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Regular Article

Comparison of brain monoamine content in three populations of *Lymnaea* that correlates with taste-aversive learning ability

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To find a causal mechanism of learning and memory is a heuristically important topic in neuroscience. In the pond snail *Lymnaea stagnalis*, the following experimental facts have accrued regarding a classical conditioning procedure known as conditioned taste aversion (CTA): (1) one-day food-deprived Dutch snails have superior CTA memory formation; (2) the one-day food-deprived snails have a low monoamine content (e.g., serotonin, dopamine, octopamine) in their central nervous system (CNS); (3) fed or five-day food-deprived snails have poorer CTA memory and a higher monoamine content; (4) the Dutch snails form better CTA memory than the Canadian TC1 strain; and, (5) the F_1 cross snails between the Dutch and Canadian TC1 strains also form poor CTA memory. Here, in one-day food-deprived snails, we measured the monoamine content in the CNSs of the 3 populations. In most instances, the monoamine content of the Dutch strain was lower than in the other two populations. The F_1 cross snails had the highest monoamine content. A lower monoamine content is correlated with the better CTA memory formation.

Key words: dopamine, *Lymnaea*, octopamine, serotonin, strain

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In invertebrate species, monoamines exert control over various behaviors such as: the fight-or-flight response; subordinate behavior, aggression, sleep, etc [1,2]. Monoamines also play key roles in mediating distinct behaviors in the pond snail, *Lymnaea stagnalis* [3–6]. They especially play an important role in associative learning and memory formation [7,8]. Recently, we established that there was a correlation between the central nervous system (CNS) monoamine content and the ability to learn and form conditioned taste aversion (CTA) memory [9–11]. In the *Lymnaea* CTA exper-

◀ ***Significance*** ▶

Pond snails learn conditioned taste aversion (CTA) and consolidate it into long-term memory. An in-bred Dutch strain has the superior CTA memory formation compared to the Canadian TC1 strain and the F_1 cross snails. We hypothesize that this is due to differences in the monoamine content between the three populations. We measured serotonin, dopamine, octopamine, their precursors and catabolites in the brain. We found that the Dutch strain had the lowest overall monoamine content. Thus, suppression of monoamine content in the brain may be a key factor in conditioned taste aversion in snails.

iments, an appetitive stimulus (e.g., a sucrose solution) is used as the conditioned stimulus (CS) and applying this CS to the lips increases the feeding response. An aversive stimulus (e.g., a KCl solution or an electric shock) is used as the unconditioned stimulus (US). The US causes the snails to immediately cease feeding. In the CTA-training procedure, the CS is paired with the US. After repeated CS-US pairings, the CS no longer elicits feeding, and this CTA persists for at least a month as long-term memory (LTM) [12,13].

We have found that one-day food-deprived snails (i.e., slightly hungry snails) learn and form CTA memory better than fed or five-day food-deprived snails (i.e., hungry snails) [14–18]. Moreover, the CNS monoamine content in one-day food-deprived snails was lower than the fed or the 5-day food-deprived snails [9–11]. Here the following monoamines were measured: (1) serotonin (5-hydroxytryptamine: 5-HT), (2) dopamine (DA) and (3) octopamine (OA). In addition, both their respective precursors and catabolites were also measured. These past observations are suggestive that good CTA learning and memory formation go hand in hand with lower CNS monoamine content.

It has been shown in *Lymnaea* that different strains have varying abilities to learn and form memory; especially as regards an operant conditioning of aerial respiratory behavior [19–25]. The Lukowiak laboratory has recently used two strains of *Lymnaea*: the Dutch strain and a freshly collected strain known as the Canadian TC1 strain. The Dutch strain is the standard in-bred laboratory strain used worldwide. This strain was established from snails originally collected from a polder in Utrecht, the Netherlands in the 1950s and continues to be maintained at Vrije Universiteit Amsterdam in the Netherlands [26] and other laboratories including both the Lukowiak and Ito laboratories. The Canadian TC1 strain was collected from a pond adjacent to the Trans-Canada Highway, Alberta, Canada (at 51.07 N, 114.39 W) [22]. A number of TC1 strain snails were transferred to Ito's laboratory in 2010s. Lukowiak and colleagues found that the Canadian TC1 strain snails have the superior memory in the aerial respiratory operant conditioning in comparison with the Dutch strain snails [22,23].

In Ito's laboratory, the CTA studies have been performed primarily using the Dutch strain, and thus the behavioral experiment parameters for CTA were optimized for that strain [27,28]. Recently, we compared the CTA learning and memory scores between the Dutch, the Canadian TC1 and the F_1 cross (between the Dutch and Canadian TC1 snails) populations. All the snails in the study were food-deprived for 1 day. The Dutch strain snails had the best CTA memory (i.e., rated as 'good'), whereas the Canadian TC1 and F_1 cross snails only exhibited 'average' CTA memory [29]. If hypothesis outlined above is correct (i.e., good CTA learning and memory formation go hand in hand with lower CNS monoamine content), we predict that the monoamine content of the Canadian TC1 and F_1 cross snails will be higher than that of the Dutch snails. We test this hypothesis here.

Materials and Methods

Snails

A more detailed account of the snails used here was described in our previous paper [29]. Briefly, we used two known strains of *Lymnaea stagnalis* Linnaeus, 1758; the Dutch strain and the Canadian TC1 strain. We also used a F_1 cross (the Dutch strain with the Canadian TC1 strain) [22,26]. All snails used here had a shell length of 20–25 mm (i.e., they are adults). The 3 populations were maintained separately in dechlorinated tap water as a substitute for pond water under a 12:12 light-dark cycle at around 20°C. All the snails were fed *ad libitum* on a kind of turnip leaf (*Brassica rapa var. peruviridis*: Komatsuna [in Japanese]) every other day [30–32]. Thus, the three populations used here developed under the same environmental conditions. In addition, they all had similar shell shape and color, however, it needs to be noted that when freshly collected the Canadian TC1 snails are much darker than the other populations when they develop in the laboratory. Furthermore, we have already confirmed that F_1 cross snails can bear eggs and that these embryos hatch from these eggs and appear to develop normally [29]. Thus, we believe that the 3 populations used in the present study have the same metabolic pathways for monoamines. As far as we observed, there were no differences in feeding responses to food between fed snails and food-deprived snails. There were no differences in feeding responses between insulin-treated snails and monoamine-treated snails. These results are supported by the data of pretests for CTA [11].

Monoamine measurements

The methods used for measuring 5-HT, DA, OA plus their precursors and catabolites were as we described previously [9–11,33]. Briefly, snails, which were one-day food-deprived, were quickly frozen using liquid N_2, and the CNS was dissected out in ice-cold *Lymnaea* saline. *Lymnaea* saline consisted of NaCl 50 mM, KCl 1.6 mM, $MgCl_2$ 2.0 mM, $CaCl_2$ 3.5 mM, and HEPES 10 mM (pH 7.9). Ten CNSs were collected from the Dutch strain and the Canadian TC1 strain snails; whereas 6 CNSs were collected from the F_1 cross snails. Each CNS was homogenized in 50 µl of ice-cold 0.1 M perchloric acid containing 5 ng of *N*-ω-methyl-5-hydroxytryptamine oxalate (NMET; Sigma-Aldrich, St. Louis, MO, USA) as an internal standard. After centrifugation of the homogenate (0°C, 21500 *g* (15000 rpm), 30 min), 40 µl of supernatant was collected. We measured the following 14 compounds in each of the three strains: (*1*) tryptophan: Trp; (*2*) 5-hydroxytryptophan: 5-HTP; (*3*) 5-hydroxytryptamine: 5-HT (serotonin); (*4*) *N*-acetylserotonin: Nac-5-HT; (*5*) 5-hydroxyindole-3-acetic acid: 5-HIAA; (*6*) tyrosine: Tyr; (*7*) tyramine: TA; (*8*) octopamine: OA; (*9*) dopamine: DA; (*10*) *N*-acetyltyramine: Nac-TA; (*11*) *N*-acetyldopamine:

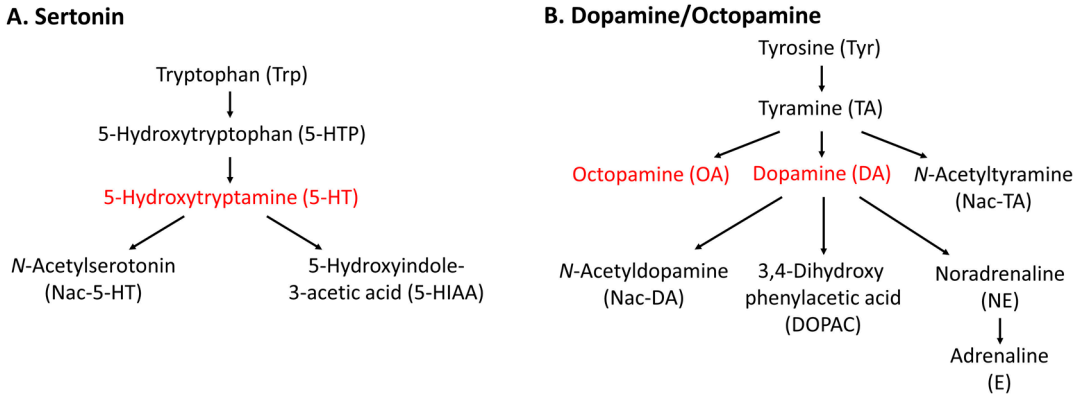

Figure 1 Metabolic pathways of monoamines. Only the monoamines that we could obtain as the HPLC peaks for the *Lymnaea* CNS are indicated. See ref. 54 for the details of these molecules.

Nac-DA; (*12*) 3,4-dihydroxy phenylacetic acid: DOPAC; (*13*) noradrenaline: NA and (*14*) adrenaline: A, using high-performance liquid chromatography with electrochemical detection (HPLC-ECD; EICOM, Kyoto, Japan). The mobile phase containing 0.18 M chloroacetic acid and 16 µM disodium EDTA was adjusted to pH 3.6 with NaOH. Sodium-1-octanesulfonate at 1.85 mM as an ion-pair reagent and CH_3CN at 8.40% (v/v) as an organic modifier were added to the mobile phase solution. The chromatographs were acquired using the computer program PowerChrom (eDAQ Pty, Denistone East, NSW, Australia). The supernatants of samples were injected directly onto the HPLC column. The data regarding 5-HTP, 5-HT, Nac-5-HT, 5-HIAA, TA, OA, DA, Nac-DA and DOPAC contents obtained from the Dutch strain have been published [9–11], but for the better comparison of the monoamine contents, we report these data here under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0) and according to the Author Rights for Scholarly Purposes permitted by the publisher.

Statistics

The data are expressed as the mean±SEM. One-way ANOVA were performed, and significant differences at $P<0.05$ were examined. Where significance was shown, the *post-hoc* Holm's test was performed.

Results

Metabolic pathways

An outline of the general monoamine metabolic pathways is shown in Figure 1. The monoamines measured in the present study were identified by the peak value recorded on our HPLC. This is the standard means for the identifying monoamines. To accomplish this, we first examined the metabolic pathway for 5-HT (Fig. 1A). We obtained the HPLC peaks for tryptophan (Trp), 5-hydroxytryptophan (5-HTP), 5-HT, *N*-acetylserotonin (Nac-5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA). Trp and 5-HTP are precursors of 5-HT, and Nac-5-HT and 5-HIAA are catabolites of 5-HT.

Second, the metabolic pathway for DA and OA was examined (Fig. 1B). The following HPLC peaks were found: tyrosine (Tyr), tyramine (TA), OA, DA, *N*-acetyltyramine (Nac-TA), *N*-acetyldopamine (Nac-DA), 3,4-dihydroxy phenylacetic acid (DOPAC), noradrenaline (NA) and adrenaline (A). Tyr and TA are precursors of OA, DA and Nac-TA. Nac-DA, DOPAC and NA are the catabolites of DA. Further, A is a catabolite of NA. In the present study, we did not find any peaks of 3,4-dihydroxyphenylalanine (L-DOPA) or *N*-acetyloctopamine (Nac-OA).

Monoamine content in the CNS

In all the samples used, the data were obtained from one-day food-deprived snails. In the Dutch snails, a 5-HT metabolite, Trp, was significantly lower ($P<0.01$) than in either the Canadian TC1 and the F_1 cross populations (Fig. 2A). The 5-HTP content in the Dutch strain was also significantly lower ($P<0.01$) than the Canadian TC1 and the F_1 cross snails (Fig. 2B). In addition, the 5-HT content of the Dutch strain snails was significantly lower ($P<0.01$) than the F_1 cross snails but was not significantly different from the Canadian TC1 strain snails (Fig. 2C). Further, the Nac-5-HT content in the Dutch strain was significantly lower ($P<0.01$) than the Canadian TC1 strain but not was significantly different from the F_1 cross snails (Fig. 2D). Finally, regarding 5-HIAA, while lower in the Dutch strain than the other two populations the differences were not significant (Fig. 2E).

Monoamines in the OA and DA metabolic pathway were also measured (Fig. 3). The Tyr content in the Dutch strain was significantly less ($P<0.05$) than in the other two populations (Fig. 3A). The TA content was not significantly different between the 3 populations (Fig. 3B). We also measured the OA content and found that it too was significantly ($P<0.01$) less in the Dutch strain compared to the other two populations (Fig. 3C). Interestingly, the DA content in the Canadian TC1 strain was significantly less ($P<0.05$) than in

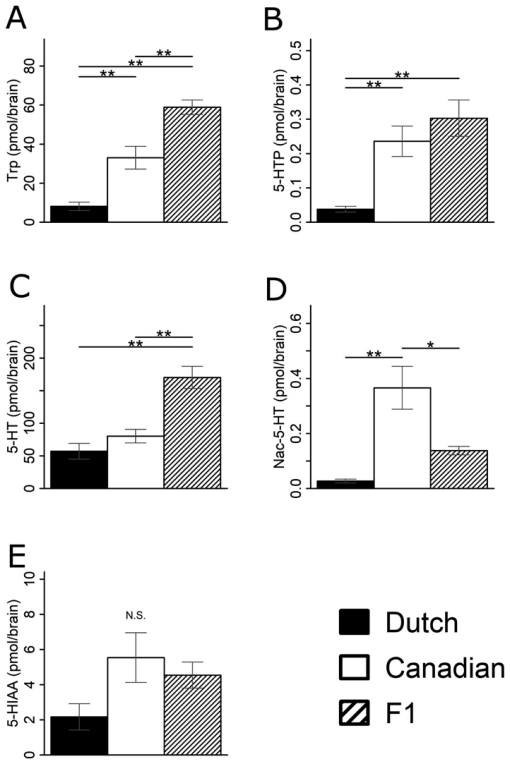

Figure 2 Monoamine contents in the 5-HT metabolic pathway in the *Lymnaea* CNS. Each CNS was isolated from the snails of three populations (i.e., Dutch, Canadian TC1 and their F_1 cross snails), and monoamines were measured independently. The number of CNSs collected from the Dutch strain and the Canadian TC1 strain snails was 10 each; and the number of CNSs collected from the F_1 cross snails was 6. The data are expressed as the mean±SEM. **P*<0.05, **P*<0.01. Trp: tryptophan; 5-HTP: 5-hydroxytryptophan; 5-HT: 5-hydroxytryptamine (serotonin); Nac-5-HT: *N*-acetylserotonin; 5-HIAA: 5-hydroxyindole-3-acetic acid.

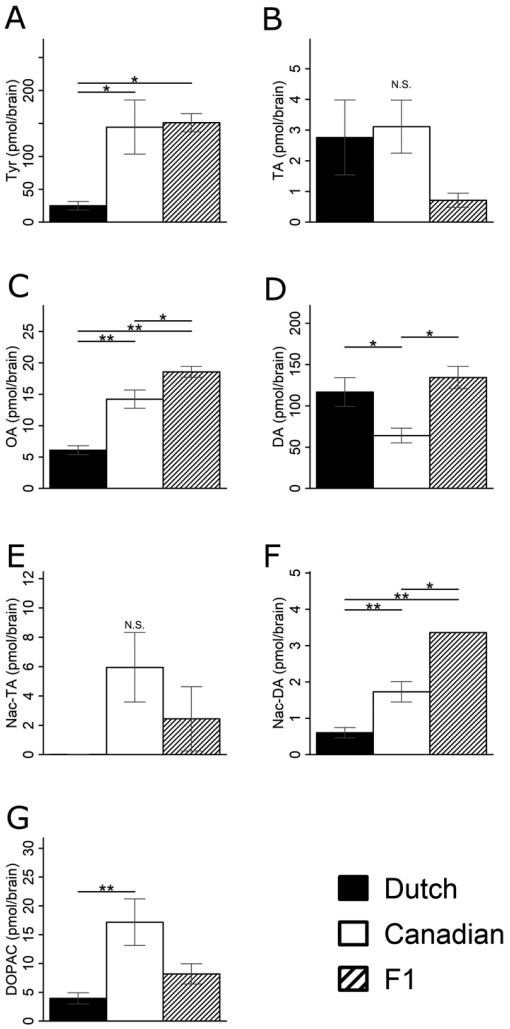

Figure 3 Monoamine contents in the OA and DA metabolic pathway in the *Lymnaea* CNS. The conditions are the same as those in Figure 2. **P*<0.05, **P*<0.01. Tyr: tyrosine; TA: tyramine; OA: octopamine; DA: dopamine; Nac-TA: *N*-acetyltyramine; Nac-DA: *N*-acetyldopamine; DOPAC: 3,4-dihydroxy phenylacetic acid.

either the Dutch strain or the F_1 cross snails (Fig. 3D). This interesting result will be expanded upon below. We were unsuccessful in obtaining a Nac-TA signal in the Dutch strain (Fig. 3E). However, when we measured Nac-DA content, we found that it was the significantly lowest (P<0.01) in the Dutch strain compared to the other two populations (Fig. 3F). Finally, we found that the DOPAC content in the Dutch strain was significantly less (P<0.01) than the Canadian TC1 strain but not significantly different from the F_1 cross snails (Fig. 3G).

Generally, OA and TA in invertebrates are thought to respectively function in a similar manner as noradrenaline (NA) and adrenaline (A) in mammals [34]. However, we found HPLC peaks in our *Lymnaea* samples that correspond to NA and A. There was no statistical difference in the NA content between the Dutch strain and the Canadian TC1 strain (Fig. 4A). However, the CNS content of A in the Dutch strain was significantly less (P<0.01) than in the other two populations (Fig. 4B).

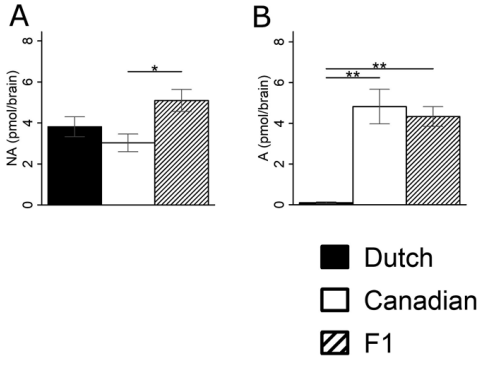

Figure 4 Noradrenaline (NA) and adrenaline (A) contents in the *Lymnaea* CNS. The conditions are the same as those in Figure 2. **P*<0.05, **P*<0.01.

Discussion

These data showed that there is a very strong correlation between CNS monoamine content and cognition; at least as regards the CTA learning and memory in *Lymnaea*. Previously, we found that insulin plays a key role in the CTA learning and memory formation and that insulin causes suppression of CNS monoamines, especially 5-HT [11]. Insulin, or more correctly a molluscan insulin-related peptide (MIP), is causatively involved in CTA-LTM in *Lymnaea* [35–37]. In the Aonuma study, we obtained the following three results [11]. (1) When snails were immersed in a 5-HT solution for 24 h, during which time snails did not have access to food, neither learning nor CTA memory formation was observed. Incidentally, one-day food-deprived snails usually exhibit good learning and memory formation. (2) An injection of insulin into one-day food-deprived snails did not alter the 5-HT content, whereas insulin injection into five-day food-deprived snails decreased the 5-HT content in the CNS. Without the injection, the 5-HT content is low in one-day food-deprived snails and high in five-day food-deprived snails. (3) When one-day food-deprived snails were immersed in a 5-HT solution for 24 h, during which time the snails did not have access to food, and then they were injected with insulin 1 h before CTA training, these snails exhibited both CTA learning and CTA-LTM formation. Thus, we concluded the followings: (A) increasing 5-HT levels in one-day food-deprived snails obstructs CTA learning and memory; and (B) insulin rescues the ability of these snails to learn and form CTA-LTM in those snails.

The relation between nutritional status (i.e., satiety or starvation) and behavioral and physiological responses involved in monoamine pathways have been studied from many perspectives in the nematode *C. elegans*. 5-HT is required for food-dependent modulation of locomotion and olfaction [38,39]. DA content also affects food-dependent changes in locomotion and sensory perception [40,41]. OA and TA play roles in mediating locomotion, aversive behavior and escape responses [42–45]. Furthermore, feeding state modulates nociception through the interaction of monoamine and neuropeptide signaling pathways [46]. Thus, it is not unreasonable to suggest that the level of food satiation alters monoamine signaling pathways in *Lymnaea* and consequently influence CTA learning and memory.

It is presently unclear to us why there is a correlation between the low monoamine content and the superior CTA learning and memory formation in *Lymnaea*. A key neuron in mediating CTA in *Lymnaea* is the cerebral giant cell (CGC), which is serotonergic [47–49]. Our finding that the 5-HT content is lowest in the Dutch snails and that the Dutch snails have the superior CTA memory does not mean that 5-HT synapses are not functional. That is, the effective 5-HT concentration at specific synapses necessary to mediate CTA may be easily achieved, when the total CNS 5-HT concentration is low. This notion receives support from related findings. A dopaminergic neuron, right pedal dorsal 1 (RPeD1), has been shown to be a necessary neuron for LTM formation, when snails receive an operant conditioning training of aerial respiration [50]. Further, the Canadian TC1 snails form LTM faster and better following the operant conditioning than the Dutch strain [22]; yet have a significantly lower DA content (Fig. 3D). Thus, when the two separate learning and memory conditions are examined in *Lymnaea*, the lower monoamine content correlates with the better learning and memory formation.

For this scenario, we advance the following hypothesis. The transporters of monoamines may be altered during CTA-LTM. However, at present we have no data either consistent with this hypothesis or negating it. We do know that there is a serotonin transporter in the CNS of *Lymnaea* [51] as well as vesicular monoamine transporter (GenBank Accession Number: AF484094). We will examine this hypothesis in our future work because this alternation is thought to occur on the basis of the various lines of indirect evidence [52,53].

In conclusion, the monoamine content in the CNS is a key factor for *Lymnaea* CTA. There remains a question why the F_1 cross strain has a high monoamine content in the CNS, because this population exhibits the poorest CTA memory of the three populations examined [29]. We will address this finding in future experiments.

Acknowledgements

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Author Contributions

H. A. measured the monoamine contents. Y. T. prepared the samples of *Lymnaea*. H. A., Y. T. and E. I. analyzed the data and prepared the figures. M. S., K. L. and E. I. wrote the manuscript.

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