

Research article

Dopaminergic neurons in the brain and dopaminergic innervation of the albumen gland in mated and virgin *Helisoma duryi* (mollusca: pulmonata)

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Abstract

Background: Dopamine was shown to stimulate the perivitelline fluid secretion by the albumen gland. Even though the albumen gland has been shown to contain catecholaminergic fibers and its innervation has been studied, the type of catecholamines, distribution of fibers and the precise source of this neural innervation has not yet been deduced. This study was designed to address these issues and examine the correlation between dopamine concentration and the sexual status of snails.

Results: Dopaminergic neurons were found in all ganglia except the pleural and right parietal, and their axons in all ganglia and major nerves of the brain. In the albumen gland dopaminergic axons formed a nerve tract in the central region, and a uniform net in other areas. Neuronal cell bodies were present in the vicinity of the axons. Dopamine was a major catecholamine in the brain and the albumen gland. No significant difference in dopamine quantity was found when the brain and the albumen gland of randomly mating, virgin and first time mated snails were compared.

Conclusions: Our results represent the first detailed studies regarding the catecholamine innervation and quantitation of neurotransmitters in the albumen gland. In this study we localized catecholaminergic neurons and axons in the albumen gland and the brain, identified these neurons and axons as dopaminergic, reported monoamines present in the albumen gland and the brain, and compared the dopamine content in the brain and the albumen gland of randomly mating, virgin and first time mated snails.

Background

Dopamine is commonly found in the molluscan central nervous system (CNS). In some gastropods, dopamine has been implicated in the regulation of many physiological activities such as feeding in *Helisoma trivolvis*[1,2], *Limax maximus*[3], *Aplysia*[4,5] and *Lymnaea stagnalis*[6], respiration in *L. stagnalis*[7,8], gill movement in

Aplysia californica[9,10], and egg laying behaviour in *L. stagnalis*[11]. Saleuddin et al. [12] have shown that dopamine stimulates protein secretion from *Helisoma duryi* albumen gland.

The albumen gland in pulmonate snails is an accessory gland of the female reproductive tract (Fig 1). It synthe-

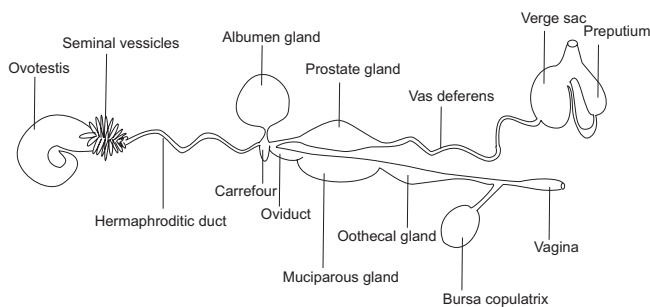


Figure 1
A diagram of the reproductive system of *H. duryi*

sizes and secretes perivitelline fluid (PVF), which is composed mainly of proteins and polysaccharides [13]. Mature oocytes are released by the ovotestis and travel via the hermaphroditic duct into the carrefour, where the albumen gland duct empties. In the carrefour the eggs are fertilized and then are coated with the PVF. The importance of the PVF lies in the fact that it is a major nutrients source for the developing embryo since the oocytes themselves contain very little vitellogenic protein. [14,15]. The secretion of the PVF and the arrival of oocytes at the carrefour must be synchronized, suggesting a precise control of the PVF release [16]. de Jong-Brink and Goldschmeding [17] identified a neuronal plexus in the duct of the albumen gland and the carrefour, which suggested that a nervous mechanism may be involved in the control of the PVF release. Furthermore, catecholamine-containing axons were identified in the albumen gland, carrefour and some other reproductive organs of *L. stagnalis* and other species of pulmonate snails [18,19]. It was also shown that the PVF secretion by the albumen gland could be stimulated by forskolin, cAMP, brain extract [16] and dopamine [12].

Dopaminergic neurons have been localized in the CNS of some snails such as *L. stagnalis*[20–23], *Helix pomatia*[24,25], *Aplysia californica*[5,26] and *Planorbis corneus*[27], and they were mapped in the buccal ganglia of *Helisoma trivolvis*[2,28] but not other ganglia. In *H. trivolvis*, Trimble et al. [28] showed that ^3H -dopamine accumulates specifically in the buccal, cerebral, pedal, left parietal and visceral ganglia, and the left pedal ganglion contains a greater amount of dopamine than the right. Furthermore, using glyoxylic acid Harris and Cottrell [29] and Syed et al. [30] identified a giant dopaminergic neuron in the left pedal ganglion in the CNS of *H. trivolvis*.

The purpose of our study was to establish possible sources of dopamine that may be regulating the secretion of the PVF from the albumen gland. Although Brisson and Collin [18] showed the presence of catecholamine-con-

taining neurons in the albumen gland and carrefour they neither specified the type of catecholamines nor their distribution within the albumen gland and the carrefour. Furthermore, since the albumen gland is known to be innervated by the CNS [17] and the localization of dopaminergic neurons in the CNS of *Helisoma* has not been studied, we focused on these investigations. In this study we describe distribution of catecholaminergic neurons and their axons in the *H. duryi* CNS and albumen gland utilizing well accepted methods that employ anti-tyrosine hydroxylase (TH) IgG and glyoxylic acid [3,20–22,24,25,31,32]. Tyrosine hydroxylase is an enzyme in the pathway of catecholamine synthesis; it converts tyrosine into DOPA and can be used to localize neurons producing catecholamines. The application of glyoxylic acid converts dopamine and other catecholamines into intensely fluorescent 2-carboxymethyl-dihydroisoquinoline derivatives [33].

Using high performance liquid chromatography with electrochemical detection (HPLC-ED) we report monoamines present in the albumen gland and the CNS, identify catecholaminergic neurons found in the albumen gland and CNS as dopaminergic, report the amount of dopamine present in these organs in randomly mating snails and compare it to that of virgin and first time mated snails to determine whether the PVF secretion caused any changes in the amount of dopamine present in these organs. We compared dopamine quantity in snails of different sexual status because research in this lab identified differences between virgin and randomly mating snails, such as differences in egg mass production and synthetic activity of the albumen gland [34]. Furthermore, known centers involved in regulating reproduction (endocrine dorsal bodies and neurosecretory caudodorsal cells) also show changes after mating [35,36]. Following from the above data we formulated a hypothesis: after mating eggs are fertilized and coated with the PVF and since dopamine stimulates the PVF release it might be spent in animals that have mated and formed an egg mass. If this hypothesis is true a difference in dopamine quantity in the albumen gland would be observed between virgin and first time mated animals. Furthermore, since the albumen gland is innervated by the CNS, dopamine is either synthesized centrally in neuronal cell bodies in the CNS and then transported along axons to the albumen gland or locally in axons in the albumen gland. Our experiments were designed to test which mechanism is valid. If first time mated animals have lower levels of dopamine in the CNS the first mechanism applies. Randomly mating animals were treated as control.

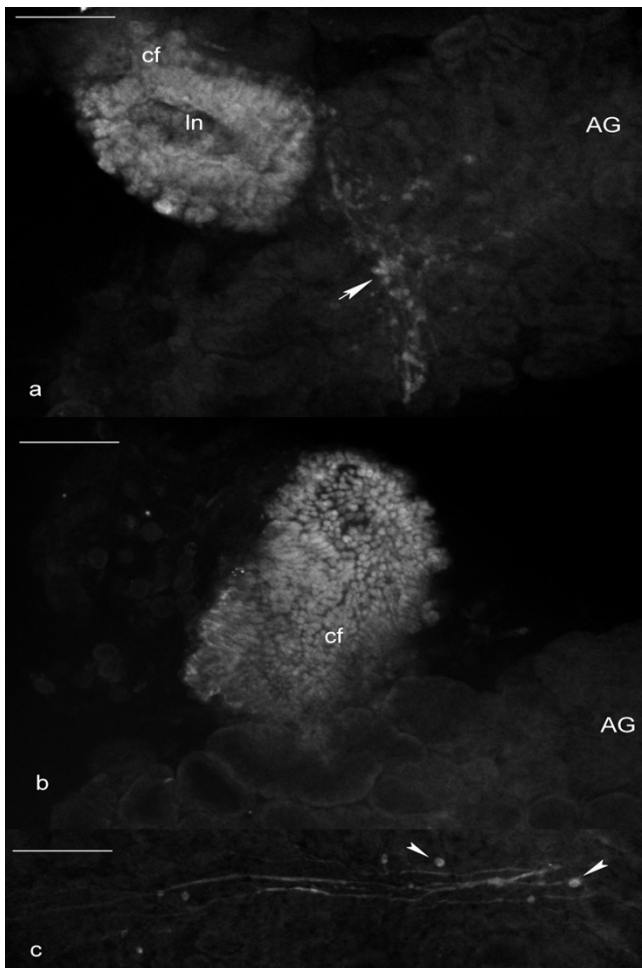


Figure 2

Confocal images of the anti-TH IgG treated tissues. TH-IR neurons are indicated with arrowheads. **a:** a part of the albumen gland (AG) with attached carrefour (cf). Lumen (ln) is clearly visible within the carrefour. TH-IR nerve endings form a tract (arrow) that originates in the carrefour. TH-IR neuronal cell bodies are seen within the tract **b:** a part of the albumen gland with attached carrefour. Closely packed together TH-IR cell bodies form walls of the carrefour. **c:** a part of the albumen gland with TH-IR axons forming the nerve tract with TH-IR neuronal cell bodies visible within the tract. Scale bar = 100 μ m.

Results

In *H. duryi* both the albumen gland (Figs. 2a,2b,2c, 3a,3b,3c) and the CNS (Figs. 4a,4b,4c, 5a,5b,5c, 6a,6b,6c, 7a,7b) contained dopaminergic neurons and fibers. In the CNS their number, size and location are recorded in Table 1. Unless otherwise stated, the mapping illustrates neurons that were found deep in the ganglia. Since HPLC analysis revealed that dopamine is the only catecholamine present in the albumen gland and there is very little norepinephrine in the brain compared with dopamine, the structures that were positive when probed

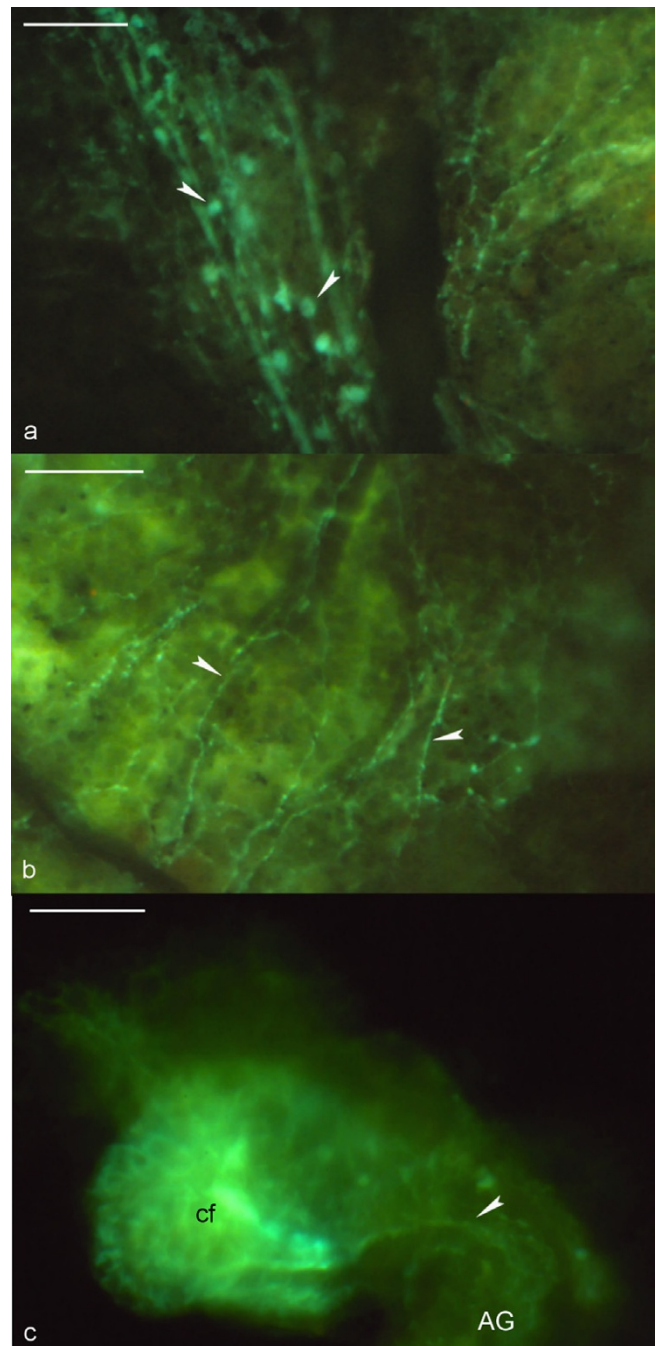


Figure 3

Fluorescence micrograph of glyoxylic acid treated tissues. **a:** the albumen gland. Dopaminergic nerve endings form a tract. Neuronal cell bodies are seen within the tract (arrowhead). **b:** the albumen gland. Dopaminergic nerve endings form varicosities (arrowheads). **c:** a part of the albumen gland (AG) with attached carrefour (cf). Dopaminergic nerve endings form a tract (arrowhead) that originates in the carrefour. Scale bar = 100 μ m.

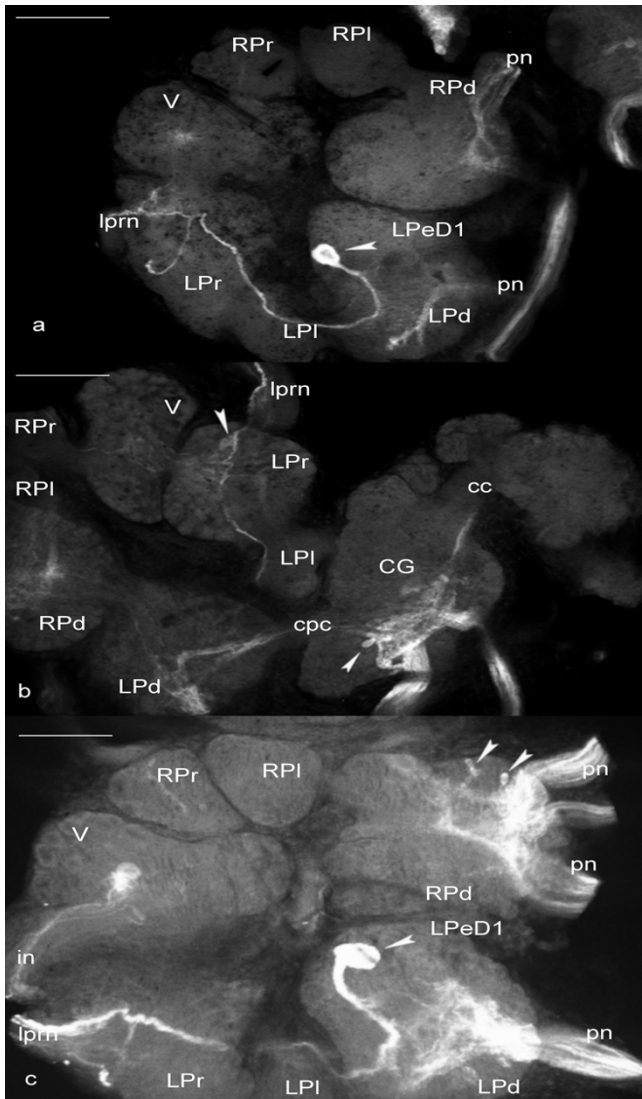


Figure 4

Confocal images of the anti-TH IgG treated CNS. TH-IR neurons are indicated with arrowheads. **a:** the CNS without cerebral ganglia. The giant dopaminergic neuron (LPeD1) is in the left pedal ganglion. TH-IR axons are visible in the ganglia and some nerves. A thick LPeD1 axon is well defined. It passes through the left pleuro-pedal and pleuro-parietal connectives giving off branches and leaving the left parietal ganglion in left parietal nerve (1 μ m). **b:** the CNS with a part of the cerebral ganglia. A TH-IR neuron is present in the left parietal ganglion. TH-IR axons are clearly visible in the ganglia and some nerves. **c:** the CNS without cerebral ganglia. The giant dopaminergic neuron (LPeD1) is in the left pedal ganglion. TH-IR axons are clearly visible in the ganglia and some nerves. A symmetrical group of three TH-IR neurons is clearly visible in the right pedal ganglion. A single TH-IR neuron is present in the visceral ganglion. The structures shown are CG-cerebral ganglia, Pd-pedal ganglia (L-left, R-right), PI-pleural ganglia (L-left, R-right), Pr-parietal ganglia (L-left, R-right), V-visceral ganglion, cc-cerebral connective, cpc-cerebro-pedal connective, in-intestinal nerve, pn – pedal nerve. Scale bar = 100 μ m.

with glyoxylic acid and anti-TH IgG are presumed to contain dopamine.

The fixation preserved the tissues probed with antibodies well. Distribution of dopaminergic neurons in *H. duryi* CNS is diagrammed in Figures 8 and 9, based on examination of 30 brains stained with glyoxylic acid and 20 brains probed with anti-TH IgG. Although some variation was found in brains stained with glyoxylic acid in the number and position of neurons, the distribution of neurons was consistent in brains probed with anti-TH IgG. The TH-immunolabeling was intense and contrasted well with the clear background causing neuronal cell bodies and axons to appear well defined. Neurons stained with glyoxylic acid were of intense fluorescence and generally contrasted well with background except for neurons in the cerebral ganglia where the presence of blue-green fluorescing axons found in the nerves leaving the cerebral ganglia interfered with the identification of the dopaminergic neurons (Fig. 6c). Blue-green fluorescing axons in the brain were of low contrast and staining intensity. The buccal ganglia were stained with glyoxylic acid only.

The distribution of dopaminergic fibers in *H. duryi* albumen gland is diagrammed in Figure 10, based on examination of 20 albumen glands stained with glyoxylic acid and 15 albumen glands probed with anti-TH IgG. The blue-green fluorescence was intense and contrasted well with the clear background whereas the intensity of TH-immunoreactivity was weaker. The number of TH-immunoreactive (TH-IR) neurons and axons found in the albumen gland was less compared to the number of blue-green fluorescing neurons and axons.

Mapping of tyrosine hydroxylase-immunoreactive neurons and comparison with glyoxylic acid-induced blue-green fluorescence

Buccal ganglia

Altogether 48–54 blue-green fluorescent neurons on the dorsal side (Fig. 7a) and 32–36 on the ventral side were observed (Figs. 7b). All the neurons occurred in bilaterally symmetrical groups. A single 15 μ m diameter neuron was found off the center towards the buccal commissure and could be seen from both ventral and dorsal surfaces. Its location suggests its similarity to the B20 neuron in *Aplysia*, which is involved in feeding behaviour [4]. Other neurons were smaller (5–10 μ m). On the dorsal surface a single neuron was located near the posterior buccal nerve and another single neuron was at the root of the ventral buccal nerve. The location of the latter neuron suggests that it is the same neuron as the N1a neuron in *H. trivolvis*[2], and the B65 neuron in *Aplysia californica*[5], which evoke the buccal motor program. Other neurons on both dorsal and ventral surfaces were located

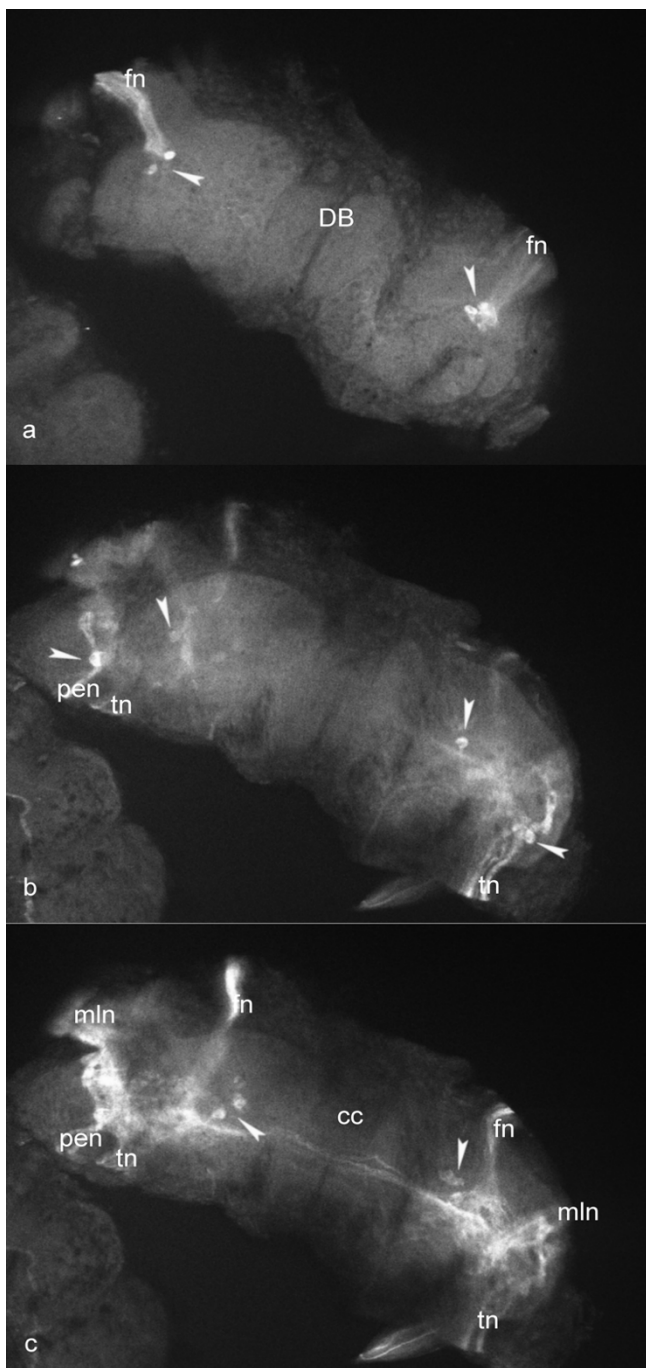


Figure 5
 Confocal images of the anti-TH IgG treated CNS. TH-IR neurons are indicated with arrowheads. **a**, **b** and **c** are a series of confocal sections through the cerebral ganglia with **a** being the first section close to the dorsal surface of the ganglia and **c** being close to the ventral surface. TH-IR axons run through the cerebral commissure (cc) and exit the cerebral ganglia in tentacular (tn), median lip (mm) penis (pen) and frontolateral (fn) nerves. TH-IR neurons are located in the vicinity of axons. Neither TH-IR cells nor axons were found in dorsal bodies (DB). Scale bar = 100 μm.

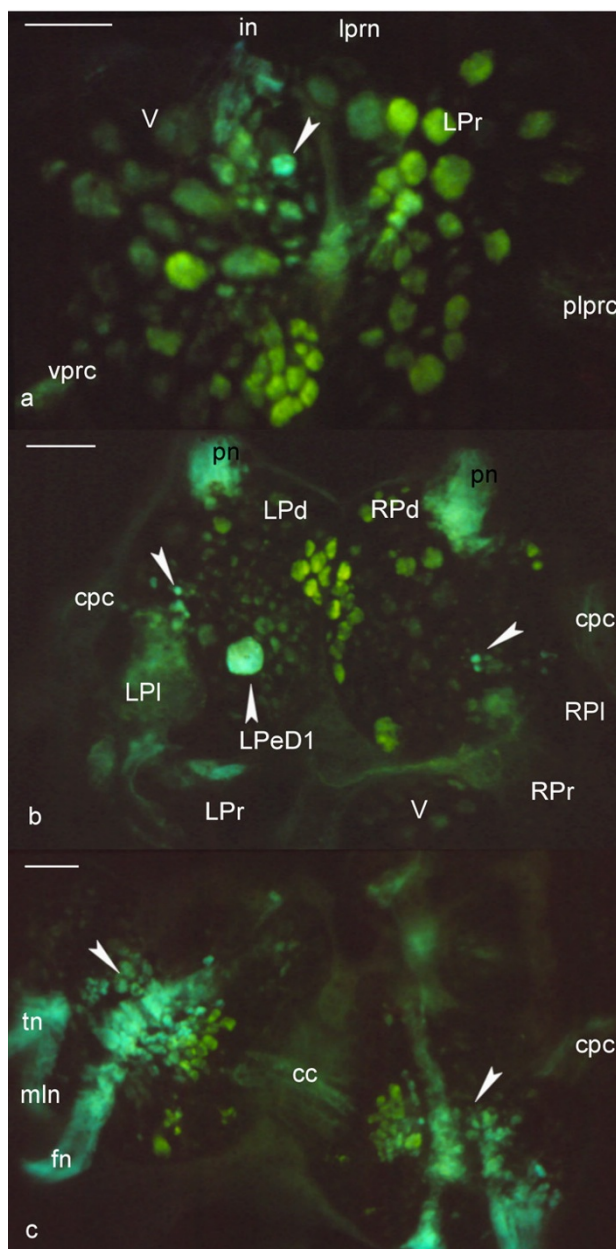


Figure 6
 Fluorescence micrograph of glyoxylic acid treated tissues. Blue fluorescing cells are indicated with arrowheads. **a**: The left parietal (LPr) and visceral (V) ganglia. A single blue-green fluorescing neuron is visible in the visceral ganglion. Blue fluorescing fibers are visible in the intestinal (in) and left parietal (lprn) nerves, visceroparietal and pleuroparietal connectives. **b**: the pedal ganglia. The LPeD1 is clearly visible in the left pedal ganglion. Groups of small dopaminergic neurons are located on the periphery. Blue fluorescing fibers are visible in the pedal nerves (pn) and cerebro-pedal connectives (cpc). **c**: the cerebral ganglia. Cerebral commissure (cc), cpc, median lip (mm), frontolateral (fn) and tentacular (tn) nerves contain blue fluorescing fibers. The structures shown are Pd-pedal ganglia (L-left, R-right), Pl-pleural ganglia (L-left, R-right), Pr-parietal ganglia (L-left, R-right). Scale bar = 100 μm.

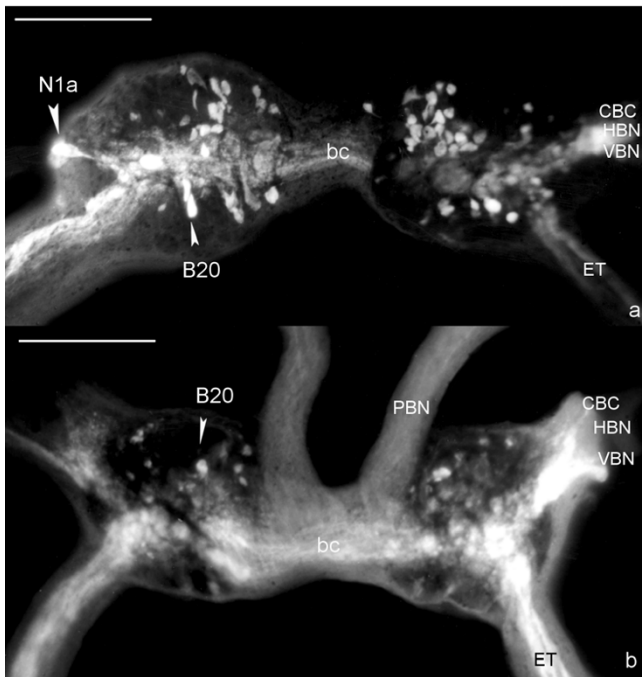


Figure 7
Fluorescence micrograph of glyoxylic acid treated buccal ganglia. **a:** dorsal surface **b:** ventral surface. The structures shown are B20 – neuron that is similar to B20 neuron in *Aplysia*[4], CBC – cerebro-buccal connective, ET – esophageal trunks, HBN-heterobuccal nerve, N1a – neuron that is involved in the control of feeding in *Helisoma*[2], PBN – posterior buccal nerve, VBN – ventral buccal nerve. Scale bar = 100 μm.

in groups composed of three to seven neurons (Fig. 7a,7b). The buccal commissure, cerebro-buccal connective, heterobuccal nerve, ventral buccal nerve, posterior

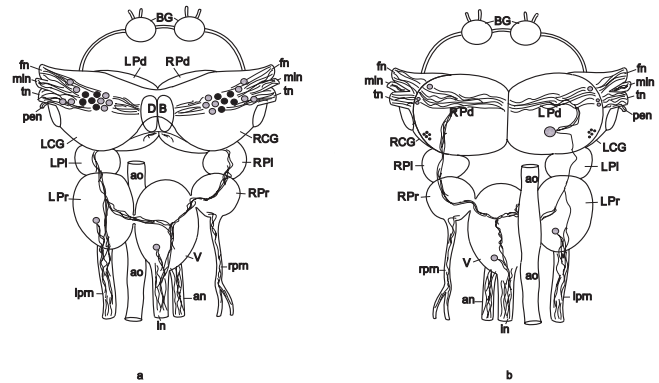


Figure 8
The distribution of dopaminergic neurons and axons in the CNS of *H. duryi*. Filled circles indicate neurons that showed green fluorescence after glyoxylic acid treatment but were not immunostained after anti-TH IgG treatment. Gray circles were both green fluorescent after glyoxylic acid treatment and TH-IR. The axons shown are those probed with anti-TH IgG. **a:** dorsal surface, **b:** ventral surface. Anterior is up. The structures shown are BG-buccal ganglia, CG-cerebral ganglia (L-left, R-right), DB-dorsal bodies, Pd-pedal ganglia (L-left, R-right), PI-pleural ganglia (L-left, R-right), Pr-parietal ganglia (L-left, R-right), V-visceral ganglion. ao – aorta, an – anal nerve, fn – frontolateral nerve, in – intestinal nerve, lprn – left parietal nerve, mln – median lip nerve, pen – penis nerve, rprn – right parietal nerve, tn – tentacular nerve.

buccal nerve and esophageal trunks contained blue-green fibers (Fig. 7a,7b). The number of fibers was high in all nerves except the posterior buccal nerve, which contained only a few axons. The buccal ganglia were stained with glyoxylic acid only due to the difficulty of manipulating them for the antiserum treatment.

Table 1: Comparison of the distribution and size of tyrosine hydroxylase immunolabeled and glyoxylic acid- induced green fluorescent neurons in ganglia of the *H. duryi* CNS.

Ganglia	Tyrosine hydroxylase-immunoreactive		Green fluorescing	
	Size (μm)	Number(Left+Right)	Size (μm)	Number min-max (Left+Right)
Buccal	Not treated with anti-TH IgG		5–15	4
			15–25	76–86
Cerebral	25–50	16	25–50	24–28
Pedal	-	-	5–15	10–12
	15–25	6	15–25	6
Parietal-pleura visceral gangli	75+	1	75+	1
	1–25–50	2	25–50	2
Total	15–75+	25	5–75+	123–139

Cerebral ganglia contained symmetrically distributed neurons, 20–30 μm in diameter. Anti-tyrosine hydroxylase antiserum revealed 16 TH-IR neurons in both left and right ganglia (Fig. 5a,5b,5c). They made up three groups: a pair of neurons was located at the root of the tentacular nerve, another pair was located at the root of the frontolateral nerve (these neurons were located close to the dorsal surface of the ganglia), and a group of 4 neurons was located in the center of the ganglia. The number of blue-green fluorescing neurons varied from 24 to 28. They were distributed in similar manner as TH-IR neurons (Fig. 6c). The cerebral commissure contained a great number of TH-IR fibers that passed through the length of the ganglia and exited in the frontolateral, median lip, penis and tentacular nerves (Figs. 5a,5b,5c). All the neurons were located in the vicinity of the TH-IR fibers. Glyoxylic acid revealed dopaminergic fibers in the frontolateral nerve, median lip nerve, tentacular nerve and cerebral commissure but no fibers were observed within the cerebral ganglia (Fig. 6c). Neither dopaminergic neurons nor axons were found in the dorsal bodies.

Pedal ganglia

The left and right pedal ganglia contained a total of 7 dopaminergic neurons that were both TH-IR and blue fluorescent (Figs. 4a,4b,4c, 6b). Six dopaminergic neurons (10–20 μm in diameter) made up a bilaterally symmetrical group located near the roots of nerves leaving the pedal ganglia. A single giant dopaminergic neuron (LPeD1) was seen in the left pedal ganglion at the pleural side of the ganglion located close to the pedal commissure (Figs. 4a,4c, 6b). The pedal commissure contained a few TH-IR fibers that passed through the length of each ganglion and exited with the nerves leaving the pedal ganglia. Neurons located in the bilaterally symmetrical group were in the vicinity of the nerve fibers. A single thick, intensely stained TH-IR LPeD1 axon passed through the left pleuro-pedal and pleuro-parietal connectives giving off branches and leaving the left parietal ganglion in the left parietal nerve (Figs. 4a,4c). Several TH-IR fibers exited the right pedal ganglion and passed through the right pleuro-pedal, pleuro-parietal, and visceroparietal connectives giving off branches, and connecting with the fibers coming from the left parietal ganglion before exiting via the anal and intestinal nerves. The cerebro-pedal connective also contained TH-IR fibers. In the glyoxylic acid treated brain an additional group composed of 5 small (5–10 μm) neurons was located near the pleuro-pedal connective (Fig. 6b). Glyoxylic acid revealed dopaminergic fibers in the pedal commissure, pleuro-pedal, cerebro-pedal connectives and the nerves leaving the pedal ganglia (Fig. 6b).

Parietal-pleural-visceral ganglia complex

Although a great number of TH-IR fibers were found throughout the parietal-pleural-visceral ganglia complex (see above) no dopaminergic neurons were seen in either right or left pleural ganglia or the right parietal ganglion (Figs. 4a,4b,4c, 6a,6b). The left parietal ganglion contained a single dopaminergic neuron 25–30 μm in diameter located in the path of the TH-IR LPeD1 axon that passed through the complex (Fig. 4b). The visceral ganglion contained a single similar size neuron, also located on the path of dopaminergic fibers going through the complex (Fig. 4c, 6a). With glyoxylic acid treatment no fibers were found within the ganglia, only in the pleuro-pedal, pleuro-parietal, visceroparietal connectives, the left and right parietal, anal and intestinal nerves.

Albumen gland

Dopaminergic nerve endings formed a uniform network that was consistent across all parts of the gland except for the central region, where the axons were parallel forming a nerve tract (Figs. 2a, 2c, 3a,3b,3c). Neuronal cell bodies were present in the vicinity of the tract. The albumen glands of virgin and mated animals showed a similar distribution of dopaminergic neurons and fibers (not shown). The origin of the nerve tract was traced to the carrefour, which was positive when probed with glyoxylic acid and anti-TH IgG (Figs. 2a,2b, 3c). A great number of dopaminergic cells that were closely packed together made up the walls of the carrefour and dopaminergic axons made up the nerve tract that went into the albumen gland.

HPLC

Among monoamines serotonin and dopamine were present in great amounts whereas norepinephrine was present in insignificant amounts in the CNS of *H. duryi* (Fig. 11). In the albumen gland dopamine was the only monoamine. The content of dopamine in the CNS and the albumen gland of randomly mating, virgin and first time mated snails is summarised in Figure 12. No significant difference was found when the concentrations of dopamine in the CNS and the albumen gland were compared between the three experimental groups ($P > 0.05$).

Discussion

Our work reported here followed the studies of a number of researchers [12,16–19]. We demonstrated the presence of the neuronal cell bodies and their axons in the *H. duryi* albumen gland and identified them as dopaminergic, described their distribution and possible origin. The analysis of biogenic amines by HPLC demonstrated that dopamine is a major catecholamine in the CNS and the only monoamine in the albumen gland. We measured the amount of dopamine in the albumen gland and the CNS of randomly mating, virgin and first time mated an-

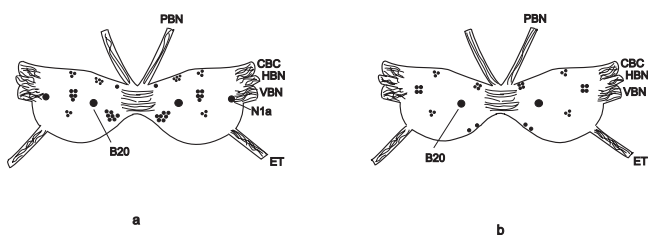


Figure 9
The distribution of dopaminergic neurons and axons in the buccal ganglia of *H. duryi*. **a:** dorsal surface, **b:** ventral surface. B20 – neuron that is similar to B20 neuron in *Aplysia*, [4], CBC – cerebro-buccal connective, ET – esophageal trunks, HBN-heterobuccal nerve, N1a – neuron that is involved in the control of feeding in *Helisoma* [2], PBN – posterior buccal nerve, VBN – ventral buccal nerve.

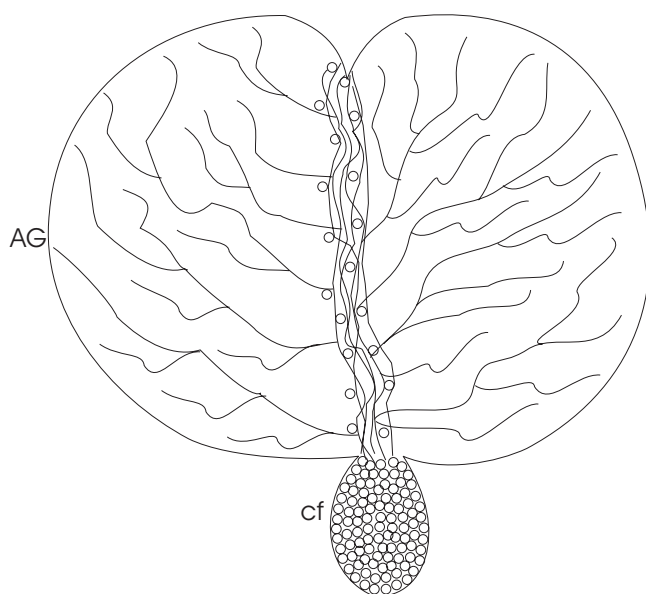


Figure 10
The distribution of dopaminergic neurons and axons in the albumen gland of *H. duryi* (not to scale). Dopaminergic nerve endings form a uniform network that is consistent across all parts of the albumen gland (AG) except for the central region, where the axons are parallel forming a nerve tract. Neuronal cell bodies (open circles) are visible in the vicinity of the tract. The nerve tract originates from the carrefour (cf). A great number of closely packed together dopaminergic cells (open circles) make up walls of the carrefour.

imals. In addition, we mapped dopaminergic neurons in the *H. duryi* CNS.

Comparison of the distribution of the dopaminergic neurons in the CNS of *Helisoma duryi* to that in other gastropods

The distribution of dopaminergic neurons and their axons in the CNS of *H. duryi* is illustrated in Figs. 8 and 9.

Since some norepinephrine was detected by HPLC it is possible that some of the mapped neurons contain norepinephrine and not dopamine. However, the amount of norepinephrine detected by HPLC was small compared with dopamine (Fig. 11), and its presence could not have interfered with our data to any great extent. Our conclusion coincides with that of other researchers who detected a significantly greater amount of dopamine compared with norepinephrine in the CNS of some other gastropods [3,24,28].

Dopaminergic neurons have been mapped in the brains of other snails [5,20–27], and they were mapped in the buccal ganglia of *Helisoma trivolvis* [2,28]. The map of the dopaminergic neurons in the buccal ganglia done by Quinlan et al. [2] is similar to the map obtained in this experiment except for a few additional neurons found in the present study. The locations of some of the neurons identified in the buccal ganglia suggest their similarity to B20, N1a and B65, well studied neurons in other snails [2,5]. Trimble et al. [28] showed that ^3H -dopamine accumulates only in the buccal, cerebral, pedal, left parietal and visceral ganglia of the *H. trivolvis* brain. Their results were consistent with the findings in our study: dopaminergic cells were found only in the buccal, cerebral, pedal, left parietal and visceral ganglia. The left pedal ganglion contained the LPeD1 neuron, which explains why Trimble, et al. [28] found the amount of dopamine in the left pedal ganglion to be greater than in the right pedal ganglion, and confirms findings of other researchers, who identified a giant neuron in the left pedal ganglion of *H. trivolvis* [29,30].

Neither dopaminergic neurons nor their fibers were found in the dorsal bodies even though the dorsal bodies are located in close proximity to the cerebral ganglia and were shown to be involved in the regulation of the albumen gland activity [37,38]. These findings are contrary to the findings of Elekes et. al. [22] who observed small dopaminergic cells and Werkman et. al. [23] who found dopaminergic axons within the dorsal bodies of *L. stagnalis*.

The number of dopaminergic neurons in the CNS of *L. stagnalis* and *Helix pomatia* was much greater than that found in *H. duryi*; however, the majority of neurons were located in the same ganglia. Distribution of dopaminergic neurons in the CNS of *H. duryi* that we mapped was almost identical to that of *Planorbis corneus* in the number of neurons, their size and position in the ganglia. The giant dopamine cell was found in the right pedal ganglion in *L. stagnalis* (but not in *Helix pomatia* or *Aplysia californica*) whereas in *H. duryi* and *Planorbis corneus* it was found in the left pedal ganglion. This difference was described in detail by other research-



Figure 11

Chromatograms showing an example of high performance liquid chromatography with electrochemical detection for the CNS and the albumen gland of randomly mating *H. duryi* (DA – dopamine, 5-HT – serotonin, NE – norepinephrine, LD – L-Dopa). NE eluted prior LD. **a**: elution profile of the CNS. Note the NE peak is higher than the LD peak, and the DA and 5-HT peaks show very clearly; **b**: elution profile of the albumen gland. Note NE and LD peaks are barely visible, 5-HT peak is absent, DA peak is very clear. A distinct peak that elutes prior to serotonin was not identified. Scale bar = 4.8 minutes.

ers [39–42] and can be explained by the fact that *L. stagnalis* is dextrally spiraled whereas *H. duryi* and *Planorbis corneus* are sinistrally spiraled leading to the restriction of some organs to one side of the snail's body. Dopaminergic fibers were found in abundance throughout the brain of *H. duryi* running in similar fashion as in *L. stagnalis*, *Helix pomatia* and *Planorbis corneus*.

Comparison of the distribution of the TH-IR neurons to the distribution of glyoxylic acid-induced blue-green fluorescing neurons

In the cerebral and pedal ganglia a few blue-green fluorescing cells did not correspond to TH-IR neurons. A plausible explanation for this discrepancy can be the overestimation of the number of blue-green cells in the

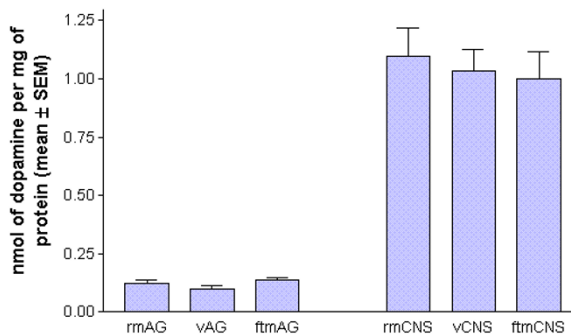


Figure 12

Bar graph showing dopamine content in the central nervous system (CNS) and the albumen gland (AG) of *H. duryi* measured by high performance liquid chromatography with electrochemical detection and expressed in nmol of dopamine per mg of protein (mean \pm SEM), sample size 6. Abbreviations: rm – randomly mating, v – virgin, ftm – first time mated snails.

cerebral ganglia due to the presence of blue-green nerves leaving the cerebral ganglia that may have interfered with identification of the dopaminergic neurons (Fig. 6c). In the pedal ganglia the neurons that TH antiserum failed to recognize were small and possibly contained insufficient amount of the enzyme for the antibodies to bind to. Nevertheless, most of the identified neurons were consistent in their location and size after the use of both techniques. Both techniques showed that all the nerves leaving the brain contained some dopaminergic fibers. More nerve fibers were localized with TH antiserum than with glyoxylic acid and they were of better contrast.

Comparison of the distribution of the dopaminergic axons in the albumen gland of mated and virgin *Helisoma duryi*

Brisson and Collin [18] and Brisson [19] demonstrated the presence of catecholamine-containing axons in the albumen gland, carrefour and some other reproductive organs in *L. stagnalis* and other species of pulmonate snails and concluded that the catecholaminergic system might be important to the carrefour region for transport and orientation of genital fluxes. In our study using HPLC-ED we provide proof that neurons and axons present in the albumen gland and the carrefour are dopaminergic and we describe their distribution in these organs. We observed a tract of dopaminergic axons that runs through the central region of the albumen gland and originates from the carrefour (Figs. 2a,2c, 3a,3c, 10). We also detected the nerve cell bodies in the vicinity of the tract. In other parts of the gland dopaminergic axons extended from the central tract and formed varicosities. de Jong-Brink and Goldschmeding [17] showed that in *L. stagnalis* the gonadal branch of the intestinal nerve,

which originates from the visceral ganglion, innervates the gonad, carrefour, albumen gland and its secretory duct. The albumen gland and its secretory duct also receive intrinsic nervous input derived from neurons in the carrefour region. Therefore, we suggest that the tract and the cell bodies observed within the tract could constitute the intrinsic innervation of the albumen gland that originates from the carrefour. Furthermore, a dopaminergic neuron was found in the visceral ganglion of the CNS of *H. duryi*, and since the albumen gland is innervated by the intestinal nerve that originates from the visceral ganglion this could be the neuron that controls the albumen gland activity. However, in order to establish this premise conclusively a thorough tracing of the axon of the dopaminergic neuron found in the visceral ganglion is required.

HPLC analysis revealed that in the CNS of *H. duryi* serotonin and dopamine were major monoamines whereas norepinephrine was present in insignificant amounts (Fig. 11). These data are in accordance with findings of other researchers [3,24,28]. In the albumen gland dopamine was the only monoamine, which is in agreement with research conducted in our laboratory by Dr. S. Mukai (unpublished results) who demonstrated that of all neurotransmitters tested only dopamine caused a significant increase in protein secretion by the albumen gland.

No marked difference was noticed when the distribution and the number of dopaminergic nerve endings in the albumen glands of randomly mating and virgin animals were compared. In addition, the dopamine concentration in both the CNS and the albumen gland of randomly mating, virgin and first time mated animals (Fig. 12) did not differ significantly between experimental groups. Since the axons of the dopaminergic neurons stained positively with anti-TH IgG and TH is an enzyme in the dopamine synthetic pathway, it is reasonable to assume that dopamine is synthesized in the axons of these cells and therefore is not transported from the cell bodies in the CNS. As a result it is perhaps not surprising that dopamine levels in the CNS did not change much following egg laying. A possible explanation for the lack of difference in the amount of dopamine present in the albumen gland is that HPLC measured the dopamine content only and as soon as dopamine is used to signal the PVF release it is either resynthesized or taken back into nerve terminals by a reuptake mechanism, which commonly occurs in molluscs [43]

Conclusions

Our results represent the first detailed studies regarding the catecholamine innervation and quantitation of neurotransmitters in the albumen gland. Earlier research in

our laboratory established the importance of dopamine in the snail's reproductive system, especially in the regulation of protein secretion by the albumen gland. Our data confirmed and extended these findings by localizing the catecholaminergic neurons and axons in the albumen gland and the CNS of *H. duryi* using glyoxylic acid and anti-TH IgG. Using HPLC-ED we identified these neurons and axons as dopaminergic, reported monoamines present in the albumen gland and the CNS, and compared the dopamine content in the CNS and the albumen gland of randomly mating, virgin and first time mated snails.

The distribution of dopaminergic neurons in the brain of *H. duryi* was similar to that of other gastropod snails. Nerve fibers found in the albumen gland formed a nerve tract with neuronal cell bodies present in the vicinity of the tract in the central region, and a uniform network in other areas of the gland. The nerve tract originated from the carrefour. The visceral ganglion contains a dopaminergic neuron and its axon runs within the intestinal nerve, suggesting that this neuron could control the albumen gland activity. No difference in dopamine quantity in the CNS and the albumen gland of snails of different mating times was observed, which can be explained by the local dopamine production within the albumen gland axons or by a reuptake mechanism.

Materials and Methods

Animals

Randomly mating *H. duryi* were taken from the stock population, which was raised in 6 L tanks containing snails of different maturity, from recently hatched youngsters to sexually mature adults. Virgin snails were isolated 2–3 weeks after hatching and placed in separate plastic containers. All animals were kept at 22°C and photoperiod of 16L/8D. Snails were maintained in dechlorinated water, which was changed once a week, and fed boiled lettuce three times per week, occasionally supplemented with fish food pellets. Virgin animals were allowed to mate and within 24 hours after mating were dissected. This group of animals was termed first time mated snails.

Immunocytochemistry

The brain and the albumen gland of mated *H. duryi* were dissected out in HEPES-buffered *Helisoma* saline (51.3 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, 5.0 mM HEPES, pH 7.4, 120 mOsm/L) and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7 at 4°C for 4 hours. Prior to fixation the albumen gland was subjected to 0.5% protease treatment in phosphate buffer. After fixation tissues were washed for 12 hours in phosphate buffered saline pH 7.2 (PBS) containing 4% Triton X-100 and then processed for immunocytochemistry. The tis-

sues were incubated with a monoclonal mouse anti-TH IgG (Incstar, Stillwater, MN) diluted 1:1000 in PBS with 5% normal goat serum and 4% Triton X-100 for 72 hours at 4°C. Prior to incubation with anti-TH IgG the albumen gland was treated with 0.2% trypsin in PBS and 0.1% CaCl₂ for 20 minutes. Then the tissues were incubated in goat anti-mouse IgG (1:100) conjugated to rhodamine (Sigma-Aldrich, Oakville, On) in PBS with 5% normal goat serum and 4% Triton X-100 for 12 hours at 4°C in the dark. Between each step tissues were washed with PBS several times. After several more rinses the tissues were mounted between two coverslips (rectangular 60 × 22 mm and circular 18 mm) in a 3:1 mixture of glycerol and PBS. Prior to mounting a circle was made on each rectangular coverslip with melted sealing wax (50% Vaseline, 50% dental wax) to prevent tissue distortions. Then the coverslips were affixed with adhesive tape to an aluminium slide (80 × 36 × 1 mm) with a 25 mm circular window so that the circular coverslip faced down within the window. In such orientation the preparation could be viewed from both sides. Specimens were viewed and photographed with a Biorad MRC 600 confocal microscope with a Krypton/Argon laser, YHS filterblock, single channel, excitation filter 568 DF10 nm, dichroic reflector 585 DRLP and an emission filter 585 EFLP nm. The location of dopaminergic neurons was drawn onto standardized maps. No staining was observed in the control experiments where the same procedure was followed except for the absence of primary antiserum.

Glyoxylic acid histochemistry

The dopaminergic neurons and their axons in the albumen gland and CNS were visualized with the glyoxylic acid-induced histofluorescence technique. The brain and the albumen gland of mated and virgin *H. duryi* were dissected out in HEPES-buffered *Helisoma* saline and pinned in a Sylgard-lined dish containing 220 mM glyoxylic acid and 40 mM HEPES, pH 7.0, and incubated for 30 minutes at 4°C. Then tissues were unpinned and arranged on glass coverslip, dried at room temperature for 30 minutes, heated at 100°C for 4 minutes and mounted in mineral oil between two coverslips (rectangular 60 × 22 mm and circular 18 mm). Prior to mounting the same procedure was followed as described above for the immunohistochemistry. The specimens were viewed and photographed with Zeiss optics (Carl Zeiss Canada, Toronto, Canada) that consisted of a mercury lamp HBO 100 W/2 with modulator, vertical illumination by the III RS condenser containing excitation filters BP 405/14, chromatic splitter FT 420, and a barrier filter LP 418. Emission spectra were measured using a Zeiss continuous interference monochromator with a range of 400–700 nm. With these filters two distinct colours were observed: yellow, indicating serotonin-containing neurons and blue-green, indicating catecholamine-containing

neurons [33]. Location of dopaminergic neurons was drawn onto standardized maps. Distribution of serotonin-containing neurons in the *Helisoma* brain was described elsewhere [31,44,45]. No staining was observed in the control experiments where the same procedure was followed except for the absence of glyoxylic acid.

HPLC-ED

Dopamine was assayed by using HPLC-ED. The CNS and the albumen gland of randomly mating, virgin and first time mated snails were dissected in HEPES-buffered *Helisoma* saline then placed in 50 µl HPLC buffer (see later) and kept on ice. The mixture was sonicated, centrifuged at 16,000 rpm for 10 minutes at 4°C and then filtered through a 0.22-µm nylon filter. Ten microliters were injected into a Brownlee RP-18 Spheri-5 HPLC column (4.6 mm × 22 cm). The mobile phase, pumped at 0.7 ml/minute, contained 75 mM NaH₂PO₄ (pH 2.75), 0.3 mM sodium octyl sulfate, 0.05 mM EDTA, 3.5% acetonitrile and 5% methanol. Detection was achieved electrochemically using an ESA model 5100 A detection system coupled to an ESA model 5010 dual coulometric detector (ESA, Inc., Bedford, MA). The first detector was set at 0.025 V to act as a screen and dopamine was detected using the second detector set at 0.2 V at a sensitivity 20 nA. A guard cell inserted before the injector valve was set at 0.5 V to preoxidize possible contaminants in the mobile phase. The output of the second detector was recorded on a Spectra Physics 4270 integrator (Spectra Physics, San Jose, CA), and dopamine was quantified using external standard method. Samples were spiked with dopamine to confirm the identity of the oxidizable substance. In addition, the I-V curve for dopamine was found to be identical for the peak eluting with authentic dopamine. Samples were also spiked with norepinephrine, L-dopa and serotonin to confirm the identity of other peaks. A Bradford protein assay (Bio-Rad, Hercules, CA) was done using human gamma globulin as a standard to determine the amount of protein in each tissue sample. The concentration of dopamine was expressed in nmol of dopamine per mg of protein per tissue.

Abbreviations in the text

CNS – central nervous system

HPLC-ED – high performance liquid chromatography with electrochemical detection

PBS – phosphate buffered saline

PVF – perivitelline fluid

TH – tyrosine hydroxylase

TH-IR – tyrosine hydroxylase-immunoreactive

Abbreviations in the figures

AG – albumen gland

an – anal nerve

bc – buccal connective

B20 – neuron that is similar to B20 neuron in *Aplysia*

BG – buccal ganglia

CBC- cerebro-buccal connective

cc – cerebral commissure

cf – carrefour

CG – cerebral ganglia

cpc – cerebro-pedal connective

DA – dopamine

DB – dorsal bodies

ET – esophageal trunks

fn – frontolateral nerve

HBN – heterobuccal nerve

5-HT – serotonin

in – intestinal nerve

L or 1 – left

LD – L-Dopa

ln – lumen

LPeD1 – giant dopaminergic neuron present in left pedal ganglion

mln – median lip nerve

N1a – neuron that is involved in the control of feeding in *Helisoma*[2]

NE – norepinephrine

PBN – posterior buccal nerve

Pd – pedal ganglion

PI – pleural ganglion

plprc – pleuroparietal connective

pn – pedal nerves

Pr – parietal ganglion

pm – parietal nerve

R or r – right

V – visceral ganglion

VBN – ventral buccal nerve

vprc – visceroparietal connective

TH-IR – tyrosine hydroxylase-immunoreactive

tn – tentacular nerve

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