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RESEARCH REPORT

Transcriptional effect of serotonin in the ganglia of *Lymnaea stagnalis*

C Benatti[1,2], C Colliva[1], JMC Blom[2,3], E Ottaviani[1], F Tascedda[1,2]

[1] Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy
[2] Center for Neuroscience and Neurotechnology University of Modena and Reggio Emilia, Modena, Italy
[3] Department of Education and Humanities University of Modena and Reggio Emilia, Modena, Italy

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Abstract

The serotonin system (5HT) is highly conserved in both vertebrates and invertebrates, and numerous evidence supports a biological link between 5HT and numerous animal function. In the present paper we evaluated the transcriptional effects of a serotonergic stimulation on selected targets involved in 5HT signalling and neurotransmission in the central nervous system of the great pond snail *Lymnaea stagnalis*. Adult snails were treated acutely (6 h) or chronically (48 h) with either 5-hydroxytrypthophan (5-HTP 1mM), the immediate precursor of serotonin, fluoxetine (FLX 1µM), a selective serotonin reuptake inhibitor, or a combination of two. The central ring ganglia were dissected and used for q-PCR gene expression analysis. Transcription was strongly induced following a chronic, but not an acute, exposure to 5-HTP in the ganglia of *Lymnaea*. In particular, *Lym*CREB1 and *Lym*P2X mRNA levels were decreased following a 6 h exposure and increased in snails receiving 5-hydroxytryptophan for 48 h. Interestingly, this effect was reduced when snails were exposed chronically to both 5-HTP and FLX, suggesting a role for SERT in mediating the effect of 5-hydroxytryptophan. These data suggest that *L. stagnalis* is suited to unravel the complexity of the serotonin signaling pathway.

Key Words: serotonin; CREB; *Lymnaea stagnalis*

Introduction

The serotonin is an ancestral complex neurotransmitter system that plays an important role in the regulation of many biological functions. Serotonin has a fundamental role in the modulation of stress-induced excitability (arousal), in the defensive behavior (Il-Han *et al.,* 2010), in the modulation of aggressive behaviors and in the control of anxiety.

Normally, for these studies, have been used small mammals (*i.e.*, rats and mice) but this approach may not be always effective and is accompanied by many ethical and economical drawbacks (Tascedda *et al.,* 2015). The high cost of these studies and the increasing difficulties in obtaining permits for experimentation prompted researchers to look for other strategies. Many researchers have attempted to solve the problem by using in vitro cell systems (Alboni *et al.,* 2013b, 2014) that have many important advantages. Unfortunately, the obtained results were often limited and inconclusive in elucidating the basis of diseases and identifying effective therapeutic strategies (Alberts, 2010). Invertebrates, thanks to their relatively simple nervous systems and to the latest technique in genome sequencing and manipulation, are becoming a useful tool for the study of neuronal physiology and for best disease process characterization (Ottaviani *et al.,* 2013; Tascedda *et al.,* 2015).

Invertebrates lack self-awareness "autonoetic consciousness" (Curren and Chalsani, 2012), emotional behaviour reduced to its individual components. In particular, the pond snail *Lymnaea stagnalis*, an aquatic pulmonate gastropod with a central nervous system (CNS) consisting of ≈20,000 neurons organized in a ring of interconnected ganglia, has proven to be an extremely useful and accessible model to study fundamental aspects of CNS function such as synaptic plasticity and associative memory. The serotonin (5-HT) neurons present in the CNS of *Lymnaea* are analogous to vertebrate 5-HT neurons that originate in the raphe nuclei. Serotonin, with specific innervation, control central pattern generators and other important circuits of the CNS. Furthermore, through integrated

Corresponding author:
Fabio Tascedda
Department of Life Sciences
University of Modena and Reggio Emilia
Via Campi 287, 41125 Modena, Italy
E-mail: fabio.tascedda@unimore.it

feedback information coming from the innervated areas, support general behavioural arousal (Andrianov *et al.,* 2015; Gillette, 2006).

In this model recent finding demonstrate a fundamental role of serotonin in the control of reproduction and behaviour (Il-Han *et al.* 2010, Ivashkin *et al.* 2015)

Dysfunction in serotonin system regulation or serotonin levels are involved in the control of many biological functions (Gillette 2006, Deneris *et al*. 2012). Understanding the transcription mechanisms associated with the hyper stimulation of the serotoninergic system is a key step to clarifying the fundamental aspects of these mechanisms. In this context, we sought to mimic the generalized activation of the serotonergic system by administering the rate-limiting 5-HT precursor 5-hydroxytryptophan (5-HTP) to freely moving animals. We used 5-HTP because studies in rodents and molluscs have proven that exposure to 5-HT or tryptophan are less effective in elevating serotonin and related molecules, with shorter amplitude and duration. It is also possible that 5-HTP disrupt homeostatically regulated serotonin levels (Lynn-Bullock *et al*., 2004; Marinesco *et al.,* 2004; Fickbohm *et al.,* 2005).

In this study, we evaluated the effects of 5-HTP on the serotonin related genes and on major intracellular systems related to serotonergic stimulation. The molecular machinery governing serotonin signaling has been cloned and characterized in *Lymnaea*. Serotonin is synthesized by tryptophan hydroxylase (LymTPH) (Koert *et al.,* 2001) in the cytoplasm of the presynaptic serotonergic neurons, vesicle monoamine transporters (LymVMAT) then package serotonin into vesicles, and upon fusion with the cell membrane, the neurotransmitter is released into the synaptic cleft and binds to specific receptors (LymHTR1 and LymHTR2) (Sugamori *et al.,* 1993; Gerhardt *et al.,*1996). The concentration of 5HT is then regulated by the action of a specific transporter (LymSERT) (Sadamoto *et al.,* 2008).

The activity of serotonin receptors produces important intracellular changes related to cAMP and Ca^{++} signalling (Poser *et al.* 2001). These pathways, in invertebrates and in mammals, are both linked to serotonergic control of related stress responses and adaptive mechanisms in response to pharmacological treatment (Kaang *et al.* 1993, Vinet *et al.,* 2003, 2004; Blom *et al.,* 2006).

One target for the serotonergic stimulation is a modifying effect on the regulation of postreceptor pathways and genes related to the cAMP cascade in particular the transcription factor cAMP response element binding protein (CREB) (Kaang *et al.,* 1993; Marinesco *et al.,* 2004). CREB is known to regulate the downstream expression of cAMP-inducible genes, and is proposed to be involved in the control of many biological functions, in the regulation of brain homeostasis and in the response to pharmacological treatment (Blom *et al*., 2002; Alboni *et al.,* 2010, 2011, 2013a). In *Lymnaea*, analogue of CREB (LymCREB1) has been cloned and characterized (Sadamoto *et al.,* 2004).

Materials and Methods

Animals and colony maintenance

Laboratory-reared freshwater pond snails, *Lymnaea stagnalis* (original stocks donated by Vrije Universiteit, Amsterdam) were maintained in aquaria at the University of Modena and Reggio Emilia (Italy) in standard laboratory conditions: 21 - 23 °C, 12:12 h light/dark cycle (on at 08:00). Adult animals having shell lengths of 20 to 25 mm were used in this experiment and were kept in 12 L tanks (30 mature snails in each) supplied with well-aerated water. They were fed pesticide-free lettuce and goldfish pellets three times a week, and the aquaria were cleaned on alternate days. Every effort was made to minimize the number of animals used and their suffering.

Pharmacological experiments

The following compounds were used for pharmacological treatments: 5-hydroxy-L-tryptophan (5-HTP) 1 mM (Sigma-Aldrich); fluoxetine hydrochloride (FLX) 1 µM (Polichimica). Solutions of specified concentrations were freshly prepared in boiled filtered water (FW) with 50 µM ascorbic acid (Sigma-Aldrich) in order to avoid 5-HTP oxidation. We incubated adult snails for 6 or 48 h without food and aeration in 2 L aquaria, 15 specimens per 400 ml of experimental solution. Untreated controls (naive adults) were left undisturbed in an equal amount of FW, a group exposed only to ascorbic acid in the same conditions was also included. After incubation the animals were anesthetized on ice for 10 min and the central ring ganglia was dissected out (buccal ganglia were excluded) and stored at -80 °C prior analysis.

Total RNA extraction, reverse transcription, and real time polymerase chain reaction

Four central ring ganglia were pooled for total RNA extraction, 4 - 6 replicates were analyzed for each group. Total RNA extraction and DNAse treatment were performed using GenElute™ Total RNA Miniprep Kit and DNASE70-On-Column DNase I Digestion Set (Sigma Aldrich) as previously described (Benatti *et al.,* 2011). Five hundred ng of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation) in 20 µl of reaction mix. mRNAs were quantified by real-time quantitative polymerase chain reaction in Roche LightCycler® 480 (Roche Diagnostics GmbH) using Power UP SYBR Green mix (Life Technologies Corporation). Specific forward and reverse primers were used at the final concentration of 300 nM (Table 1). Single PCR products were subjected to a heat dissociation protocol as previously described (Caraci *et al.,* 2016). Cycle threshold (Ct) value was determined by the LightCycler® 480 Software (Roche Diagnostics GmbH).

Statistical analysis

For quantitative evaluation of changes the comparative ΔΔCt method was performed, using as calibrator the average levels of expression of control snails. The stability of mRNA expression of two reference genes (elongation factor 1-alpha, *Lym*EF1α

Table 1 Nucleotide sequence of the forward and reverse primers used for Real-Time PCR

Gene Bank Accession	Target	Product Length	Type sequence	Ct (25 ng)
AB041522.1	*Lymnaea stagnalis* cAMP responsive element binding protein, *Lym*CREB1	*180 bp [49-229]*	Fw GTCAGCAGGGAATGGTCCTG Rv AACCGCAGCAACCCTAACAA	25
JX524180.1	*Lymnaea stagnalis* P2X receptor, *Lym*P2X	*150bp [1005-1155]*	Fw GGGATCGTCTTCGTGGTGA Rv AGTTCCTGGCCTTCAACAGAT	24
AJ238276.1	*Lymnaea stagnalis* neuropeptide Y, *Lym*NPY	*188 bp [432-620]*	Fw ACTCTTGGTGTCACTGCTCG Rv CTTGCGCCGTTTCTCTTTCC	17
L06803.1	*Lymnaea stagnalis* serotonin receptor 1, *Lym*HTR1	*126 bp [893-1019]*	Fw ACTATCTCATCCTGTCCTTG Rv GATATCCACATGTCACACAC	23
U50080.1	*Lymnaea stagnalis* serotonin receptor 2, *Lym*HTR2	*115 bp [884-999]*	Fw ACACCTGGAGTATTCTCATC Rv GAAGTAGTTGGTCACGTTCT	23
FX185022	*Lymnaea stagnalis* serotonin transporter, *Lym*SERT	*177 bp [726-903]*	Fw ATACCGTACCTTGTCATGTT RvTGTTGTAGTACCAGGAGACA	20
AF129815.1	*Lymnaea stagnalis* tryptophan hydroxylase, *Lym*TPH	*179 bp [238-417]*	Fw AGGATACAGTCTACCGACAG Rv TGAGTTCACGGAAAACTATT	18
AF484094.1	*Lymnaea stagnalis* vesicular monoamine transporter, *Lym*VMAT	*172bp [529-701]*	Fw AACGTGTACATGACTGTGAC Rv AAGCCAGTAAACATTGGTAT	22
DQ278441.1	*Lymnaea stagnalis* elongation factor 1-alpha, *Lym*EF1α	*150bp [7-157]*	Fw GTGTAAGCAGCCCTCGAACT Rv TTCGCTCATCAATACCACCA	16
X15542.1	Snail, beta-tubulin, *Lym*TUB	*127 bp [92-219]*	Fw GAAATAGCACCGCCATCC Rv CGCCTCTGTGAACTCCATCT	16

The accession number, the size (bp) of the PCR product obtained by amplification of the cDNA (mRNA) are given for each target. As indication of the relative abundances of each target average Ct values in adult snails (25 ng, n = 4).

and beta-tubulin, *Lym*TUB) was assessed using Normfinder®, *Lym*TUB was the most stable gene across groups and was used for gene normalization. Statistical analyses were performed using an analysis of variance (One-way ANOVA). Significant changes were determined by Tukey post-hoc test (with $p < 0.05$ significance level).

Results

Effect of an exposure to 5-HTP for 6 or 48 h on the expression levels of analogues of CREB1 and P2X in the CNS of L. stagnalis

Lymnaea CREB1 (*Lym*CREB1) is a homolog of mammalian CREB that is expressed in the CNS of *Lymnaea* and is involved in synaptic facilitation (Sadamoto *et al.,* 2004, 2010). One way ANOVA revealed a main effect of both an acute and a prolonged exposure to 5-HTP [$F(4;21) = 5.282$, $p = 0.004$ and $F(4;30) = 13.896$, $p < 0.0001$ respectively; Fig. 1A]. In particular, post hoc analysis showed that the expression of CREB1 was significantly induced in snails exposed for 48 h to 5-HTP with respect to all the other treatment regimens ($p < 0.001$). The effect of 5-HTP was reduced in presence of FLX: *Lym*CREB1 mRNA levels of the 5-HTP/FLX group were significantly higher than the control group ($p < 0.05$), while being significantly lower than the group receiving 5-HTP alone ($p < 0.01$). In contrast, following a 6 h exposure we observed a significant decrease of *Lym*CREB1 expression in the group exposed either to 5-HTP or FLX and to the combination of the two compounds with respect to control ($p < 0.05$) (Fig. 1A).

A similar trend was observed for *Lym*P2X (Fig. 1B); this purinergic receptor was recently identified and cloned in the CNS of *Lymnaea* (Bavan *et al.,* 2012). Prolonged exposure to 5-HTP resulted in an overall significant increase in *Lym*P2X mRNA in ganglia [$F(4;30) = 22.506$, $p < 0.0001$]. Again, this effect was still present when snails were exposed to both FLX and 5-HTP, but was significantly reduced with respect to the increase observed following 5-HTP alone ($p < 0.0001$) (Fig. 1B). When considering the effects of a 6 h treatment, one way ANOVA revealed a main effect [$F(4;20) = 6.257$, $p = 0.002$; Fig. 1B], indeed, P2X expression was reduced by 5-HTP, FLX, and their combination with respect to control ($p < 0.05$).

Effect of a 5-HTP exposure on the expression levels of components of the serotonergic system in the CNS of L. stagnalis

To date, two 5-HT receptor genes have been cloned in *Lymnaea*: *Lym*HTR1 and *Lym*HTR2 (Sugamori *et al.,* 1993; Gerhardt *et al.,* 1996). *Lym*HTR1 expression levels were affected by a 6 h exposure to 5-HTP [$F(4;18) = 5.714$, $p = 0.004$; Fig. 2A], while no effect was observed when treatment was protracted up to 48 h [$F(4;29) = 1.609$, $p = 0.199$; Fig. 3A]. In particular post hoc test revealed that FLX 1 µM was able to decrease the expression

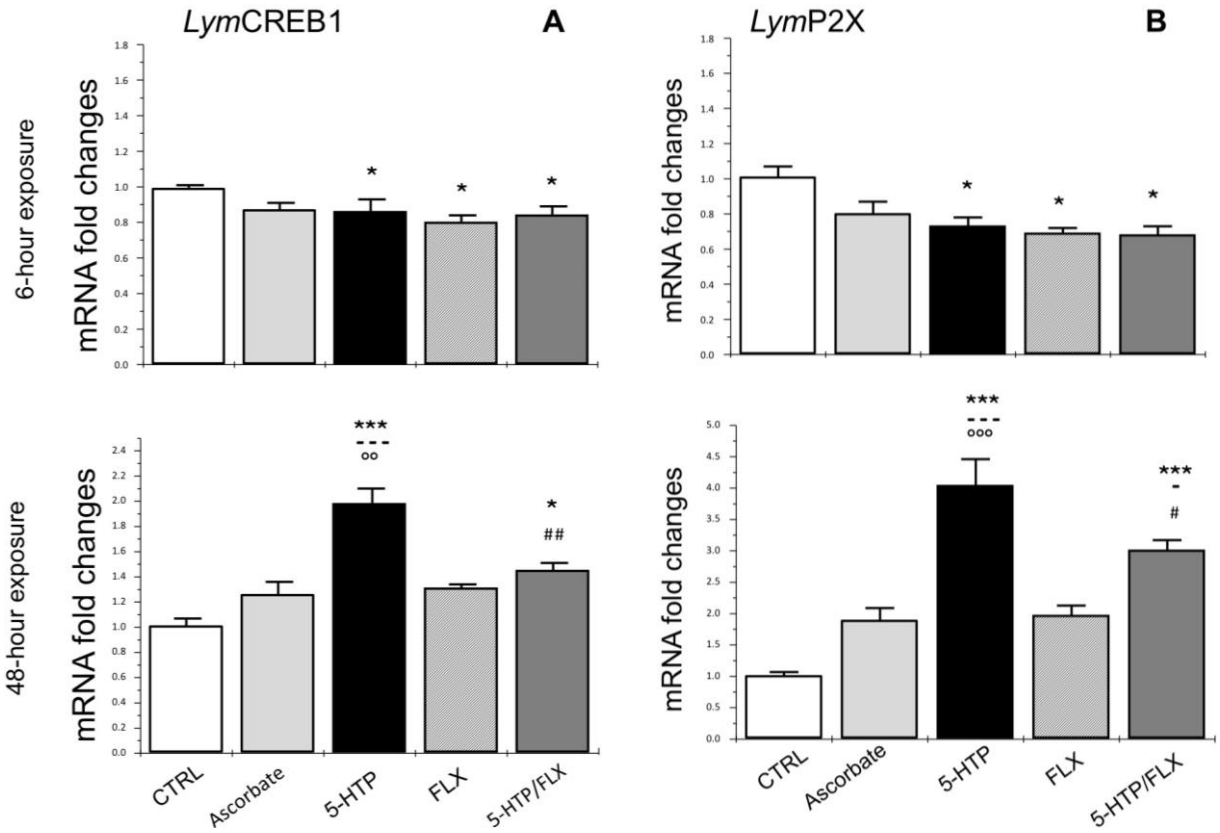

Fig. 1 Effect of an exposure to 5-HTP for 6 or 48 hs on the expression levels of analogues of CREB1 and P2X in the CNS of *Lymnaea stagnalis.* Adult snails were incubated in 1 mM 5-HTP, 1 µM FLX, or a combination of the two (5-HTP/FLX) for 6 or 48 h. Untreated adults (CTRL) and a group exposed only to ascorbic acid (Ascorbate) in the same conditions were also included. *LymCREB1* (A) and *LymP2X* (B) mRNA expression in the ganglia, with *LymTUB* as endogenous control, were measured by Real-time PCR. N = 4 - 7 pools of 3 snails each. Data are represented as means ± S.E.M. and were analyzed with ANOVA followed by Tukey. ***$p < 0.0001$, **$p < 0.01$,*$p < 0.05$ vs CTRL; $p< 0.0001$, $p < 0.05$ vs Ascorbate; °°° $p < 0.0001$, °° $p < 0.01$ vs FLX; ## $p < 0.01$, # $p < 0.05$ vs 5-HTP.

levels of *Lym*HTR1 with respect to both control groups ($p < 0.05$). This down-regulation was blunted in the group exposed to 5-HTP, alone ($p = 0.072$) or in combination with FLX ($p = 0.015$). On the other hand, the expression levels of the other serotonergic receptor, *Lym*HTR2, were not altered on our experimental conditions [F (4;19) = 1.140, $p = 0.368$ for 6 h; Fig. 2B, and F (4;29) = 2.380, $p = 0.075$ for 48 h exposure; Fig. 3B].

Sadamoto and co-workers (2008) identified and characterized the localization of the of the serotonin transporter in *Lymnaea*: *Lym*SERT. In our experimental conditions, no effect on *Lym*SERT mRNA levels was observed following a 6 h treatment regime [F (4;21) = 2.010, $p = 0.130$; Fig. 2C], while exposure to 5-HTP for 48 h significantly increased the expression levels of serotonin transporter with respect to untreated controls [F (4;29) = 3.043, $p = 0.033$; Fig. 3C].

We also evaluated in ganglia the transcriptional effect of a serotonergic stimulation on the rate-limiting enzyme in the synthesis of serotonin: tryptophan hydroxylase (*Lym*TPH) (Koert *et al.,* 2001). A main effect was revealed in animals experiencing a 48 h exposure to 5-HTP [F (4;30) = 8.662, $p < 0.0001$; Fig. 3D], while no effect was observed following a 6 h treatment [F (4;18) = 1.887, $p = 0.157$; Fig. 2D]. In particular, *Lym*TPH mRNA was significantly higher in the ganglia of ascorbic acid-exposed snail with respect to the levels found in both the 5-HTP exposed groups (alone ($p = 0.007$) or in combination with FLX ($p < 0.0001$) and in untreated control snails ($p = 0.017$) (Fig. 3D).

Vesicular monoamine transporter (*Lym*VMAT) mRNA levels were not affected in snails exposed to 5-HTP, FLX or their combination for 6 h with respect to control groups [F (4;19) = 2.058, $p = 0.127$] (Fig. 2E). On the other hand, one-way ANOVA revealed a main effect of a chronic treatment with 5-hydroxytryptophan on VMAT expression [F (4;26) = 11.300, $p < 0.0001$] (Fig. 3E). We observed a significant decrease of *Lym*VMAT mRNA in animals exposed to 5-HTP for 48 h, alone or in combination to FLX, with respect to control groups ($p < 0.01$) or to FLX-treated snails ($p < 0.01$).

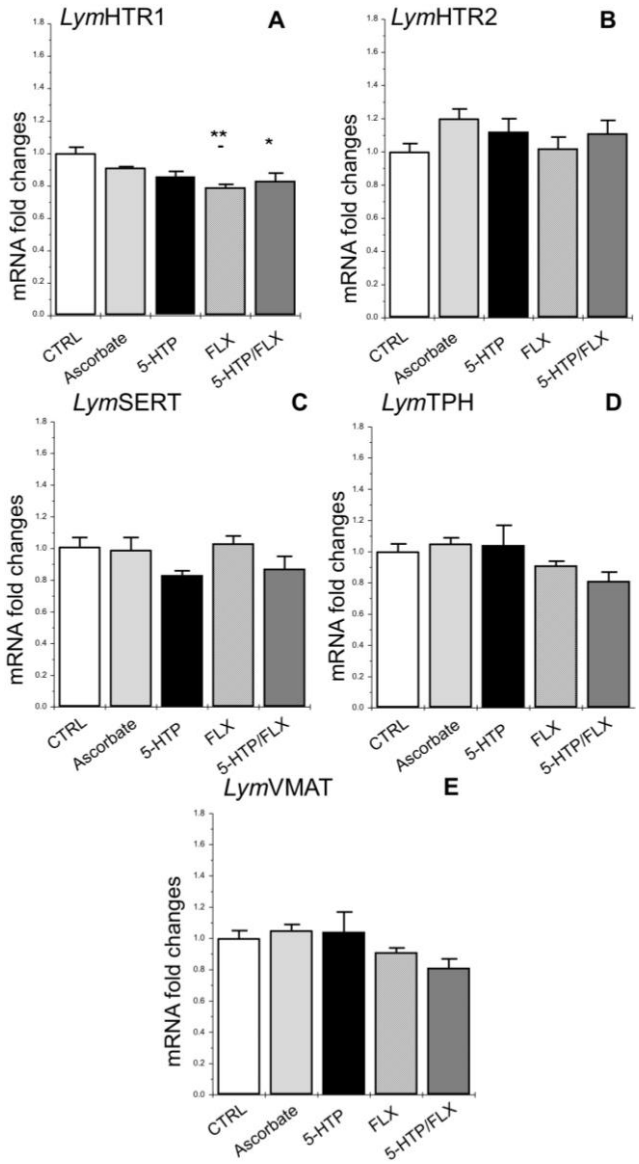

Fig. 2 Effect of a 6-h 5-HTP exposure on the expression levels of components of the serotonergic system in the CNS of *Lymnaea stagnalis.* Adult snails were incubated in 1 mM 5-HTP, 1 µM FLX or a combination of the two (5-HTP/FLX) for 6 h. Untreated adults (CTRL) and a group exposed only to ascorbic acid (Ascorbate) in the same conditions were also included. *Lymnaea stagnalis* serotonin receptors [*LymHTR1* (A), *LymHTR2* (B)] and transporter [*LymSERT* (C)], tryptophan hydroxylase [*LymTPH* (D)], vesicular monoamine transporter [*LymVMAT* (E)] mRNA expression in the ganglia, with *LymTUB* as endogenous control, were measured by Real-time PCR. N = 4 - 7 pools of 3 snails each. Data are represented as means ± S.E.M. and were analyzed with ANOVA followed by Tukey. $^{**}p < 0.01$,$^{*}p < 0.05$ vs CTRL; $p < 0.05$ vs Ascorbate.

Discussion

Here we demonstrated that specific transcription was strongly induced following a prolonged, but not an acute, exposure to 5-HTP in the ganglia of *Lymnaea*. In particular, *Lym*CREB1 and *Lym*P2X mRNA levels were increased in snails receiving 5-HTP for 48 h, and decreased following a 6 h exposure. Interestingly, this effect was reduced when snails were exposed chronically to both 5-HTP and FLX, suggesting a role for SERT in regulating the effects of 5-HTP.

Previous studies in vertebrates and invertebrates have shown that a treatment with 5-HTP is able to increase serotonin content in the CNS, in both serotonergic and non-serotonergic regions (Gartside *et al.,* 1992; Lynn-Bullock *et al.,* 2004; Fickbohm *et al.,* 2005). Moreover, studies on isolated neuron in *Lymnaea* have demonstrated that the precursor acts only indirectly through its conversion to serotonin and its effects are mediated by enhanced serotonin release, activation of its receptors and modulation of electrical activity (Dyakonova *et al.,* 2009).

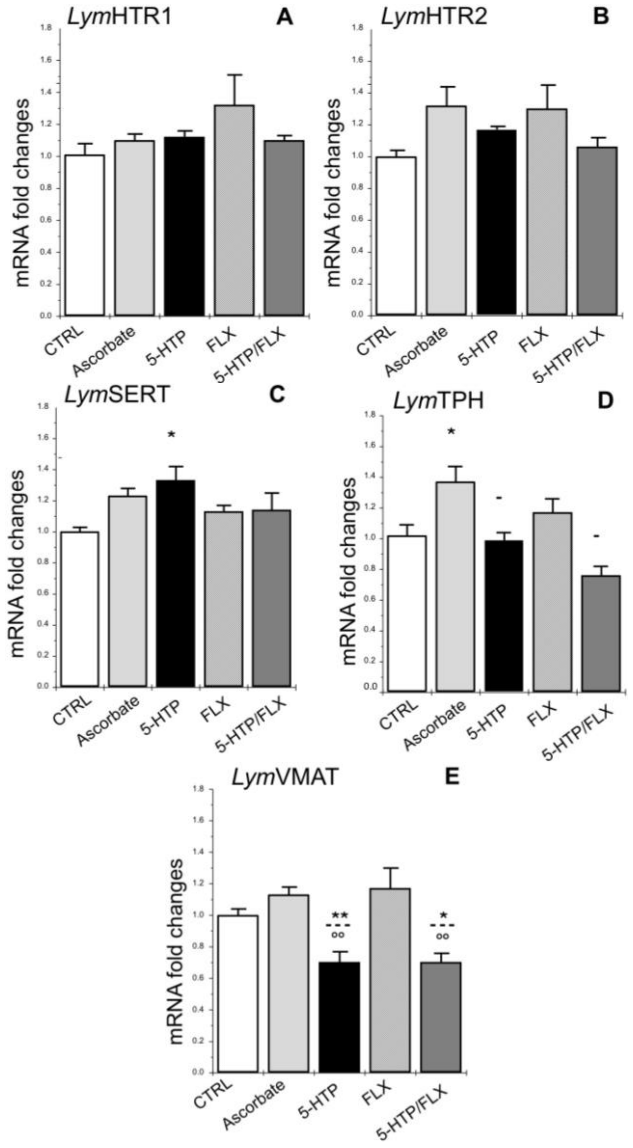

Fig. 3 Effect of a 48-h 5-HTP exposure on the expression levels of components of the serotonergic system in the CNS of *Lymnaea stagnalis.* Adult snails were incubated in 1 mM 5-HTP, 1 μM FLX or a combination of the two (5-HTP/FLX) for 48 h. Untreated adults (CTRL) and a group exposed only to ascorbic acid (ASC) in the same conditions were also included. *Lymnaea stagnalis* serotonin receptors [*LymHTR1* (A), *LymHTR2* (B)] and transporter [*LymSERT* (C)], tryptophan hydroxylase [*LymTPH* (D)], vesicular monoamine transporter [*LymVMAT* (E)] mRNA expression in the ganglia, with LymTUB as endogenous control, were measured by Real-time PCR. N = 4 - 7 pools of 3 snails each. Data are represented as means ± S.E.M. and were analyzed with ANOVA followed by Tukey. ***p* < 0.01,**p* < 0.05 vs CTRL; *p* < 0.0001, *p* < 0.05 vs Ascorbate; °° *p* < 0.01 vs FLX.

In our experimental conditions we demonstrated that 48 h exposure to serotonin precursor is able to influence also gene expression of selected targets and that some of these effects where significantly diminished in the presence of FLX, a selective inhibitor of serotonin transporter. In the group exposed to FLX alone for 48 h no alterations of gene expression of the evaluated targets were observed. This effect is in agreement with the results of Yu RL and collaborators (2008) that have shown that in *Aplysia,* 24 h treatment of paired pleural-pedal ganglia, induced the mRNA of CREB gene and protein.

In *Aplysia*, treatment with 5-HTP has been demonstrated to potentiate serotonergic activity (Marinesco *et al.,* 2004), which, in turn, may increase intracellular levels of cAMP and cause a rapid and transient induction of CRE-responsive genes (Kaang *et al.,*1993). This effect is mediated by the phosphorylation of CREB at Ser^{119}, this transcription factor is a key component in regulating synaptic plasticity both in physiologic and pathologic

conditions (Kaang *et al.,* 1993; Sadamoto *et al.* 2010, Blom *et al.,* 2002; Alboni *et al.,* 2011). We observe a strong increase in CREB mRNA levels following a 48 h serotonergic stimulation in the CNS of *Lymnaea* which could be the basis for major behavioral changes induced by serotonin in different invertebrate models (Il-Han *et al.,* 2011; Andrianov *et al.,* 2015).

The LymP2x gene acts similarly to CREB. Currently, in the literature, there is no direct evidence of a link between the transcription of this receptor and the activity of the serotoninergic system. We can hypothesize that an increase in the number of receptors can lead to an increase in intracellular Ca^{++} and a strengthening of CREB's transcriptional activity. The link between CREB and Ca^{++} is widely demonstrated both in mammalian and invertebrate models (Poser and Storm 2001; Ghosh-Roy *et al.,* 2010).

Studies on knockout mice have established the importance of VMAT2 in regulating catecholamine and serotonin levels, and their release from neurons following a depolarizing stimulus (Eiden and Weihe, 2011). Similarly, in our model a long lasting serotonergic stimulation caused a significant down-regulation in LymVMAT expression, this effect was still present even when snails were exposed to the combination 5-HTP/FLX, suggesting that this effect does not depend on SERT functionality. It is possible that the increased activity of serotonergic neurons evoked by exposure to 5-HTP may induce this down-regulation.

Conclusions

Our experiments show that stimulation of the serotoninergic system can induce specific transcriptional changes in the ganglia of *L. stagnalis*. Furthermore, these data suggest that *Lymnaea* ideally suited to unravel the complexity of the serotonin signaling pathway and may represent a good model to provide new insights on how serotonin can modulate different biological functions and its role in brain homeostasis.

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