has no such effect; it rather inhibits the activity of this enzyme (Cuperus, pers. comm.). As mentioned, the axon terminals of the CDC were stained with anti-met-enkephalin. Whether this indicates structural identity of the snail's ovulation hormone with met-enkephalin remains to be investigated.

Of most of the peptidergic cells identified in this study it can be stated that they either have locations other than those of the AB/AY positive cells, or that they occur not only in these locations, but also in others, so that it is impossible to readily identify any of them with the classical peptidergic cells. For example, anti-vasopressin/vasotocin positive cells were only found in the pedal ganglia; in these ganglia AB/AY positive cells are not present (Wendelaar Bonga 1970). Anti-oxytocin and anti-PP positive cells, on the other hand, occur in the parietal and visceral ganglia in locations that are comparable to those of the Yellow Green Cells (YGC). However, since anti-oxytocin as well as anti-PP positive cells occur also in the pedal ganglia and in other ganglia, e.g., the cerebral, that do not contain YGC, it seems unlikely that it is YGC that were stained with these antisera. Yet, this possibility can not be excluded entirely. As mentioned, the cells stained with a particular antiserum may in fact comprise more than one cell type. The same seems to be true for certain AB/AY positive cell types. Thus, it has been shown that only two of the Yellow Cells (YC) are anti-ACTH positive, indicating that the YC consist of at least two cell types (Boer et al. 1979). Furthermore, it should be mentioned that possibly a number of anti-insulin positive cells are identical with particular YGC. In all preparations stained with anti-insulin, positive perikarya were observed in the cerebral and pedal ganglia. In one case only additional cells were found in the visceral and parietal ganglia and within nerves of these ganglia. (It is not clear why these latter cells were not observed in the other preparations, but possibly they

contained too low a concentration of anti-insulin positive material.) These cells in the parietal and visceral ganglia and in their nerves may well be identical with particular YGC, as according to Wendelaar Bonga (1970) cells occurring in visceral nerves are YGC. Since the number of YGC in the visceral and parietal ganglia is much larger than the number of anti-insulin positive cells, this would either mean that the YGC consist of at least two cell types (YGC/anti-insulin positive cells and YGC/anti-insulin negative cells), or that only a limited number of anti-insulin cells was stained. It would further indicate that there are two types of anti-insulin positive cells, viz. anti-insulin YGC (parietal and visceral ganglia, visceral nerves) and anti-insulin/AB/AY-negative cells (cerebral and pedal ganglia). These observations and considerations indicate that further studies may reveal an even greater diversity among the classical as well as the new peptidergic cells in the CNS of the pond snail than is suggested by the results obtained so far.

Compared to vertebrates, invertebrates possess few endocrine organs. Neurosecretory cells, on the other hand, are numerous (e.g., Joosse 1979), indicating that many physiological processes in these animals are regulated by neurohormones. In view of this fact it is likely that a number of the new peptidergic cells are neuroendocrine cells. On the other hand, it cannot be excluded that others are "more conventional" neurons. On the basis of the morphological evidence presented here, a neuroendocrine nature is feasible for the anti-glucagon, -insulin, -met-enkephalin, -somatostatin and -PP positive cells. These neurons project their fibers, just like the classical peptidergic cells, to the peripheries of commissures, connectives, or nerves, i.e., to areas from which neurosecretory products are released into the haemolymph (Wendelaar Bonga 1970; Roubos 1973). Other new peptidergic cells show rather the topographic relationship of conventional neurons. The fibers of, e.g., anti-vasopressin, -vasotocin, -oxytocin, -GIP positive cells seem to terminate synaptically on other neurons. This strongly suggests that the products contained in these fibers act as neurotransmitters. Whether the division of the new peptidergic cells into "neuroendocrine" and "conventional" neurons is absolute, is not clear. The fact that morphological and functional studies in vertebrates have indicated that BAP may act not only as (neuro)hormones, but also as neurotransmitters (e.g., De Wied and Gispen 1977; Buijs and Swaab 1980) suggests that further studies may well show that of a group of neurons producing a given peptide, only some are "neuroendocrine", i.e., release their product into the haemolymph.

It is attractive to suppose that BAP with hormonal activity have comparable functions in vertebrates and invertebrates. There are in fact reports that support this hypothesis (e.g., insects: insulin, vasopressin, e.g., Duve et al. 1979; Kramer et al. 1980; molluscs: insulin, Plisetskaya et al. 1978). It seems, however, quite possible that the respective BAP have other hormonal functions in invertebrates than in vertebrates. It has been shown, for example, that TRH in *L. stagnalis* is involved in osmoregulation (Grimm-Jørgensen 1979).

No immunostaining was obtained with anti-CCK, anti- β -endorphin and with anti-neurophysin I and II. With the antisera used positive results have been obtained in vertebrate material (Van Leeuwen, pers. comm.). This suggests that substances resembling these peptides are not present in the CNS of *L. stagnalis*. In this respect a study of the peripheral nervous systems seems of interest. Preliminary

observations have shown the occurrence of anti-vasotocin positive cells in the connective tissue of the stomach. Also the fact that positive fibers (e.g., anti- α -MSH fibers in the buccal ganglion) seem to enter the CNS rather than to leave it suggests the presence of peptidergic neurons in the peripheral nervous system (cf. Van Noorden et al. 1980).

The variety of positively-immunostained perikarya and fibers, and their wide and mainly non-overlapping distribution in the CNS of *L. stagnalis* supports the hypothesis that invertebrates possess many substances that are structurally related to vertebrate BAP and suggests that they may play an important part in regulatory processes.

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Accepted December 3, 1980