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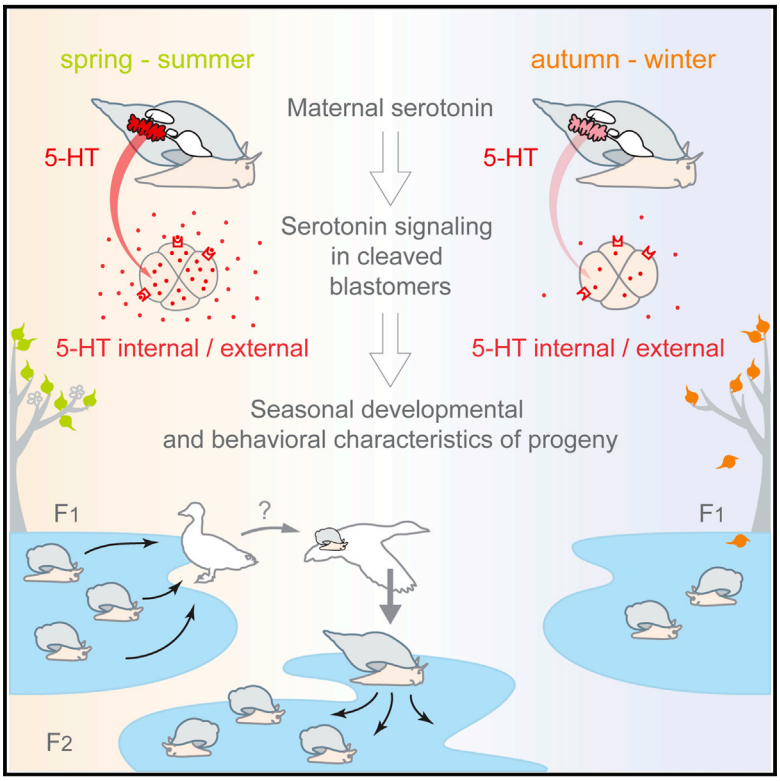
Serotonin Mediates Maternal Effects and Directs Developmental and Behavioral Changes in the Progeny of Snails

Graphical Abstract



Highlights

- Snail uterus shows variation of serotonin levels according to changing seasons
- Serotonin-based signal modulates developmental dynamics and behavior of juveniles
- Balance of intra- and extracellular serotonin is a key for relay of maternal signal
- Serotonylation of proteins is necessary for transmission of maternal effects

Authors

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In Brief

Ivashkin et al. reveal that maternally derived serotonin tunes the developmental dynamics and behavior of snail offspring under changing environmental conditions. The balance of intra- and extracellular serotonin exclusively during the non-neural stage of development, as well as serotonylation of proteins, is crucial for the transmission of a serotonin-based non-genetic signal.

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Serotonin Mediates Maternal Effects and Directs Developmental and Behavioral Changes in the Progeny of Snails

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SUMMARY

Many organisms survive in constantly changing environments, including cycling seasons. Developing embryos show remarkable instant adaptations to the variable environmental challenges they encounter during their adult life, despite having no direct contact with the changing environment until after birth or hatching. The mechanisms by which such non-genetic information is transferred to the developing embryos are largely unknown. Here, we address this question by using a freshwater pond snail (*Lymnaea stagnalis*) as a model system. This snail normally lives in a seasonal climate, and the seasons define its locomotion, feeding, and reproductive behavior. We discovered that the serotonergic system plays a crucial role in transmitting a non-genetic instructive signal from mother to progeny. This maternal serotonin-based signal functions in embryos during a short time window at exclusively early pre-neural developmental stages and modulates the dynamics of embryonic and juvenile growth, feeding behavior, and locomotion.

INTRODUCTION

External environmental factors significantly influence organisms at a non-genetic level and, through this effect, impact their subsequent progeny. The progeny may retain the non-genetic memory up to three generations after the parents have been exposed to changes in temperature, nutrition, dietary composition, hypoxia, photoperiod, social environment, or predator activity (Burton and Metcalfe, 2014). Behavioral programs that are sensitive to parental effects and are exceptionally important for survival include feeding behavior (Van Allen and Rudolf, 2013), escape from predators (Allan et al., 2014), learning, and dispersion (Massot and Clobert, 1995).

In general, the non-genetic transfer of information to progeny is widespread in nature and varies in terms of molecular mechanisms (Dias and Ressler, 2014; Duckworth et al., 2015). Although the exact molecular mechanisms underlying maternal effects, especially on developmental plasticity, remain mostly unknown, some have recently been documented. For example, Ben Dantzer, Andrew McAdam, and their co-authors demonstrated that higher maternal glucocorticoid levels in wild mammals result in accelerated growth of progeny (Dantzer et al., 2013).

It is worth mentioning that early developmental stages are especially crucial and are key for the implementation of parental effects (Burton and Metcalfe, 2014). Many embryonic features that will influence the resulting fitness and future reproductive success of progeny are initiated during early development (Uller, 2008). Moreover, there is a huge number of animals for which the gametogenesis and early cleavage stages represent the final moments of guaranteed contact between parent and offspring and, thus, the window of time allowing the transmission of non-genetic adaptive information to progeny.

The serotonergic system is specifically known for its importance during early developmental stages in a multitude of animal forms. Serotonin (5-HT) modulates the maturation of oocytes and sperm in the reproductive systems of both vertebrates and invertebrates (Buznikov et al., 1993; Cerdá et al., 1995; Stricker and Smythe, 2000). 5-HT is also responsible for the correct development of left-right asymmetry in amphibian embryos (Beyer et al., 2012; Fukumoto et al., 2005). It appears evolutionarily before the formation of the first neurons and is a well-recognized component of ancient and archetypical signaling systems. Additionally, 5-HT plays an important role in the regulation of propagation cycles because it is concomitantly involved in such controls at the level of the nervous system (Tiwari et al., 2006) and reproductive system (Buznikov et al., 1993).

One of the largest challenges is represented by the question of how environmental information and behavioral experiences are

encoded and transmitted from the nervous system to the gonads of parents and subsequently to progeny (Dias and Ressler, 2014). 5-HT and the serotonergic system play important roles both inside and outside of the nervous system and thus represent plausible candidates for connecting experiences rooted in parental neural structures with developmental dynamics in progeny that later affect multiple aspects of their adaptive fitness as adults.

To address serotonergic-system-related mechanisms that mediate the non-genetic information transfer and parental effects, we selected the freshwater pond snail *Lymnaea stagnalis* as a model organism that traditionally survives under constantly changing climatic conditions. The locomotion, feeding, and fertility of *L. stagnalis* snails demonstrate season-dependent characteristics in natural habitats (Dogterom et al., 1985). Concomitantly, climatic seasonal cycles provide an array of separable factors that may cause distinct parental effects (Chotai et al., 2001; Rickard et al., 2012).

L. stagnalis can be defined as a very successful mollusk with incredible flexibility that can be found across the Holarctic region. Previously, it has been shown that *Lymnaea* snails may disperse under natural condition to distant water habitats by riding on the feathers and inside the gut of waterfowl and similar birds (Boag, 1986; Kawakami et al., 2008; van Leeuwen et al., 2012). Therefore, the fast locomotion of juveniles increases their chances for such dispersion by clinging to feathers exclusively during the summer season (birds do not migrate large distances; therefore, the snails do not dry in the air). Unlike many other animals, *Lymnaea* snails may disperse to the new water habitats only during juvenile stages, during which the intensity of locomotion is strictly proportional to successful encounters with the feathers or stomach of birds. The possible benefits of maternal effect-driven phenotypic adjustments may include more efficient dispersion, feeding, survival skills, and fertility rates that are tuned according to different season-dependent features such as the availability of birds, food, periods of lower temperature, or danger of predators.

Taken together, these ecological features and cycling parameters, as well as adaptive flexibility, render *L. stagnalis* an excellent model for the study of the role of 5-HT in refining the flexibility of offspring surviving under different environmental conditions. Here, we address the role of the serotonergic system in transmitting a non-genetic adaptive signal as a principle and also provide insight regarding the molecular mechanism mediating the reception of parental information in *L. stagnalis*.

RESULTS

Seasonal Oscillations of 5-HT within the Female Reproductive System Correlate with Offspring Characteristics

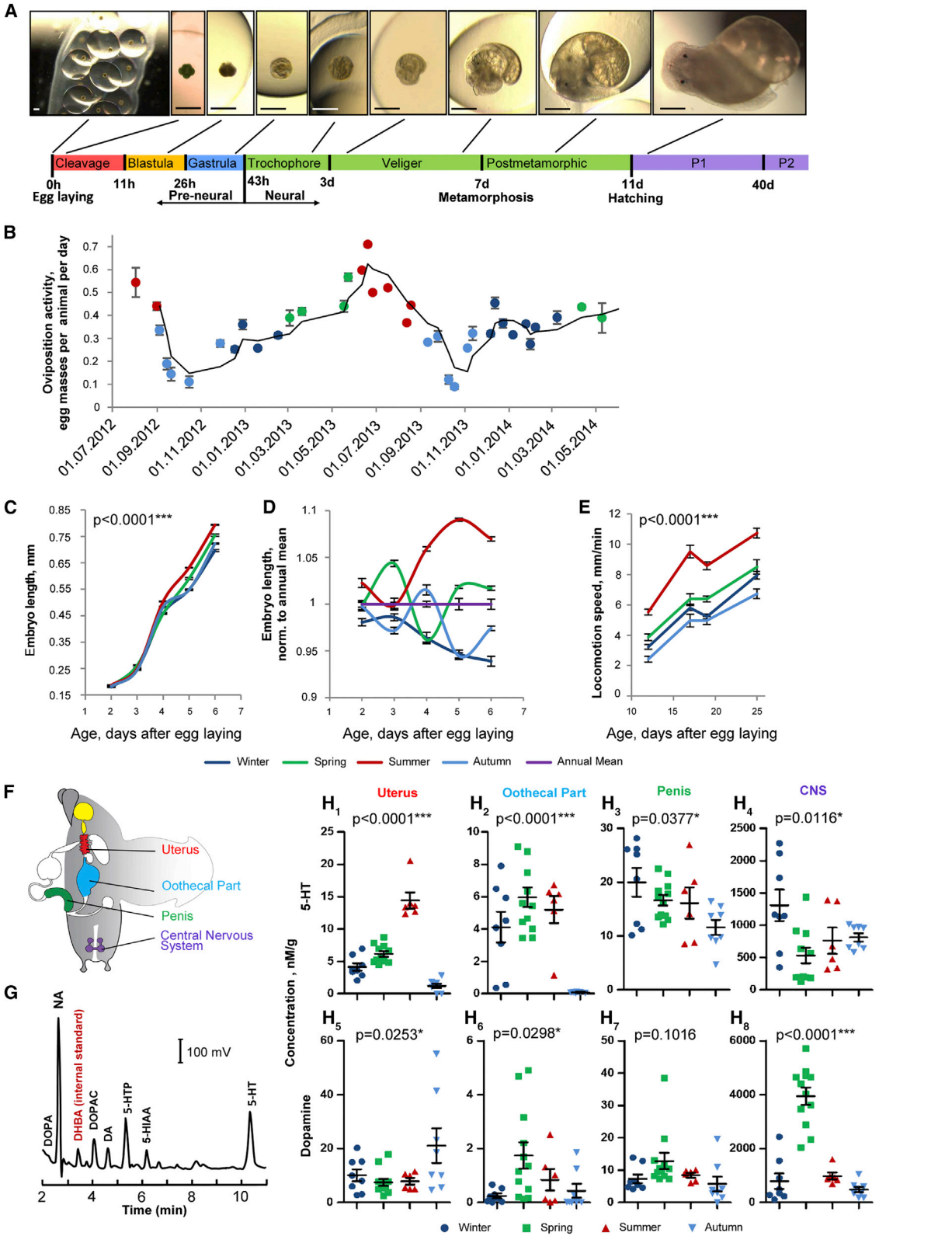
Under natural conditions, the life of this type of snail depends heavily on changing temperatures and the availability of food. Thus, locomotion, feeding, and fertility demonstrate season-dependent characteristics under natural situations (Dogterom et al., 1985). Laboratory culture of *L. stagnalis*, despite the maintenance of stable conditions for over 40 years, still demonstrates variations in oviposition activity and embryonic developmental dynamics according to the changing seasons of the year (Figures 1B–1E). We found that oviposition activity gradually increased from winter to summer and strongly decreased by autumn (Figure 1B). While the overall embryonic development from oviposition until hatching (Figure 1A) was approximately equivalent (11 ± 0.5 days), the fastest embryo development occurred during summer and in spring. In contrast, the slowest rate of development was observed during autumn and in winter (Figure 1C). Embryos that developed during opposite seasons demonstrated phase shifts of oscillating embryonic developmental dynamics. The length of the embryo at each stage normalized to the annual mean expressed striking season-dependent patterns: the summer wave mirrored the winter valley, while the spring increases on the third and sixth days after egg laying mirrored the corresponding autumn dips (Figure 1D). In addition, we observed heterochrony-like effects: premetamorphic (trochophore and veliger stages, 2–4 days after egg laying) embryos demonstrated the fastest and slowest development during spring and autumn, respectively, while the fastest and slowest development during the metamorphic stages (5–6 days after egg laying) occurred during summer and winter, respectively. Immediately after hatching, juvenile *Lymnaea* snails search for new habitats by taking advantage of intensive terrestrial locomotion (Boag, 1986). We discovered that, during the entire period of active locomotion (12–25 days after egg laying), summer-born juvenile individuals were exceptionally efficient at dispersion. In contrast, the snails that hatched during the autumn, winter, and spring tended to remain in their local environment (Figure 1E). Thus, offspring born in different seasons demonstrated defined variations in developmental dynamics and locomotion-based dispersion behavior.

To address the potential mechanism that might mediate these season-dependent effects, we tested the possibility that monoamines serve as an adaptive signal from mother to progeny. Previously, several research teams have demonstrated that variations in monoamines correlate with seasonal behavior (Chotai et al., 2003). Moreover, the serotonergic system controls oviposition, locomotion, and the developmental tempo in molluscs (Glebov et al., 2014; Voronezhskaya et al., 2004; Winlow and Haydon, 1986). Thus, we directly examined whether 5-HT and other monoamines constitute a signaling system that translates seasonal information in *L. stagnalis*.

First, we discovered that the levels of 5-HT and dopamine (DA) cycle in the uterus (pars contorta), oothecal region of the oviduct (oothecal part), penis, and CNS, according to different seasons (Figures 1F–1H_8). The peak level of 5-HT in the uterus (Figure 1H_1) correlated with high oviposition activity and the fastest embryonic development during summer. We never observed comparable correlations of DA levels in the reproductive system. However, in the CNS, the DA level appeared to be higher in the spring compared with the other seasons (Figure 1H_8).

5-HT Accumulates in Serotonergic Cells of the Female Reproductive System after Ectopic Application of 5-HTP

In the following experiments, we identified a dense network of 5-HT-containing cell bodies and their long projections in the



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uterus (Figures 2A–2E), while other parts of the snail reproductive system (oothecal part, oviduct, and penis) showed exclusively 5-HT-immunoreactive projections (Figures 2I, 2K, and 2M). Numerous 5-HT-positive cell bodies and interconnected fibers were located directly underneath the epithelium along the uterus duct folds (Figures 2B–2E), representing the key location in which the membrana interna and membrana externa are secreted to cover zygotes (Koene, 2010). Some 5-HT-immunoreactive processes appeared to supply the surrounding muscle fibers (Figures 2B and 2E). In the oothecal region and penis, 5-HT-containing fibers were found only in association with muscles (Figures 2I and 2M).

To assess whether the cells constituting the serotonergic plexus in the uterus could specifically and selectively modulate the amount of 5-HT depending on the amount of the available biochemical precursor, we incubated mother snails in a range of 5-HTP (the immediate biochemical precursor of 5-HT) concentrations, which resulted in a highly selective and strong increase (Figure 2O$_1$) in intracellular 5-HT by up to 25-fold, specifically in the serotonergic cells of the uterus (Figures 2F–2H). In contrast, the oothecal region and CNS showed maximum increases by 2-fold that were not statistically significant (Figures 2O$_2$ and 2O$_4$). No changes were detected in the penis (Figure 2O$_3$). The results of high-pressure liquid chromatography (HPLC) further confirmed the immunochemical findings (Figures 2I–2N). Concomitantly, the level of DA slightly decreased in the uterus (Figure 2O$_5$) and did not change in any of the reproductive organs (Figures 2O$_6$ and 2O$_8$). Of note, the 5-HTP-responsive serotonergic plexus in the posterior part of the uterus (Figures 2F–2H) represents the location of closest contact between maternal tissues and the zygote. Thus, the discovered serotonergic plexus likely mediates the transmission of 5-HT-based signals to progeny.

Characteristics of the Progeny Shift in Response to Experimental Modulation of Maternal 5-HT

Next, we questioned whether the increased amount of 5-HT in the reproductive system of the mother could affect the behavior of the progeny. To achieve this goal, we incubated the mother snails in 5-HTP, removed it from the cultivation medium by washing, and analyzed developing embryos and juveniles after hatching (Figure 3A). We found that the oviposition activity of the mother snails increased. In addition, other features that are known to be related to 5-HT, such as embryonic developmental dynamics (Glebov et al., 2014), rotation of the embryos inside the egg (Goldberg et al., 2011), and feeding activity (Elliott and Susswein, 2002), as well as their locomotion-based dispersion (Winlow and Haydon, 1986), were altered in the experimental progeny. Furthermore, the amount of released egg masses increased by up to 5- to 6-fold during the initial 12 hr and was maintained at levels that were 1.5–2 times higher during the subsequent week (Figure 3B). It decreased to control levels only within the second week after treatment. Concomitantly, the number of eggs per egg mass did not differ from the controls (data not shown). The embryonic rotations under the experimental conditions accelerated 1.5–1.8 times (Figure S1A; Movie S1). The speed of juvenile locomotion appeared to be elevated 1.2- to 2-fold on the first day after hatching (12 days after egg laying) and maintained at higher than normal levels for up to 2 weeks (25 days after egg laying; Figures 3C$_1$–3C$_3$; Movie S2), in parallel with a significant increase in escape behaviors (crawling above the water line; Figures 3D$_1$ and 3D$_2$). HPLC analysis demonstrated a content of 5-HT that was 1.5 times higher in the head complex of 12-day-old experimental juveniles compared with age-matched control animals (Figure 3F). In contrast, the food consumption of the experimental juvenile snails decreased up to 25% simultaneously with their growth retardation (Figures S1B$_1$–S1C$_2$). The percentage of naturally occurring exogastrulae, a rare (less than 0.01%) season-specific (only in summer) developmental abnormality, increased up to 4% after the incubation of mother snails with 5-HTP (Figure 3G). Historically, researchers have utilized Li^+-induced exogastrulation as a tool to discover some key morphogenetic mechanisms in *L. stagnalis* (Raven, 1958). In our experiments, the induction of exogastrulae by incubating the mothers with 5-HTP (Figures 5C–5F) appeared in an all-or-nothing manner. All of the embryos that successfully passed the gastrulation stage demonstrated normal subsequent development (Figure S4B). The 5-HTP-induced exogastrulae always demonstrated a normal distribution of ciliated and non-ciliated ectodermal cells (Figures 5G and 5H). Most importantly, the developmental

Figure 1. Seasonal Changes in Oviposition Activity, Embryonic Developmental Dynamics, Juvenile Locomotion, and Monoamine Concentrations in Different Organs of *L. stagnalis*

(A) *Lymnaea stagnalis* normal development from the zygote stage through cleavage, blastula, gastrula, trochophore, and veliger stages up to the post-metamorphic and adult-like juvenile snail (P1 and P2). Scale bars, 250 μm.

(B) Monitoring of oviposition activity in laboratory animals living under stable conditions (based on the colony of 300 mature animals). Note the season-correlated propagation cycles.

(C and D) Seasonal embryonic growth curves. (C) Length of 2- to 6-day-old embryos born in winter, spring, summer, and autumn. (D) Normalization to the annual mean size. Note the specific pattern of growth rate changes in spring, summer, autumn, and winter. n = 105–130 for each point, two-way ANOVA followed by the Bonferroni post hoc test.

(E) Speed of terrestrial locomotion related to the locomotion-based dispersion of juveniles born in different seasons, mm/min. Note the increased rate of locomotion during summer. n = 50–150 for each point, two-way ANOVA followed by the Bonferroni post hoc test.

(F) Organs of *L. stagnalis* analyzed for monoamine content.

(G) Characteristic chromatogram for the detection of monoamines in the uterus.

(H$_1$–H$_8$) HPLC measurements of 5-HT (H$_1$–H$_4$) and dopamine (H$_5$–H$_8$) content in the uterus (H$_1$ and H$_5$), oothecal region (H$_2$ and H$_6$), penis (H$_3$ and H$_7$), and CNS (H$_4$ and H$_8$) during different seasons. Note the high 5-HT levels in the uterus during summer. n = 6–10, three animals in each measurement; one-way ANOVA followed by Bonferroni's multiple comparison post-test.

Data are given as mean ± SEM. See also Figure S7.

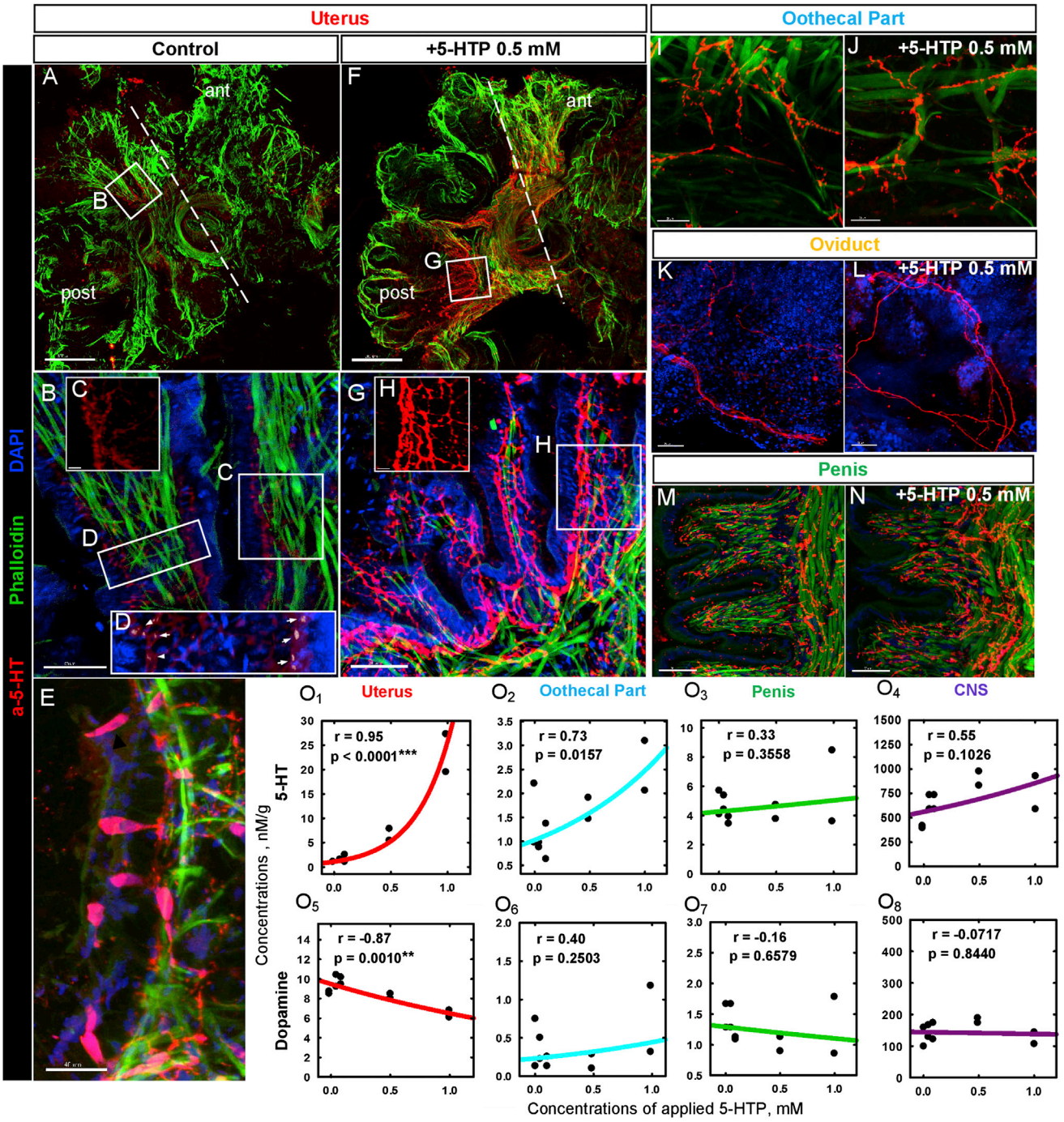


Figure 2. Changes in Serotonin and Dopamine Levels in Different Organs of *L. stagnalis* after 5-HTP Treatment

(A–N) Localization of 5-HT in different tissues: in the uterus (A–H), oothecal region (I and J), oviduct (K and L), and penis (M and N) in both control and 5-HTP-treated mature snails (48-hr incubation). Staining with antibody against 5-HT (red), phalloidin (green), and DAPI (blue). Note the 5-HT$^+$ fibers forming a dense network in the uterus. (D) Arrows denote the nuclei of 5-HT$^+$ cells (white) visualized by the co-localization of 5-HT with DAPI. (E) High magnification of 5-HT$^+$ cells located in the posterior region of the uterus. Note the 5-HTP-induced enhancement of anti-5-HT immunostaining specifically in the uterus (F–H), post and ant represent posterior and anterior parts of the uterus, respectively. Scale bars, 300 μm for (A) and (F); 50 μm for (B), (G), and (K–N); 10 μm for (C) and (H); 40 μm for (E); and 30 μm for (I) and (J).

(O_1–O_8), HPLC measurements of 5-HT (O_1–O_4) and DA (O_5–O_8) in the uterus (O_1 and O_5), oothecal region (O_2 and O_6), penis (O_3 and O_7), and CNS (O_4 and O_8) after incubation in a range of 5-HTP concentrations (0.05–1 mM). Note the significant increase in 5-HT content and decrease in DA content specifically in the uterus. Three animals per each point were assessed, and the experiments were repeated.

dynamics of embryos born in autumn from mothers treated with 5-HTP dramatically differed from the normal autumn seasonal developmental dynamics, with two dips on days 3 and 5. In fact, the developmental pattern of the autumn-born treated embryos accelerated from 4 to 6 days in a manner similar to that of embryos born in the summer to naive mothers (Figure 3E_2; compare with Figure 1D). In agreement with this finding, the developmental pattern of embryos born in the spring from 5-HTP-treated mothers also exhibited patterns similar to the embryos born in the summer from naive mothers (compare Figures S1E_1 and S1E_2).

In contrast to the effects of 5-HTP, incubation of mother snails in chlorpromazine (CPZ) induced depletion of 5-HT within the serotonergic plexus in the uterus (Figures S2A_1–S2C_1). It also caused a dramatic decline in 5-HT content in the oothecal region (Figures S2C_2) and in the CNS (Figure S2C_4), as well as a decline in DA in the uterus (Figure S2C_5). As a result, the embryonic growth of the first generation decelerated (Figure S2E_1). However, the 5-HT level in the experimental juveniles did not differ from that in the controls (Figure S2D), and only a transient decrease in juvenile locomotion could be detected from days 25–27 (Figure S2E_2). The second generation derived from the CPZ-treated snails showed no differences compared with the controls (Figures S2F_1 and S2F_2).

All of the aforementioned changes appeared to be expressed in a concentration-dependent manner. Taken together, these results demonstrated that experimental increases (but not decreases) in 5-HT in the uterus of mother snails resulted in developmental and behavioral shifts in the characteristics of their progeny from low 5-HT seasons (autumn and spring) to a high 5-HT season (summer; Figure S1F).

Next, we examined the stability of the maternal effects on the scale of two succeeding generations of progeny. The results clearly demonstrated increased migratory and fertility capacities of the first-generation offspring of the mothers with increased 5-HT (Figures 3C_1–3C_3 and 3I_1–3J; Figure S1D). These capacities may well provide for the dispersion phase that is immediately followed in nature by the local expansion phase necessary for conquering a new habitat after the successful arrival. By contrast, the second-generation progeny displayed an increase in survival (Figure S3B) accompanying normal dispersion rates compared with the control (Figure S3C). Thus, this second generation may ensure survival in the new habitat following the arrival and primary expansion phase.

To better understand the relationship of the ecological features with maternal signals, we experimentally simulated the classical overcrowding situation in a relatively small habitat. 5-HTP-treated and control snails were exposed to conditioned water from overcrowded tanks. The 5-HTP-treated embryos hatched up to 4-fold faster (Figure S1G) and survived better (Figures 3I_1 and 3I_2) compared with the control population in response to the conditioned water. In combination with increased locomotion skills, these phenomena may lead to higher chances of spreading to a new habitat if they are interpreted within the ecological context.

Taken together, the present experimental data strongly support the capacity for seasonal fluctuations of 5-HT in the uterus of a mother snail to significantly change the developmental and behavioral characteristics of the progeny.

Pre-neural Developmental Stages Represent a Unique Time Window for the Transmission of 5-HT-Based Signaling

In the following steps, we addressed which developmental stages were sensitive to 5-HT and which molecular mechanisms were responsible for 5-HT-mediated adaptive variations in *L. stagnalis*. First, we found that 5-HT was present and uniformly distributed among blastomeres beginning at the two-blastomere stage and subsequently during cleavage until the late blastula stage (Figures 4A–4E). At the veliger stage, 5-HT was already restricted to the cells and processes of the developing nervous system (Figure 4F). The incubation of eggs in tryptophan significantly raised the level of 5-HT within 12- to 16-blastomere embryos, while the same incubation did not change the 5-HT level at the blastula stage (Figure 4G_3). Incubation in 5-HTP (immediate 5-HT precursor) led to a 4- to 8-fold increase in the level of 5-HT within all embryonic cells at all investigated stages. However, the addition of external 5-HT to the culture medium increased the intracellular level of 5-HT only after the early blastula stage (Figures 4G_1–4G_3). The application of ASP^+ (specific fluorescent indicator of monoamine uptake) to living embryos confirmed that the 5-HT was taken up from the external medium by all blastula cells and not cells from cleaved embryos (four-blastomere stage). This uptake was blocked by the membrane 5-HT transporter (SERT) inhibitor citalopram (Figures 4H_1–4H_3). Therefore, the main components of the 5-HT synthesis and uptake systems were already present at the pre-neural (cleaved blastomeres and blastula) early developmental stages of the *L. stagnalis* embryo.

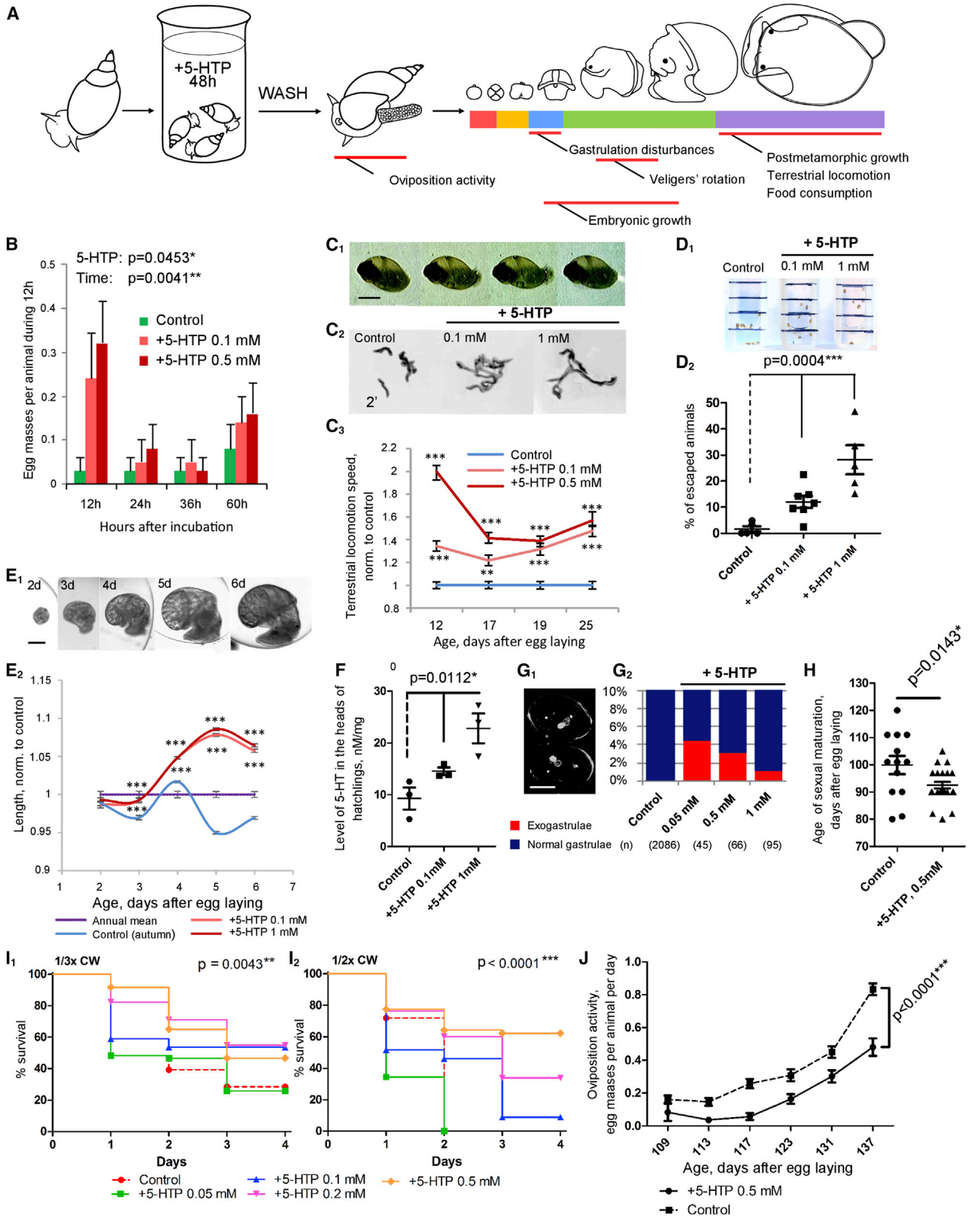
Second, the direct incubation of eggs with 5-HTP resulted in an increased proportion of exogastrula rather than normal gastrula formation (Figure S4). The concentration-dependent characteristics of the observed effect (Figures S4A and S4C) allowed us to examine specific time windows of embryo sensitivity to exogenous 5-HT. Apparently, only the cleavage stages before blastula formation were sensitive to 5-HT manipulation. The treatment of embryos at later time points never resulted in such an effect (Figure 5A; Figure S4B).

Finally, we found significant increases up to 1.5 times in the locomotion-based dispersal of juvenile snails after 5-HTP treatment exclusively. This effect was concentration-dependent and was maintained up to 25 days after egg laying (Figures 6D and 6E_1). In contrast, the same treatment during neural developmental stages (veliger) did not result in increased locomotion-based dispersal (Figures 6D and 6E_2).

Thus, manipulations of the serotonergic system exclusively during pre-neural stages specifically affected certain developmental characteristics and behaviors of juvenile individuals in a delayed manner.

The Balance of Extra- and Intracellular 5-HT in an Embryo Is Important for Transducing the 5-HT-Based Signal

Further experiments demonstrated that the equilibrium of pre-neural 5-HT inside/outside of the cell played a prominent role in regulating the developmental characteristics and future behavior



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of snails. External 5-HT did not enter the embryo at the sensitive early cleavage stage (Figures 4G_1 and 4G_3); correspondingly, the addition of 5-HT to the cultivation medium had no effect on normal development (Figure S4D), despite the presence of membrane receptors to 5-HT (Figures S6B and S6C). However, the precursors of 5-HT (5-HTP and tryptophan) entered the developing embryo at the early cleavage stage (Figures 4G_1 and 4G_3), and their conversion to 5-HT was obligatory for exogastrula formation (Figures S4B and S4D). Consistently, 5-HTP demonstrated a profound concentration-dependent effect on the formation of exogastrulae (Figure S4C). To test the hypothesis that the equilibrium of external and internal 5-HT is important for exogastrula formation rather than only the increase in internal 5-HT, we added both precursor (5-HTP or tryptophan) and 5-HT to the cultivation medium. Indeed, the external 5-HT, which demonstrated no effect when administered alone, was a strong enhancer of the exogastrulation caused by 5-HTP. Additionally, it functioned as an indispensable co-initiator of exogastrulation when administered together with tryptophan (Figure S4D). The biological meaning of this finding can be explained by the observation that snail embryos secrete 5-HT into their surrounding media. This phenomenon was confirmed by measuring the concentrations of 5-HT by ELISA after a 4-hr incubation of non-treated embryos in a small volume. Indeed, the embryos secreted 5-HT, and this process was robustly enhanced by the addition of 5-HTP (Figure S5B). Thus, external 5-HT might also serve as a ''quorum-sensing'' molecule to control further adaptations for the later dispersion of young animals and the population density.

Because membrane-localized transporters are critical for maintaining the equilibrium of extracellular and intracellular 5-HT, we assessed whether SERT plays a role in normal development. The results showed that the combined incubation of embryos in 5-HTP and SSRIs (serotonin-specific reuptake inhibitors) strongly decreased the percentage of 5-HTP-induced exogastrulae (Figure S5A). Additionally, we examined the efflux of 5-HT from embryos and found that a SSRI (citalopram) inhibited this process (Figure S5B). Thus, SSRI-mediated SERT inhibition did not allow the secretion of freshly synthesized intracellular 5-HT back into the medium, thus elevating the concentration of 5-HT inside of the cells. As a result, blockage of 5-HT efflux with a subsequent reduction of the external 5-HT concentration normalized development.

To evaluate the mechanism of external 5-HT reception, we took advantage of numerous pharmacological agonists and antagonists of 5-HT receptors. We cultivated snail embryos at cleavage stage in a mixture of agonists or antagonists and examined their effects using 5-HTP-induced exogastrulation as a model. We discovered that some agonists stably increased the percentage of exogastrulae, while some antagonists reduced the proportion of exogastrulae (Figure S6A). Noteworthy, the agonists and antagonists that demonstrated an effect on exogastrulation are known to target 5-HT second-type receptors in vertebrates. Indeed, we confirmed the expression of 5-$HT2_{Lym}$ in all blastomeres during the 5-HT-sensitive cleavage stage and the early neural stage (Figures S6B–S6H). Concomitantly, activation of adenylate cyclase and protein kinase A (PKA) (Figure S6J), as well as high concentrations of Ca^{2+} (Figure S6I), induced exogastrulation. Inhibition of SERT by citalopram (and the subsequent increase in intracellular 5-HT) together with the application of dibutyryl-cAMP (mimics activation of membrane receptors) also caused exogastrulation (Figures 5B_1 and 5B_2). These data highlight that the activity of 5-HT receptors during the pre-neural stage is important for the 5-HT-mediated effects on subsequent snail development. Taken together, the experiments involving components of the intracellular signal transduction support the importance of the intra- and extracellular 5-HT balance for correct developmental processes (Figure 7).

Figure 3. Application of 5-HTP to Adult Mothers Induces Changes in the Progeny

(A) General scheme of the experimental design.

(B) Concentration-dependent increase in oviposition activity in mothers treated with 5-HTP. n = 30 snails, two-way ANOVA followed by the Bonferroni post hoc test. Data are given as mean ± SEM.

(C_1) Phases of juvenile terrestrial locomotion. Scale bar, 0.5 mm.

(C_2) Typical locomotion tracks of juvenile snails from control and treated groups, four juveniles per projection.

(C_3) Speed of terrestrial locomotion of autumn-born juveniles. n = 30–60 for each point, **p < 0.01; ***p < 0.001, unpaired Student's t test. Data are given as mean ± SEM.

(D_1) Example of the escape behavior test performed with the juveniles on day 17.

(D_2) Percentage of escaping juveniles in the control and experimental groups. n = 15–20 animals for each time point, one-way ANOVA followed by Dunnett's multiple comparison post-test. Data are given as mean ± SEM.

(E_1) Two 6-day-old embryos. Scale bar, 0.5 mm.

(E_2) Embryonic length normalized to the annual mean. Note the dips at days 3 and 6 in control embryos and the developmental acceleration at 4–6 days in the experimental embryos. n = 120–150 for each point. ***p < 0.001, unpaired Student's t test. Data are given as mean ± SEM.

(F) HPLC measurements of 5-HT content in the head complex of 12-day-old control and experimental animals. n = 3 measurements, 10 animals in each sample;, one-way ANOVA followed by Dunnett's multiple comparison post-test. Data are given as mean ± SEM.

(G_1) 5-HTP-induced exogastrulae. Scale bar, 0.5 mm.

(G_2) Percentage of exogastrulae in the progeny of 5-HTP treated snails.

(H) Sexual maturation (age of first oviposition) in the control and experimental groups. n = 13 groups, 15 animals in each group, unpaired Student's t test. Data are given as mean ± SEM.

(I_1 and I_2) Survival of the juveniles in water conditioned by overcrowded adults. CW, conditioned water. See Supplemental Experimental Procedures for details. n = 70–90 for each group at the starting point, chi-square test.

(J) Oviposition activity of the first-generation snails. n = 195 in the control group and 370 in the experimental group; two-way ANOVA followed by Bonferroni's post hoc test. Data are given as mean ± SEM.

See also Supplemental Experimental Procedures, Figures S1–S3, and Movie S2.

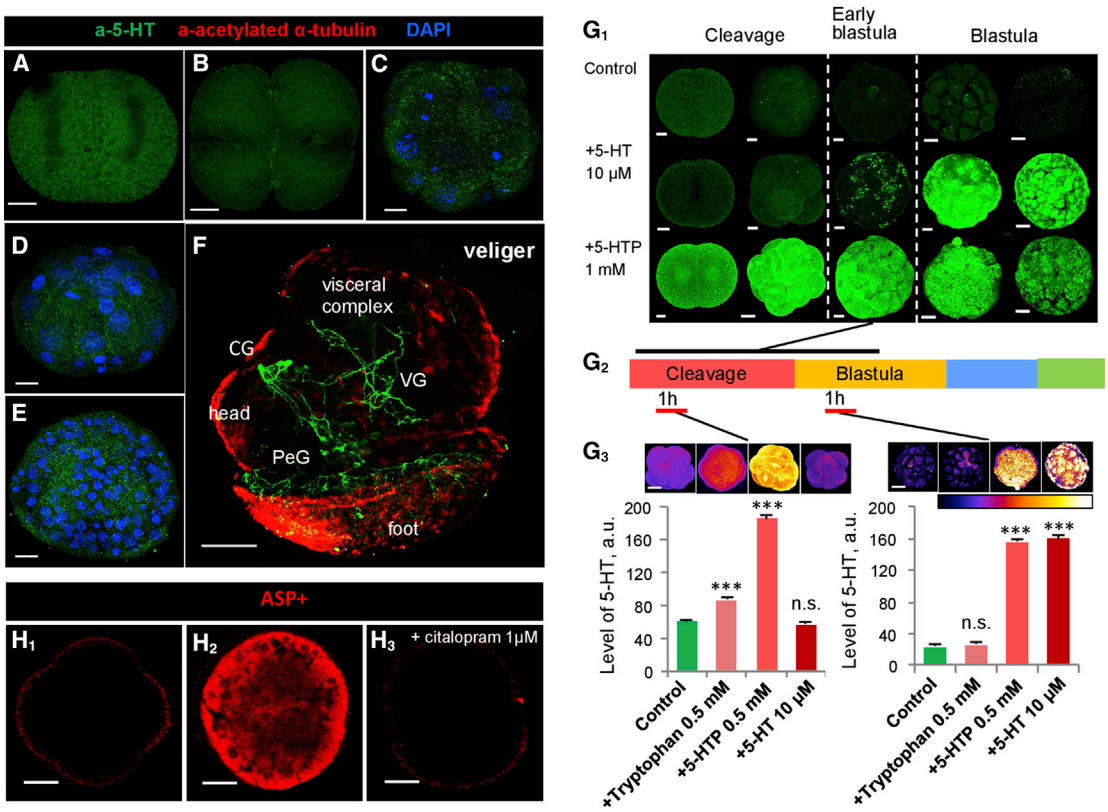

Figure 4. Characterization of the Serotonergic System during Different Developmental Stages of the *L. stagnalis* Embryo

(A–F) Distribution of 5-HT (green) at two (A) and four (B) blastomeres, early cleavage (C), early (D), and late (E) blastula, and late veliger (F) stages. Red indicates ciliary structures, and blue indicates nuclei. CG, cerebral ganglion; PeG, pedal ganglion; VG, visceral loop ganglia. Scale bars, 20 μm in (A–E) and 100 μm in (F).

(G_1) 5-HT staining (green) of embryos at subsequent developmental stages (from the two-blastomere stage up to the blastula) after incubation with 5-HT or 5-HTP. Scale bars, 20 μm.

(G_2) Design of the experiments presented in (G_1) and (G_3). h, hours.

(G_3) Relative brightness of 5-HT staining (color coded) and relative level of 5-HT at the cleavage and blastula stages after incubation with tryptophan, 5-HTP, or 5-HT. n = 30. ***$p < 0.001$; n.s., non-significant, unpaired Student's t test, Data are given as mean ± SEM. a.u., arbitrary units.

(H_1–H_3) SERT-specific uptake of ASP^+ (red) by live embryo at the four-blastomere (H_1) and blastula (H_2) stages. Note that the four-cell embryo does not take up ASP^+, while the uptake of ASP^+ during the blastula stage is blocked by the SERT-inhibitor citalopram (H_3). Scale bars, 20 μm.

We further demonstrated that mother-derived 5-HT induced delayed long-term developmental and behavioral effects by activating embryonic receptors during the early cleavage stage. To demonstrate this finding, we designed a rescue experiment in which we combined the incubation of mothers in 5-HTP with the subsequent incubation of released eggs in mianserin (Figure 6A), a non-selective and highly potent 5-HT_2 receptor antagonist that is a known inhibitor of the 5-HT_{2Lym} receptor (Gerhardt et al., 1996). Minimal concentrations of mianserin were selected (0.5 μM and 1 μM) to avoid effects on the control animals. As a result, the typical shift in developmental dynamics and increased juvenile locomotion in the progeny of experimental mothers were negated in embryos that were treated with mianserin (Figures $6B_1$ and $6B_2$). Therefore, blocking the activity of 5-HT at the level of the receptors prevented changes in behavior that were induced by high concentrations of 5-HT in the reproductive system of the mother. Finally, we examined whether seasonal cycles affect 5-HT-mediated developmental outcomes that were induced during the pre-neural cleavage stage. We discovered a correlation between the sensitivity to 5-HT measured as the exogastrula incidence rate and the specific time of year (Figure $5I_1$). The maximum and minimum sensitivities to 5-HT corresponded to the seasonal maximum and minimum oviposition activities, respectively (Figure $5I_2$).

The hormonal system controlling fluctuating egg production includes hormonal peptides produced by neurons in the snail CNS (Koene, 2010). Similarly, the population density of squirrels correlates with maternal glucocorticoids that are also associated with growth-related maternal effects (Dantzer et al., 2013). We tested the possibility of controlled 5-HT levels and maternal effects in snails via the peptidergic hormonal system using cooling stress to manipulate the levels of released hormones according to a published methodology (Hodasi, 1976) (Figure S7A). The results demonstrated that the levels of 5-HT and DA did not change in the uteruses of snails that were affected by temperature (Figures $S7B_1$–$S7B_8$). In addition, when the

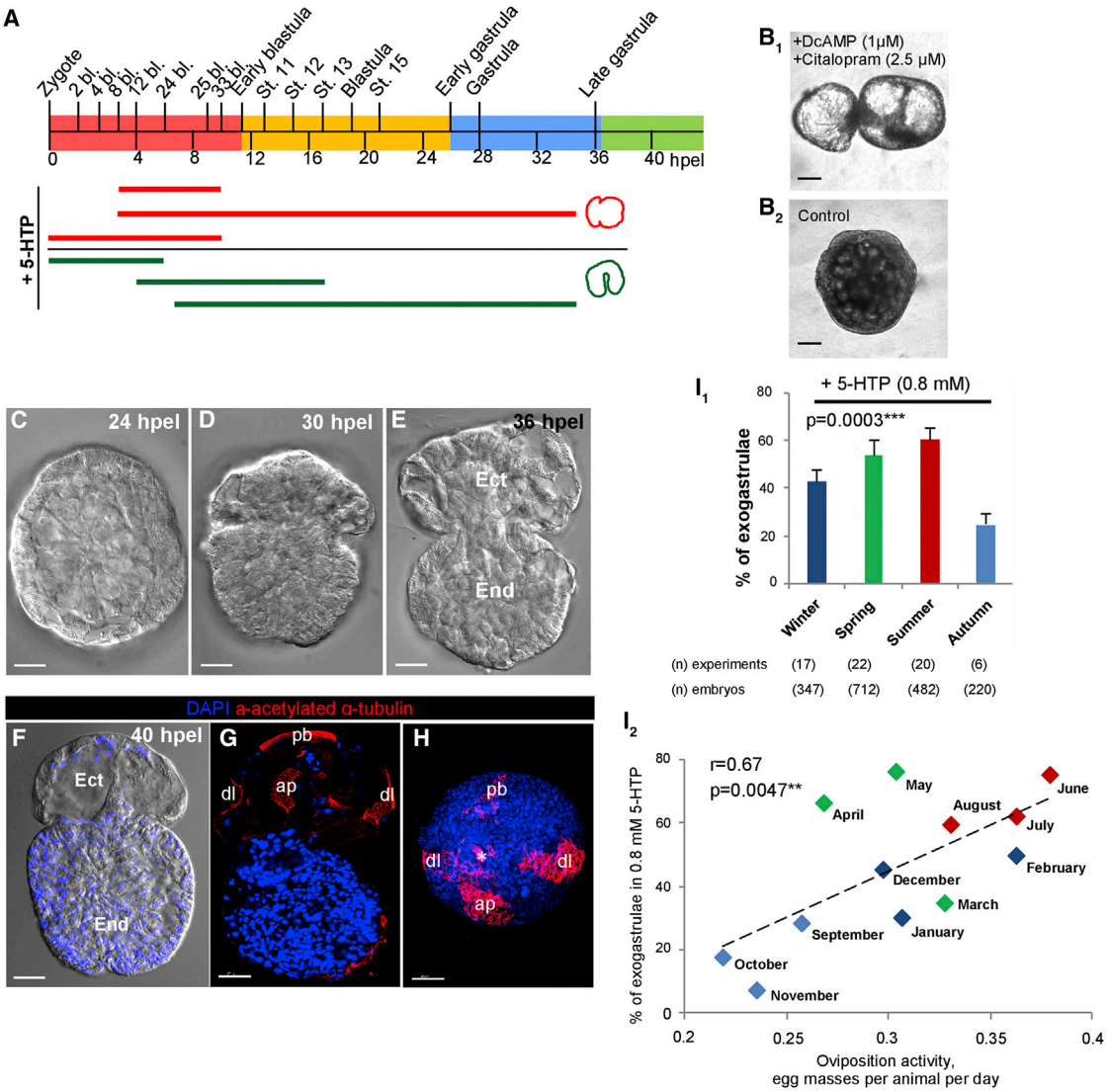

Figure 5. 5-HTP-Induced Exogastrulation

(A) Application of 5-HTP during defined time windows in *L. stagnalis* development results in exogastrula formation (red) or does not affect gastrulation (green). Note that the interval of treatment starting from 12 blastomeres (bl.) to 33 blastomeres is both essential and sufficient for the induction of the irreversible malformation. n = 1,200 embryos in 39 experiments. hpel, hours post-egg laying.

(B_1 and B_2) The simultaneous increase in the intracellular serotonin and cyclic AMP (cAMP) level (induced by citaloplam and dibutyryl-cAMP) at the 12- to 33-blastomere stage results in exogastrula formation (B_1) in the same manner as the application of 5-HTP. (B_2) Control embryo, early trochophore stage.

(C–E) Differential interference contrast (DIC) images of subsequent stages (24, 30, and 36 hr post-egg laying; hpel) of 5-HTP-induced gastrulation disruption. End, endodermal part; Ect, ectodermal part.

(F) Dumbbell-shaped exogastrulae, combined DIC image and DAPI nuclear staining (blue).

(G and H) Immunochemical detection of surface ciliary fields (red) and nuclei (blue) in exogastrulae (G) and normal gastrulae (H). Four types of ciliary structures are well differentiated in both exogastrulae and normal gastrulae: two dorsolateral bands of cilia (dl), an apical plate (ap), and a pedal band of cilia (pb). The asterisk indicates the ciliated stomodeum. End, endodermal part; Ect, ectodermal part.

(I_1) Proportions of 5-HTP-induced exogastrulae induced by the same concentration of applied 5-HTP correlate within the changing seasons. One-way ANOVA followed by Dunnett's multiple comparison post-test. Error bars indicate mean ± SEM.

(I_2) Correlation between the incidence rate of exogastrulae and the seasonal oviposition activity of the mother snails. n = 1,200 embryos.

Scale bars, 50 µm for (C)–(H). See also Figures S4–S6.

mothers recovered from the cooling stress and started to lay eggs, their progeny did not show any developmental differences from the controls (Figures S7C_1 and S7C_2). Importantly, developing embryos of *L. stagnalis* from wild populations also demonstrated coherent season-dependent developmental patterns (Figures S7D_1–S7D_3), which might have important adaptive effects. These results suggest that cold temperatures and the snail hormonal system do not regulate the maternal signal that

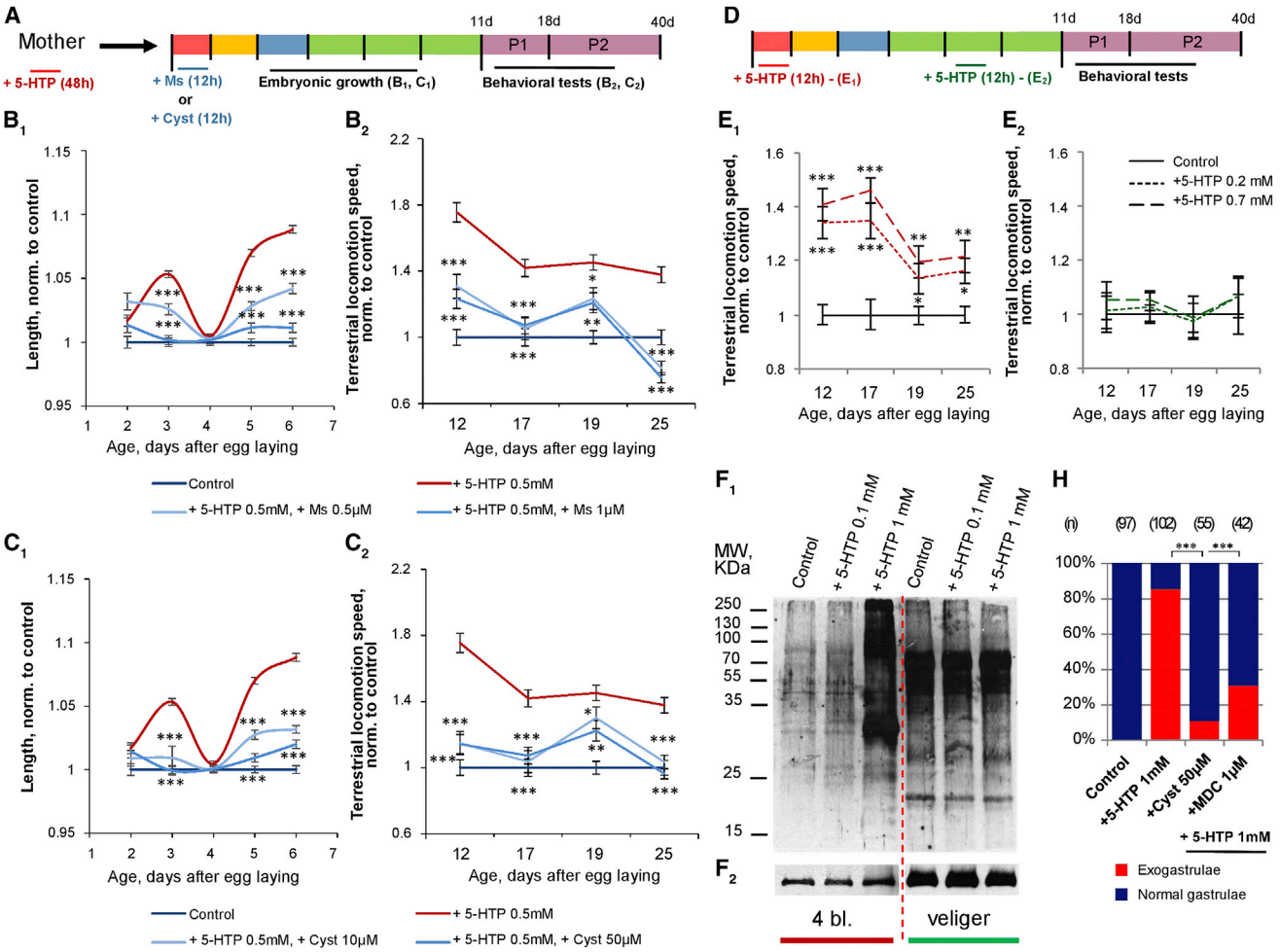

Figure 6. Elevated Intracellular Serotonin and Activation of Membrane 5-HT Receptors Induce Serotonylation that Is Required for the Transduction of Maternal Effects

(A) Design of the experiment. The application of 5-HTP to mothers was followed by the subsequent inhibition of 5-HT receptors (application of mianserin; Ms) or transglutaminase activity (application of cystamine; Cyst) in early embryos.

(B_1–C_2) Developmental dynamics (B_1 and C_1) and speed of terrestrial locomotion in juveniles. (B_2 and C_2). Note the dose-dependent reduction of changes induced by maternal serotonin in mianserin and cystamine-treated progeny. Comparisons among the 5-HTP and 5-HTP +Ms and 5-HTP +Cyst-treated groups. n = 120–170 for each point. *p < 0.05; **p < 0.01; ***p < 0.001, unpaired Student's t test. Data are given as mean ± SEM.

(D) Design of the experiment. 5-HTP was applied during the 12-hr time window of the cleavage (non-neuronal) or veliger (neuronal) stages.

(E_1 and E_2) Speed of terrestrial locomotion of juveniles after 5-HTP treatment during the cleavage (E_1) and veliger (E_2) embryonic stages. Comparisons between control and 5-HTP. n = 90–120 for each point. *p < 0.05; **p < 0.01; ***p < 0.001, unpaired Student's t test. Data are given as mean ± SEM.

(F_1) Visualization of serotonylated proteins by western blot analysis using a polyclonal antibody against 5-HT during the cleavage (4 bl.) and veliger embryonic stages. Note the concentration-dependent increase in protein serotonylation after incubation in 5-HTP exclusively during the early cleavage stage. MW, molecular weight.

(F_2) Reference for the corresponding samples (α-tubulin).

(G) Inhibition of transglutaminase activity by cystamine (Cyst) and monodancyl cadaverine (MDC) leads to a reduction of the 5-HTP-induced rate of exogastrula formation. Proportion of exogastrulae to normal gastrulae. ***p < 0.001, chi-square test.

is responsible for modifying the developmental patterns of progeny according to the time of year. We also assessed whether melatonin affected developmental outcomes in a manner similar to that of 5-HT. Melatonin is a key molecule in many cycling processes in both vertebrates and invertebrates (Tosches et al., 2014). However, the direct application of melatonin, DA precursor L-DOPA, a number of antagonists of different 5-HT receptor types (tropanyl, WAY-100,135, SB-269970, GR-113808, zacopride, or NAN-190; concentration range, 1 μM–1 mM), or 5-HT receptor agonists (5-CT, 5-MeO-DMT, 8-OH-DPAT, αMS, 2-Br-LSD, DOI, 5-HTQ, and mCPP; concentration range, 0.1–100 μM), separately or in any combination, to early embryos had no effect compared with the control, including the proportion of 5-HTP-induced exogastrulae.

A prominent question concerns how the early pre-neural receptive mechanisms translate the intracellular signal up to later neural stages and affect the behavior of juveniles. To gain insight into the potential mechanism underlying the delayed effects, we

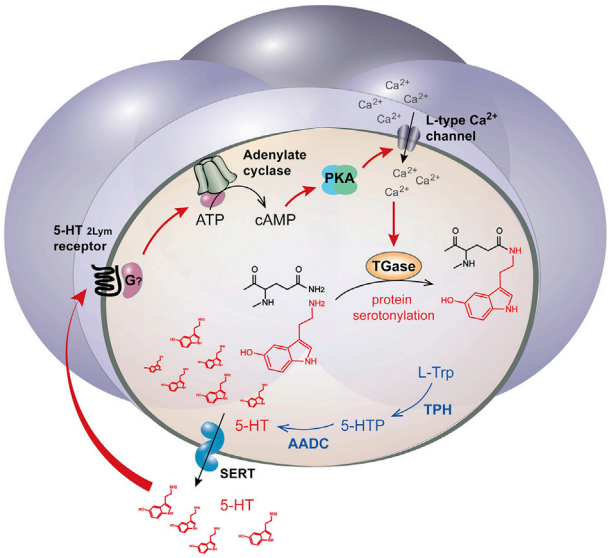

Figure 7. Proposed Model for the Reception of Serotonin-Based Maternal Signals

Maternal 5-HT activates 5-HT_{2Lym} membrane receptors to initiate a cAMP/PKA-dependent cascade of intracellular reactions that, in turn, results in long-lasting changes inside the blastomeres. These long-lasting changes include post-translational modifications of proteins, for example, covalent binding of serotonin to proteins (i.e., serotonylation catalyzed by transglutaminase; TGase) in the presence of high intracellular concentrations of Ca^{2+} (entering through L-type Ca^{2+} channels). Serotonylation is active exclusively in the early non-neural (cleavage) stages and requires two coinciding events: high serotonin levels inside the cell and stimulation of membrane serotonin receptors. AADC, aromatic L-amino acid decarboxylase; L-Trp, L-tryptophan; TPH, tryptophan hydroxylase.

questioned whether specific post-translational protein modifications might mediate the delayed intracellular signal. Serotonylation, a recently discovered novel post-translational modification (Walther et al., 2011), was found to be important in *L. stagnalis* to achieve the maternal 5-HT-based signal. Our results demonstrated the serotonylation of specific proteins, which was elevated exclusively during early development (cleavage) under experimental conditions (high 5-HT inside and outside of the cell concomitantly, resulting in receptor stimulation; Figure 6F_1). Maternal 5-HT-based effects were negated by the inhibition of transglutaminase, a key enzyme in the covalent binding of 5-HT to proteins (Figures 6C_1 and 6C_2). Transglutaminase inhibition also prevented 5-HTP-induced exogastrulation (Figure 6G). In line with this, the expression and activity of transglutaminase in oocytes and early embryos highlights the important role of this protein in the regulation of development (Kim et al., 2001).

Altogether, our findings reveal a novel serotonin-based mechanism that bridges adaptive developmental and behavioral characteristics of progeny with season-dependent maternal serotonin via mother-to-progeny communication at early pre-neural embryonic stages.

DISCUSSION

The presence of 5-HT in the gonads of adult animals together with the activity of 5-HT in very early embryos renders 5-HT a potential factor mediating the integration of organisms belonging to different generations. The evidence for such integration comes from different model systems, including, for example, a classical mouse experiment in which the female progeny of *Tph1*$^{-/-}$ females became sterile independently of the progeny genotype (Côté et al., 2007). The important challenge in this field is related to the previous inability to discriminate the roles of neural and non-neural 5-HT in early embryos. In the present study, we accepted this challenge and provided evidence for a novel and unique role of pre-neural 5-HT in modulating the characteristics of progenies.

Importantly, the early serotonergic system is conservative among many phylogenetically distant groups of animals. Keeping this in mind, we took advantage of a flexible model organism that develops outside of the mother's body from the zygote stage in a periodically changing environment. For example, the photoperiodic cycles in these snails can be influenced by variations in temperature (Dogterom et al., 1985), food consumption, and other factors (Wayne, 2001). In addition to these fast adaptive skills, *L. stagnalis* snails also exhibit reproductive, behavioral, and developmental cycles under standard laboratory conditions. It turned out that the speed of terrestrial locomotion during the first 2 weeks after hatching, i.e., the most critical period for the dispersion of juveniles (Boag, 1986), was fastest and slowest during the summer and autumn seasons, respectively. In parallel, in human rural populations, the season of a mother's birth defines the size of her fetuses (Rickard et al., 2012), while the behavioral patterns of humans living in a seasonal climate correlate with the season of birth (Chotai et al., 2001). Similarly, experimental increases in the concentration of 5-HT in snail mothers inevitably led to a number of effects in the progeny. These effects included elevated levels of 5-HT in the offspring together with some changes in parameters linked to monoamine concentrations. Unexpectedly, embryos from treated mothers demonstrated developmental dynamics, growth rates, and behaviors that differed from those of the non-treated controls. These parameters shifted from those that were originally appropriate for the current season to different parameters. For example, the progeny of treated mothers demonstrated "summer characteristics" in autumn and in spring. Such effects cannot be explained by the accumulation of 5-HT in embryos starting from treatment stages and up to the juvenile or adult stage, in which stored 5-HT would play a role. Application of 5-HT at the earliest neural embryonic stages had nothing in common with pre-neural treatment experiments when it comes to the resulting delayed consequences and manifestations.

Importantly, not all of the developmental and growth-related features changed in the progeny of treated animals; rather, only selected characteristics were modified. This finding supports a low probability of a general systemic metabolic boost driven by 5-HT, but it provides evidence for the refinement of only selected parameters. For instance, under some experimental conditions, the developmental speed of the premetamorphic stages (2–4 days after egg laying) was slower, while the metamorphic stages (4–6 days after egg laying) were accelerated. The idea of a general boost in metabolism was also barely compatible with the very narrow time window during pre-neural stages in which 5-HT induced strongly delayed effects

in progeny. Externally applied 5-HT or biochemical precursors (5-HTP and tryptophan) during the earliest neural developmental stages resulted in a completely different set of effects that contrasted with those resulting from the pre-neural time window. Importantly, the experimental elevation of 5-HT in the reproductive system of mother snails, as well as the treatment of early embryos during pre-neural stages, induced equivalent delayed effects. The rescue experiment revealed that such delayed effects could be efficiently abolished by the inhibition of 5-HT receptors in embryos during the cleavage stage.

In *L. stagnalis*, the periodicity of the locomotion and other behavioral traits coincided with the reproductive cycles according to the changing seasons. We suggest that the hierarchical behavioral programs switch depending on the conditions and corresponding environmental signals. For example, fast embryo growth and increased locomotion may lead to the efficient dispersal of snails to new water habitats via waterfowl. Concomitantly, the increased locomotion, which is necessary for efficient dispersion, may inhibit efficient food consumption because the juvenile snails do not have sufficient time to remain in one place and obtain enough food. Therefore, the fast early locomotion of juveniles increases the chances for bird-mediated dispersion during the summer season. Consistent with this finding, we clearly observed an increase in the locomotion of *Lymnaea* juvenile snails, specifically in the summer and after 5-HT treatment (imitating the maternal summer signal). Moreover, crowding of parental snails also induced maternal effects that impacted specific characteristics of the progeny. Such mechanisms may provide explanations for the extremely successful expansion of *Lymnaea* across the world, given that these snails do not have actively swimming larvae.

The seasonal amounts of monoamines may vary in gonads, in which 5-HT and DA directly participate in the maturation of gametes and also in nerve endings inside of the reproductive ducts, where monoamines participate in the ejection of gametes into the external environment (Fong et al., 2003). Our long-term monitoring experiments in combination with 5-HT-related manipulations demonstrated that 5-HT-dependent seasonal oscillations in the adult reproductive system correlated with the developmental and behavioral characteristics of the progeny. Importantly, we confirmed the seasonal dynamics of 5-HT in the female reproductive system of adult *L. stagnalis* and demonstrated the presence of the previously unknown serotonergic plexus in the subepithelial layer of the uterus. The incubation of snails in 5-HTP coincided with a specific elevation of the level of 5-HT only in those plexus-forming serotonergic cells. These results suggested that the plexus might be the source of the 5-HT released into the snail reproductive system and accumulated in the zygote. Mammals, despite being dramatically different from mollusks, also possess 5-HT-releasing machinery in the reproductive system. For example, the placenta serves as a transient 5-HT-producing factory that supplies the embryonic forebrain with freshly synthesized 5-HT (Bonnin and Levitt, 2011). In addition to the nerve endings, numerous mast cells that are localized to the mammalian oviduct represent the source of 5-HT inside the mammalian reproductive system (Amenta et al., 1992).

Despite a large amount of experimental evidence confirming the importance of maternal 5-HT for the offspring, the exact mechanisms underlying signal transduction and reception were previously unknown. According to our results, some of the maternal effects can be transmitted to the second generation. Here, we demonstrated that the adaptive non-genetic signal can be represented by 5-HT that is received directly from the mother and deposited into the oocyte or zygote, as well as by the 5-HT synthesized de novo from biochemical precursors in early embryos or by a combination of both (Figure 7). Primary germ cells and oocytes express SERT, which allows them to capture and transport maternal 5-HT into the cell. Concomitantly, both of the enzymes required for 5-HT synthesis may be expressed and active in early embryos (Amireault and Dubé, 2005; Buznikov et al., 2003). We confirmed that 5-HT is routinely present in cleaved blastomeres up to the blastula stage in *L. stagnalis*. All of the blastomeres efficiently and equally performed the second step of 5-HT synthesis from 5-HTP using the AADC enzyme. Similar to this situation in snails, metabolism and the levels of tryptophan in the uterus of the mother control the amount of 5-HT available to developing mouse embryos (Doherty et al., 2011). Consistent with this finding, we discovered that elevation of 5-HT during early cleavage stages causes a very special developmental abnormality: the formation of exogastrulae. We found that it was convenient to use 5-HT-induced exogastrulation as a model to examine the mechanistic interactions between different components of the serotonergic system during sensitive developmental intervals. Exogastrulation started to dominate when high concentrations of intracellular 5-HT were counterbalanced by high concentrations of extracellular 5-HT from neighboring cells (after being produced in those cells from precursors) or supplied by other sources in the immediate environment. Thus, the equilibrium between extracellular and intracellular 5-HT during a defined time window at the cleavage stage, which occurs long before the development of the nervous system, is crucial for properly adapted development. Extracellular and intracellular pathways converging to relay the 5-HT-based signal seem to be important for other delayed long-term effects in developmental dynamics and in larval or juvenile behaviors (Figure 7).

It is generally accepted that 5-HT acts via the activation of specific 5-HT receptors and the corresponding intracellular pathways (Roth, 2008). However, complementary mechanisms should participate in mediating the delayed effects inside a cell, in addition to the initial receptor-based input. In our experiments, the combination of increased 5-HT level inside the cleaved blastomeres and activation of membrane 5-HT receptors appeared to be necessary for the emergence of delayed developmental and behavioral effects. In mammalian tissues, the long-term effects of 5-HT have been demonstrated in the regulation of insulin secretion from pancreatic β-cells, in the release of alpha-granules from platelets, and in vascular smooth muscle contraction. The mechanisms underlying these 5-HT-induced processes involve receptor activation, the operation of membrane and vesicular transporters (SERT and VMAT), and covalent bonding of 5-HT (serotonylation) to various proteins (Walther et al., 2011). However, the mechanisms underlying the intracellular molecular imprinting of the 5-HT-based signal remain enigmatic and require further systematic investigation utilizing the power of genetic and epigenetic approaches. Here, we focused on the role of serotonylation in maternal effects in snails to highlight the importance

of post-translational protein modifications in the intracellular transmission of the non-genetic 5-HT-based signal. This is a non-canonical effect of 5-HT, in which an increase in the concentration of 5-HT inside the cell together with receptor activation may drive more efficient serotonylation of proteins (Figure 7). The serotonylation of specific proteins may impinge on their stability, lifetime, and regulatory properties involved in developmental control. This logic, if true, may explain the intracellular transmission of the maternal signal from early pre-neural embryonic cells to the cells that are present during later neural stages, during which the retained signal is translated into the developing phenotypic outcomes. Our results reveal for the first time the role of serotonylation in embryonic development.

Taken together, our data reveal the role of mother-derived 5-HT in adjusting snail progeny to the appropriate phases of the cycling environment. Because the serotonergic system is highly conserved between evolutionarily distant animal groups, our results also raise the possibility of a universal strategy that connects neuronal-activity-dependent behavioral experiences with information-relaying processes in the reproductive system and, through this, with offspring.

EXPERIMENTAL PROCEDURES

The great pond snail *Lymnaea stagnalis L.* is a freshwater aquatic pulmonate gastropod mollusk from the family *Lymnaeidae*. It is widely distributed in many countries of the Holarctic region. To characterize differences in the behavior of seasonal and artificially manipulated snails, we used the spontaneous terrestrial locomotion test (newly hatched and juveniles 17, 19, and 25 days after egg laying), water escape behavior (the number of animals crawling above the water line), embryonic rotation and food consumption essays. We obtained confocal images of individual immunostained embryos for 5-HT quantification (n = 20 for each point). To investigate active specific 5-HT transport in early embryos, we used the ASP^+ uptake assay in addition to immunochemical analysis. A double-blind protocol was used for all of the experiments. For details regarding embryonic development, staging, animal maintenance, embryo culturing, and each method used in this study, please refer to the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.022.

AUTHOR CONTRIBUTIONS

E.I., M.K., and E.V. performed all of the experiments and designed the study. L.N., V.M., and O.K. performed some of the experiments. I.A. designed the study and wrote the manuscript. E.I. and M.K., as well as I.A. and E.V., contributed equally to this work. All authors discussed the results and commented on the manuscript.

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