

RESEARCH ARTICLE

Serotonin-induced brain glycogenolysis in rainbow trout (*Oncorhynchus mykiss*)

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SUMMARY

In this study, we evaluated the serotonin-mediated control of cerebral glycogen levels in the rainbow trout, *Oncorhynchus mykiss*. Intracerebroventricular (i.c.v.) administration of serotonin (5-HT) to normoglycemic trout (time and dose response) decreased glycogen levels in the brain and increased brain glycogen phosphorylase activity (time response). In hypoglycemic fish (that had been fasted for 5 and 10 days), there was a time-dependent decrease in brain glycogen levels; under these conditions, i.c.v. administration of 5-HT also reduced the brain glycogen content in fish that had been fasted for 5 days. In fish with local cerebral hypoglycemia (induced by 2-DG administration), the glycogen levels decreased and, as above, i.c.v. administration of 5-HT also lowered the glycogen content. In hyperglycemic fish, 5-HT did not affect glycogen levels. Administration of receptor agonists 5-HT1A (8-OH-DPAT), 5-HT1B (anpirtoline and CP93129) or 5-HT2 (α -m-5-HT) decreased the brain glycogen levels. This effect was antagonized by the administration of receptor antagonists 5-HT1A (WAY100135 and NAN190), 5-HT1B (NAS181) and 5-HT2B/C (SB206553). Administration of the receptor agonists (\pm)-DOI (5-HT2A/2C), *m*-CPP (5-HT2B/2C), BW723C86 (5-HT2B) and WAY 161503 (5-HT2C) led to decreases in the levels of brain glycogen. We found that 5-HT is involved in the modulation of brain glycogen homeostasis in the rainbow trout, causing a glycogenolytic effect when fish are in a normoglycemic or hypoglycemic state, but not when they are in a hyperglycemic state. 5-HT1A, 5-HT1B, 5HT2B and 5-HT2C-like receptors appeared to be involved in the glycogenolytic action of 5-HT, although the effect mediated by 5-HT1A or 5-HT1B was apparently stronger.

Key words: glycogen, brain, serotonin, trout.

Received 25 January 2012; Accepted 23 April 2012

INTRODUCTION

In vertebrates, the brain is one of the most active organs in the body and consumes a large amount of energy for the maintenance of neural activity (Magistretti et al., 1993). Glucose is the primary fuel for the adult brain, and in teleosts as well as in other vertebrates a continuous supply of glucose from the blood is essential for normal brain functioning (Soengas and Aldegunde, 2002). In general, the two most common circumstances that lead to a cerebral glucose deficiency are hypoglycemia, caused by a decrease in supply of glucose to the brain, and increased neuronal activity, which produces a sudden increase in energy demand so that local glucose levels are unable to satisfy the demand (Swanson et al., 1992; Swanson and Choi, 1993; Wender et al., 2000; Pellerin et al., 2007).

Although the potential physiological importance of the brain glycogen stores has not been fully elucidated, it appears that when brain glucose levels decrease, the glycogen stores are used as an emergency energy substrate (Choi et al., 2003). They therefore act as a physiological energy source available to provide additional fuel to the brain when systemic and cerebral levels of glucose fall. In fish, it is possible that brain glycogen breakdown is also one of the mechanisms used to provide energy to the brain and protect against hypoglycemia. As observed in mammals, the brain glycogen content in fish changes in response to glucose or food deprivation (Soengas et al., 1996; Soengas and Aldegunde, 2002; Sangiao-Alvarellos et al., 2003; Polakof et al., 2006).

In mammals, glycogen content is regulated by numerous factors, such as glucose, hormones and neurotransmitters, indicating the

participation of these factors in the metabolic response to glycemic or hormonal state and brain activation (Cambray-Deakin et al., 1988; Magistretti, 1988; Forsyth et al., 1996; Oz et al., 2007). Comparatively few studies have been carried out in fish as regards the effects of hormones or neurotransmitters on brain glycogen content. It has been reported that administration of melatonin (Soengas et al., 1998a), glucagon (Magnoni et al., 2001), noradrenaline (Sangiao-Alvarellos et al., 2003), arginine vasotocin (AVT) (Sangiao-Alvarellos et al., 2004) or growth hormone (GH) (Sangiao-Alvarellos et al., 2006) decreases cerebral levels of glycogen whereas administration of adrenaline increases these levels (Foster et al., 1993). In the mammalian brain, serotonin (5-HT) is one of the neurotransmitters involved in the modulation of brain glycogen stores, stimulating brain glycogenolysis both *in vitro* (Quach et al., 1982) and *in vivo* (Darvesh and Gudelsky, 2003). However, no studies have addressed the possible role of 5-HT in controlling the brain glycogen content in fish. Nonetheless, there is some indirect evidence that indicates a possible link between 5-HT and brain glycogen: (i) in fish, different types of stress can modify brain serotonergic activity (Winberg and Nilsson, 1993; Winberg and Lepage, 1998; Gestó et al., 2008) in a manner similar to that observed in mammals (Joseph and Kennet, 1981; Singh et al., 1994; Filipenko et al., 2002; Khanbabian et al., 2002), in which changes in 5-HT activity are consistent with changes in brain glycogen content (Cruz and Diniel, 2002; Herzog et al., 2008) and (ii) in a previous study (Tubío et al., 2010) we demonstrated that peripheral 5-HT is involved in the modulation of hepatic glycogenolysis in rainbow trout.

In this context, we hypothesized that, as in mammals, 5-HT may be involved in the mechanisms that control energy homeostasis in the fish brain and thus participate in the modulation of brain glycogen levels. To test our hypothesis, we investigated the following: (i) the effect of 5-HT on brain glycogen levels under different physiological conditions – normoglycemia, fasting-induced hypoglycemia, cerebral hypoglycemia induced by administration of 2-DG, and hyperglycemia – given that in rainbow trout the homeostatic system for brain glucose metabolism is more sensitive to hypoglycemia than hyperglycemia (Soengas and Aldegunde, 2004), (ii) the effect of 5-HT on the activity of the enzyme glycogen phosphorylase (GPase), which is one of the key enzymes of glycogenolysis, and (iii) the serotonin receptors involved in the above.

MATERIALS AND METHODS

Animals

Immature rainbow trout (*Oncorhynchus mykiss* Walbaum; body mass, 70–90 g) were obtained from a commercial trout farm (Soutorredondo, Noia, NW Spain) and transported to the experimental aquarium in the Faculty of Biology (Universidad de Santiago de Compostela). Fish were acclimated in 300 litre tanks for at least 4 weeks in running dechlorinated water (temperature $13\pm 1^\circ\text{C}$, pH 6.7 ± 0.3) with continuous aeration, and maintained under a 12 h:12 h light:dark photoperiod (lights on at 08:00 h). Fish were fed to satiety once daily, in the morning (at 10:00 h), with commercial dry pellets and were not fed in the 24 h prior to experiments. All experiments were carried out in the morning to avoid possible effects of circadian variations. Fish were anesthetized before handling, injection or sampling, with 2-phenoxyethanol (0.5 ml l^{-1}). The experiments comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and are in accordance with local national guidelines (decree 1201/2005, BOE 252) for animal experimentation.

Drugs used and drug administration

Serotonin (5-hydroxytryptamine creatinine sulphate), 2-deoxy-D-glucose (2-DG), (\pm)-DOI hydrochloride (5HT2A/2C receptor agonist) and WAY100135 dihydrochloride (5-HT1A receptor antagonist) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The following drugs were obtained from Tocris (Cookson, Bristol, UK): 8-hydroxy-DPAT hydrobromide (8-OH-DPAT; 5-HT1A receptor agonist), NAN190 hydrobromide (5-HT1A receptor antagonist), anpirtoline hydrochloride (5-HT1B receptor agonist), CP93129 dihydrochloride (5-HT1B receptor agonist), NAS-181 (5-HT1B receptor antagonist), α -methyl-5-hydroxytryptamine maleate (α -m-5-HT; 5-HT2 receptor generic agonist), *m*-CPP hydrochloride (5-HT2B/2C receptor agonist), SB206553 hydrochloride (5-HT2B/2C receptor antagonist), WAY161503 hydrochloride (5-HT2C receptor agonist) and BW723C86 hydrochloride (5-HT2B receptor agonist).

Glucose, 2-DG, 5-HT and 5-HT agonists and antagonists were dissolved in saline (SAL; 0.6% w/v NaCl), except for BW723C86, which was initially dissolved in DMSO, and 8-OH-DPAT and NAN190, which were initially dissolved in ethanol. In every case, the desired final concentration was achieved by dissolving the corresponding solution in SAL (the final DMSO content was 15% and the final ethanol content was 1–15% for 8-OH-DPAT and 3% for NAN190; VEH, vehicle). Drug solutions were administered by intraperitoneal (i.p.) injection in 300 μl of saline, or intracerebroventricular (i.c.v.) injection in 2 μl of saline or VEH per fish, as previously described (Aldegunde and Mancebo, 2006; Tubío et al., 2010). Briefly, i.c.v. administration was carried out with a

10 μl Hamilton microsyringe and a 25 gauge needle. For i.c.v. administration, each fish was placed dorsal-side upwards and the tissue covering the skull was removed by scraping, to facilitate rapid visualization of the injection point, on the midline at the level of the anterior point of union of the lobes of the optic tectum. A small hole was made at this point (with a 25 gauge needle) and, immediately after the needle was placed in the hole, leading to the third ventricle at the level of the preoptic nuclei, the microsyringe was held in place while the 2 μl dose was infused slowly; the needle was then held in position for 5–10 s to allow for clearance.

Effect of i.c.v. administration of 5-HT on brain glycogen levels and GPase activity

In the first part of the study, a group of experiments (Experiments 1–5) were carried out to investigate the action of 5-HT on brain glycogen levels in fish under different conditions of glycemia. Doses and times were chosen on the basis of the results of previous studies carried out in our laboratory (Ruibal et al., 2002; Tubío et al., 2010).

Experiment 1 was carried out to study the effect of 5-HT on brain glycogen levels in normoglycemic fish. Initially, we conducted a dose–response study using the following doses of 5-HT: 0, 5.12, 12.8, 32, 80 and 200 $\mu\text{g kg}^{-1}$. The fish were decapitated and the brain removed from each fish 60 min after i.c.v. administration of 5-HT ($N=10$ fish per dose). A time–response study was then conducted, to examine the effect of the i.c.v. administration of a dose of 5-HT (12.8 $\mu\text{g kg}^{-1}$); in this case, the brains were removed 20, 60, 120 and 180 min after administration of the 5-HT ($N=8$ –10 fish per group and time).

Experiment 2 was carried out with hypoglycemic fish, in which the hypoglycemia was induced by fasting for 5 or 10 days. A dose of 5-HT (12.8 $\mu\text{g kg}^{-1}$) was administered i.c.v. Brain samples were obtained 60 min after administration ($N=8$ –10 fish per group and time) for analysis of brain glycogen content. In Experiment 3, local cerebral hypoglycemia was induced in normoglycemic fish by i.c.v. administration of 2-DG. Three treatments were established: (1) SAL, (2) 2-DG (300 $\mu\text{g kg}^{-1}$) and (3) 2-DG (300 $\mu\text{g kg}^{-1}$) + 5-HT (12.8 $\mu\text{g kg}^{-1}$) (in this case, both drugs were administered together, by the i.c.v. route). Brain samples were removed after 120 min for later determination of glycogen levels ($N=8$ –10 fish per group).

In Experiment 4, hyperglycemia was induced in fish by i.p. administration of glucose (500 mg kg^{-1} in 300 μl). Two treatments were administered: (1) glucose (500 mg kg^{-1} , i.p.) + SAL (i.c.v.) and (2) glucose (500 mg kg^{-1} , i.p.) + 5-HT (12.8 $\mu\text{g kg}^{-1}$, i.c.v.). Intracerebroventricular administration of the corresponding drug was performed immediately after i.p. administration of glucose (anesthetized fish). Brain samples were removed 0, 30, 60 and 120 min after treatment ($N=8$ –10 fish per bar) for analysis of glycogen levels.

The aim of Experiment 5 was to investigate the effect of 5-HT on GPase activity in normoglycemic fish. Two treatments were considered: (1) SAL (i.c.v.) and (2) 5-HT (12.8 $\mu\text{g kg}^{-1}$, i.c.v.). Brain samples were removed 20, 60, 120 and 180 min after drug administration ($N=6$ –7 fish per bar) for determination of enzyme activity.

Effect of i.p. and/or i.c.v. administration of different pharmacological agents

Another set of experiments (Experiments 6–13) was conducted in order to determine the possible involvement of 5-HT1 and 5-HT2 receptors in the glycogenolytic effect of 5-HT. Bearing in mind that there are no drugs specifically designed for serotonergic receptors on fish, this study was carried out using serotonergic agonists and antagonists designed for mammals. As most of the drugs were being

used for the first time in fish (rainbow trout), the doses used were based on those used in mammals (Papp and Willner, 1991; Swedberg et al., 1992; Ichikawa and Meltzer, 1995; Ochi and Goto, 2000; Schreiber et al., 2000; Rodriguez de Fonseca et al., 2001; Ahlander-Lüttgen et al., 2003; De Deurwaerdere et al., 2004; Ginawi et al., 2005; Egashira et al., 2007).

Experiments 6 and 7 were carried out to study the possible involvement of the 5-HT_{1A} receptor subtype in the glycogenolytic action of 5-HT. The 5-HT_{1A} receptor agonist 8-OH-DPAT was administered i.p. In Experiment 6, two treatments were established: (1) VEH and (2) 8-OH-DPAT (0.1, 0.5 and 1 mg kg⁻¹). The brains were removed for later determination of glycogen 0, 30, 60, 90 and 150 min after i.p. injection ($N=9-10$ fish per group). In Experiment 7, the effect of the i.p. administration of two different 5-HT_{1A} antagonists, WAY100135 and NAN190, on the glycogenolytic action of 8-OH-DPAT was evaluated. The following treatments were administered: (1) SAL + VEH, (2) SAL + 8-OH-DPAT (1 mg kg⁻¹), (3) WAY100135 (3 mg kg⁻¹) + 8-OH-DPAT (1 mg kg⁻¹), (4) VEH + VEH, (5) VEH + 8-OH-DPAT (1 mg kg⁻¹) and (6) NAN190 (3 mg kg⁻¹) + 8-OH-DPAT (1 mg kg⁻¹). In this experiment, brains were removed from the fish 150 min after i.p. injection of the agonist. The time between administration of the antagonist and the agonist was 30 min ($N=8-10$ fish per group).

Experiments 8 and 9 were carried out to investigate the role of the 5-HT_{1B} subtype receptors on the glycogenolytic action of 5-HT. Two 5-HT_{1B} agonists, anpirtoline and CP93129, were used, and both were administered i.p. Different treatments were established: (1) SAL, (2) anpirtoline (0.5, 1 and 2 mg kg⁻¹), (3) SAL and (4) CP93129 (0.5, 1.5 and 5 mg kg⁻¹). In all cases, the brains were removed for later determination of glycogen 30, 90 and 150 min after the i.p. injection ($N=9-10$ fish per group). In Experiment 9, the effect of the 5-HT_{1B} antagonist NAS-181 on the glycogenolytic action of anpirtoline or CP93129 was evaluated. Different treatments were administered: (1) SAL + SAL, (2) SAL + anpirtoline (2 mg kg⁻¹), (3) NAS-181 (4 and 8 mg kg⁻¹) + anpirtoline (2 mg kg⁻¹), (4) SAL + SAL, (5) SAL + CP93129 (5 mg kg⁻¹) and (6) NAS-181 (4 and 8 mg kg⁻¹) + CP93129 (5 mg kg⁻¹). In these experiments, the brains were removed from the fish 90 and 150 min after i.p. injection of the agonist. The time between the first (antagonist) and second (agonist) injection was 30 min ($N=8-10$ fish per group).

Experiments 10–13 were carried out to study the possible involvement of 5-HT₂ receptors. In Experiment 10, the 5-HT₂ generic receptor agonist α -m-5-HT was administered i.c.v. Two treatments were established: (1) SAL and (2) α -m-5-HT (10 and 30 μ g kg⁻¹). The brains were removed 30, 90 and 150 min after i.c.v. injection ($N=8-10$ fish per group) for later determination of glycogen. Experiment 11 was carried out to investigate the effect of the 5-HT_{2B/C} antagonist SB206553 on the glycogenolytic action of α -m-5-HT. The following treatments were administered: (1) SAL (i.p.) + SAL (i.c.v.), (2) SAL + α -m-5-HT (10 μ g kg⁻¹, i.c.v.) and (3) SB206553 (4 and 8 mg kg⁻¹, i.p.) + α -m-5-HT (10 μ g kg⁻¹, i.c.v.). Brain samples were removed from the fish 90 min after i.c.v. injection of the agonist. The time between the first (antagonist) and second (agonist) injection was 30 min ($N=8-10$ fish per group).

In Experiment 12, the agonists 5-HT_{2A/2C} [(\pm)-DOI] and the 5-HT_{2B/2C} (*m*-CPP) were administered i.p. The groups established were: (1) SAL, (2) (\pm)-DOI (1 and 3 mg kg⁻¹), (3) SAL and (4) *m*-CPP (2 and 5 mg kg⁻¹). The brains were removed 30, 90 and 150 min after i.p. injection ($N=8-10$ fish per group) for later determination of glycogen levels. Finally, in Experiment 13, the agonists 5-HT_{2B} (BW723C86) and 5-HT_{2C} (WAY161503) were administered i.p.

Different treatments were established, as follows: (1) VEH, (2) BW723C86 (2 and 8 mg kg⁻¹), (3) SAL and (4) WAY161503 (2 and 4 mg kg⁻¹). Samples of brain were obtained 30 and 90 min after i.p. injection ($N=8-10$ fish per group) for later determination of glycogen levels.

Tissue sampling and analytical procedures

Fish were anesthetized with 2-phenoxyethanol (0.5 ml l⁻¹) and killed by decapitation. Brains were quickly removed, frozen in liquid N₂ and stored at -80°C . To analyze brain glycogen levels, tissues were homogenized by ultrasonic disruption in ice-cooled 0.6 mol l⁻¹ HClO₄ and neutralized with 1 mol l⁻¹ NaHCO₃. The neutralized homogenate was centrifuged (9000 g for 2 min at 4°C), and samples of the supernatant were used for glycogen analysis. Glycogen levels were assessed by the method of Kepler and Decker (Kepler and Decker, 1974), with minor modifications. Brain glucose obtained after glycogen breakdown was determined enzymatically with a commercial kit (Spinreact, Sant Esteve de Bas, Spain) adapted to a microplate format.

Determination of GPase activity was based on the methods described by Moon et al. (Moon et al., 1989) and Foster et al. (Foster et al., 1993), with minor modifications. Brains were homogenized by ultrasonic disruption in ice-cooled homogenization solution 1:10 (w/v) comprising 50 mmol l⁻¹ imidazol; 100 mmol l⁻¹ NaF; 5 mmol l⁻¹ EDTA-Na; 5 mmol l⁻¹ EGTA; 0.1 mmol l⁻¹ PMSF and 15 mmol l⁻¹ β -mercaptoethanol; pH 7.5. The homogenate was centrifuged (9000 g for 2 min at 4°C) and samples of the supernatant were stored at -80°C for further analysis. Total GPase activity was determined in 50 mmol l⁻¹ phosphate buffer (pH 7.0) containing 0.5 mmol l⁻¹ NADP; 2.5 mmol l⁻¹ AMP; 5.0 μ mol l⁻¹ glucose-1,6-bisphosphate; PGM 2 U tube⁻¹ and G6DPH 5 U tube⁻¹. GPase *a* activity was determined using the same solution as before with 5 mmol l⁻¹ caffeine. Caffeine inactivates the inactive, or *b*, form of GPase and thus provides a direct measure of the active, or *a*, form of the enzyme. GPase *a* activity was defined as the activity in the presence of caffeine, and total GPase as activity without caffeine. Protein determination was performed by the method of Bradford (Bradford, 1976). Enzyme activity was expressed as mU mg⁻¹ protein.

Statistical analysis

All data are presented as means \pm s.e.m. Statistical significance was evaluated by one-way ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. All analyses were carried out with commercial software (SigmaStat v. 2.0, Systat Software, San Jose, CA, USA).

RESULTS

In fish that received i.c.v. injections of different doses of 5-HT (0, 5.12, 12.8, 32, 80 and 200 μ g kg⁻¹) there was a significant ($P<0.05$) decrease in brain glycogen levels 60 min after administration (Fig. 1A). There was also a time-dependent ($P<0.05$) decrease (Fig. 1B) in brain glycogen levels 20, 60, 120, and 180 min after 5-HT administration (12.8 μ g kg⁻¹). No significant variations in brain levels of glycogen were observed in fish injected with saline solution.

Compared with control fish (Fig. 2), there was a significant ($P<0.05$) decrease in brain glycogen levels in hypoglycemic fish (Fig. 2). After 5 days of fasting, administration of 5-HT induced a significant ($P<0.05$) decrease in glycogen levels. This effect was not observed after 10 days of fasting. The administration of 2-DG (300 μ g kg⁻¹) induced a significant ($P<0.05$) decrease in the levels

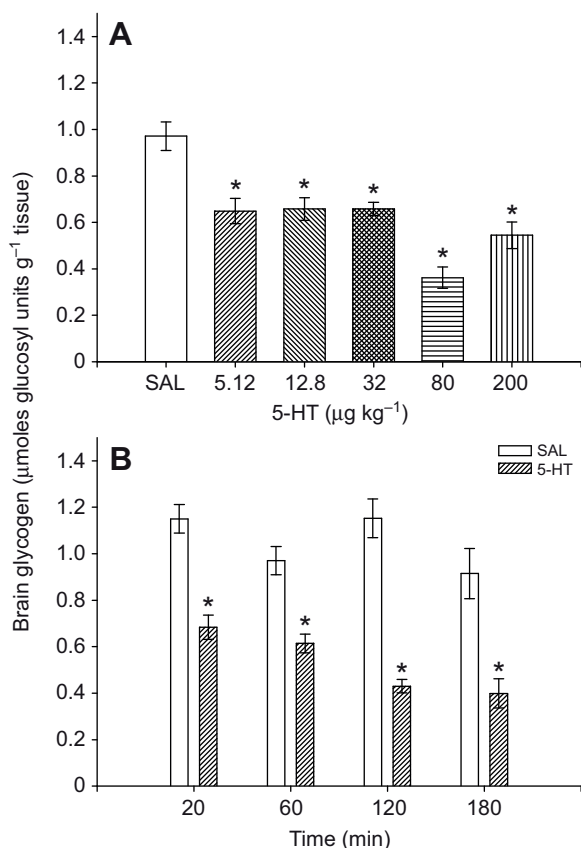


Fig. 1. Effect of intracerebroventricular (i.c.v.) administration of 5-HT on brain glycogen levels in normoglycemic rainbow trout. (A) Brain glycogen levels 60 min after i.c.v. administration of SAL or different doses of 5-HT. Each value is the mean \pm s.e.m. of 10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the saline control group. (B) Brain glycogen levels at different times after i.c.v. administration of saline or 5-HT ($12.8 \mu\text{g kg}^{-1}$). Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective saline control. There were no significant differences ($P > 0.05$) between the saline control groups. SAL, saline; 5-HT, serotonin.

of brain glycogen (Fig. 3). The administration of 5-HT to fish with local brain hypoglycemia (2-DG + 5-HT; $12.8 \mu\text{g kg}^{-1}$) resulted in a significant decrease ($P < 0.05$) in the glycogen levels relative to the control and to the 2-DG groups.

In the fish in which hyperglycemia was induced by i.p. administration of glucose (500 mg kg^{-1}), there was no significant variation in the levels of brain glycogen after 30, 60 and 120 min administration of 5-HT ($12.8 \mu\text{g kg}^{-1}$ 5-HT) (Fig. 4). Likewise, there was no significant variation in brain glycogen levels among fish treated only with glucose.

The i.c.v. administration of 5-HT ($12.8 \mu\text{g kg}^{-1}$) produced an increase in GPase activity (total enzyme activity), which was significant ($P < 0.05$) 20 and 60 min after 5-HT administration (Fig. 5). The observed increase in the activity after 120 and 180 min was not significant.

The i.p. administration of different doses (0.1, 0.5 and 1 mg kg^{-1}) of the 5-HT_{1A} receptor agonist 8-OH-DPAT induced decreases in the levels of brain glycogen, which were significant ($P < 0.05$) 150 min after the dose of 0.5 mg kg^{-1} (Fig. 6B) and 90 and 150 min after administration of the highest dose of 1 mg kg^{-1} (Fig. 6C). Pretreatment with antagonists of the 5-HT_{1A} receptor, WAY100135 (3 mg kg^{-1}) (Fig. 7A) and NAN190 (3 mg kg^{-1}) (Fig. 7B) blocked the

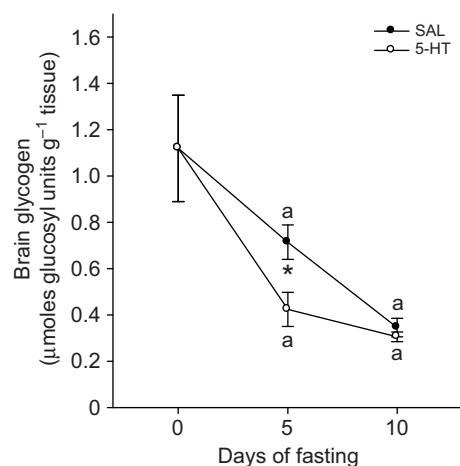


Fig. 2. Brain glycogen levels 60 min after intracerebroventricular (i.c.v.) administration of saline or 5-HT ($12.8 \mu\text{g kg}^{-1}$) in hypoglycemic rainbow trout. Hypoglycemia was achieved by fasting the animals for 5 or 10 days. Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) between fish treated with 5-HT and the respective saline control groups. The same letters indicate significant differences ($P < 0.05$) relative to the 0 days control group. SAL, saline; 5-HT, serotonin.

observed decrease in the glycogen levels induced 150 min after the injection of the 5-HT_{1A} agonist 8-OH-DPAT (1 mg kg^{-1}). The treatments with the antagonists (WAY100135 + 8-OH-DPAT and NAN190 + 8-OH-DPAT) did not result in any significant differences ($P < 0.05$) relative to the respective controls.

The i.p. administration of different doses of the 5-HT_{1B} receptor agonists anpirtoline (0.5 , 1 and 2 mg kg^{-1}) and CP93129 (0.5 , 1.5 and 5 mg kg^{-1}) induced significant ($P < 0.05$) decreases in the levels of brain glycogen (Fig. 8). In the first case, significant differences ($P < 0.05$) were observed with the highest dose of anpirtoline (2 mg kg^{-1}) 30, 90 and 150 min after administration, and with the other doses after 150 min (Fig. 8A). In the case of the agonist CP93129, significant differences ($P < 0.05$) were observed 90 and

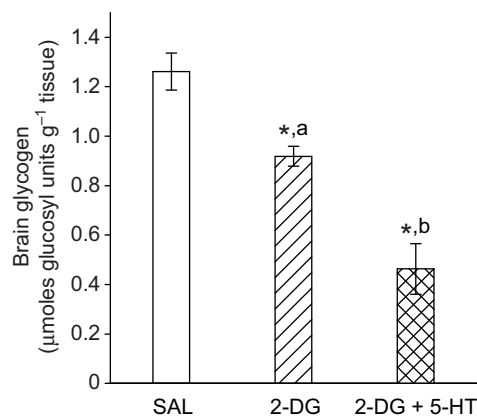


Fig. 3. Brain glycogen levels 120 min after intracerebroventricular (i.c.v.) administration of saline or 5-HT ($12.8 \mu\text{g kg}^{-1}$) in normoglycemic rainbow trout with local brain hypoglycemia induced by i.c.v. administration of 2-DG ($300 \mu\text{g kg}^{-1}$). Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the saline control group. Bars with different letters indicate significant differences ($P < 0.05$). SAL, saline; 2-DG, 2-deoxyglucose; 5-HT, serotonin.

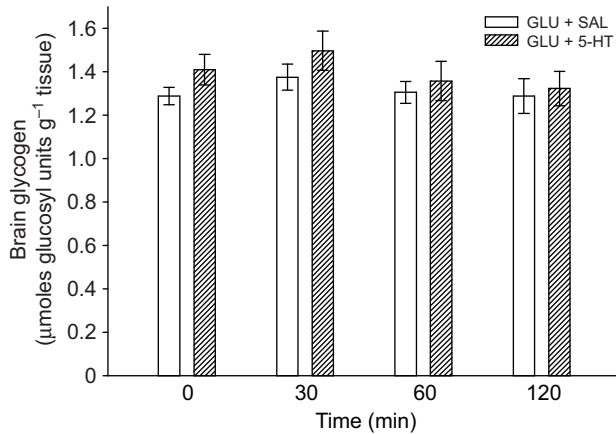


Fig. 4. Brain glycogen levels at different times after intracerebroventricular (i.c.v.) administration of saline or 5-HT ($12.8 \mu\text{g kg}^{-1}$) in hyperglycemic rainbow trout. Hyperglycemia was achieved by intraperitoneal (i.p.) administration of glucose (500 mg kg^{-1}). Each value is the mean \pm s.e.m. of 8–10 fish per group. GLU, glucose; SAL, saline; 5-HT, serotonin.

150 min after the highest dose of 5 mg kg^{-1} (Fig. 8B). Pretreatment with different doses of the 5-HT_{1B} receptor antagonist NAS-181 (4 and 8 mg kg^{-1}) blocked the decrease in the glycogen levels induced 90 and 150 min after injection of the 5-HT_{1B} agonists: anpirtoline (2 mg kg^{-1}) and CP93129 (5 mg kg^{-1}) (Fig. 9). Administration of antagonist + agonists – NAS-181 + anpirtoline (Fig. 9A) and NAS-181 + CP93129 (Fig. 9B) – did not produce significant differences ($P < 0.05$) relative to the respective controls. However, administration of NAS-181 + CP93129 (Fig. 9B), resulted in incomplete blocking of the agonist effect, as 150 min after administration of the agonist, significant ($P < 0.05$) differences in brain glycogen levels were observed relative to the control.

The i.c.v. administration of different doses (10 and $30 \mu\text{g kg}^{-1}$) of the 5-HT₂ receptor agonist α -m-5-HT induced significant ($P < 0.05$) decreases in the levels of brain glycogen 90 min post-administration (Fig. 10). At the other times studied (30 and 150 min),

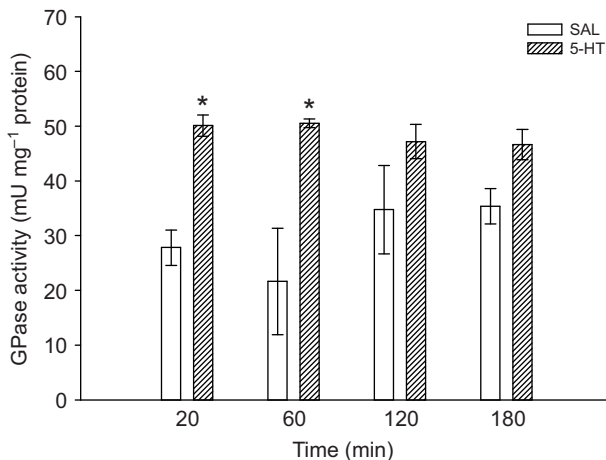


Fig. 5. Brain glycogen phosphorylase (GPase) at different times after intracerebroventricular (i.c.v.) administration of saline or 5-HT ($12.8 \mu\text{g kg}^{-1}$) in normoglycemic rainbow trout. Glycogen phosphorylase activity is expressed in mU mg^{-1} protein. Each value is the mean \pm s.e.m. of 6–7 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective saline control group. There were no significant differences ($P > 0.05$) between saline control groups. SAL, saline; 5-HT, serotonin.

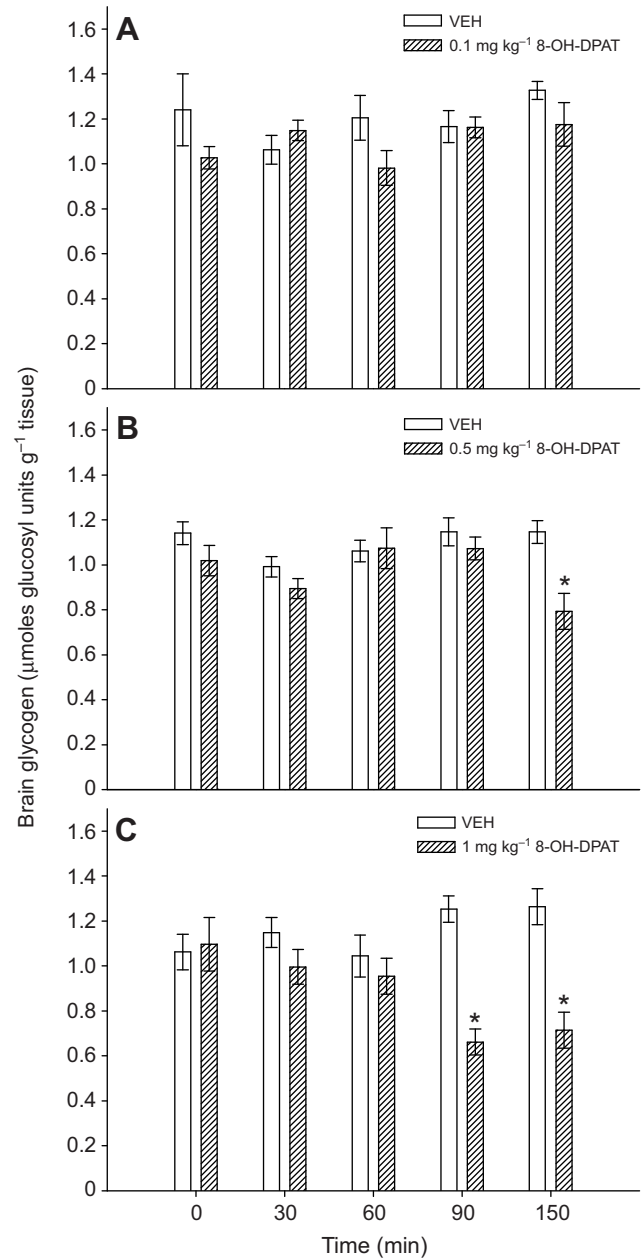


Fig. 6. Effect of intraperitoneal (i.p.) administration of different doses of 8-OH-DPAT (5-HT_{1A} receptor agonist) on brain glycogen levels. (A) Effect of 0.1 mg kg^{-1} 8-OH-DPAT; (B) effect of 0.5 mg kg^{-1} 8-OH-DPAT; and (C) effect of 1 mg kg^{-1} 8-OH-DPAT. Brain glycogen levels were measured 0, 30, 60, 90 and 150 min after administration of 5-HT agonist or vehicle. Each value is the mean \pm s.e.m. of 9–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective control group. VEH, vehicle; 8-OH-DPAT, 8-hydroxy-DPAT hydrobromide.

the decreases were not significant. Pretreatment with different doses of the 5-HT_{2B/2C} receptor antagonist SB206553 (4 and 8 mg kg^{-1}) blocked the decrease in the glycogen levels induced 90 min after the injection of the agonist α -m-5-HT ($10 \mu\text{g kg}^{-1}$) (Fig. 11). In other words, treatment with antagonist + agonist (SB206553 + α -m-5-HT) did not result in significant differences ($P < 0.05$) between these groups and their respective controls.

The i.p. administration of different doses of the 5-HT_{2A/2C} receptor agonist (\pm)-DOI (1 and 3 mg kg^{-1}) and the 5-HT_{2B/2C}

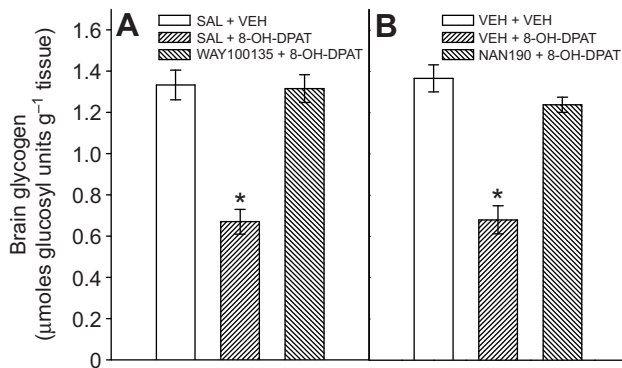


Fig. 7. Effects of NAN190 and WAY100135 (5-HT_{1A} receptor antagonists) on the glycogenolytic action of the 5-HT_{1A} receptor agonist 8-OH-DPAT (1 mg kg⁻¹). (A) Effect of pretreatment with WAY100135 (3 mg kg⁻¹); (B) effect of pretreatment with NAN190 (3 mg kg⁻¹). WAY100135 and NAN190 were administered intraperitoneally (i.p.) 30 min before the 5-HT_{1A} agonist, and the brain glycogen was measured 150 min after i.p. administration of the agonist. Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the control group. VEH, vehicle; 8-OH-DPAT, 8-hydroxy-DPAT hydrobromide; NAN190, NAN190 hydrobromide; WAY100135, WAY100135 dihydrochloride; SAL, saline.

receptor agonist *m*-CPP (2 and 5 mg kg⁻¹) induced significant ($P < 0.05$) decreases in the levels of brain glycogen (Fig. 12); in the first case, with the agonist (\pm)-DOI (Fig. 12A), only 90 min after the highest dose of 3 mg kg⁻¹, and in the case of the other agonist *m*-CPP, 30 and 90 min after the highest dose of 5 mg kg⁻¹ (Fig. 12B).

The i.p. administration of different doses of the 5-HT_{2B} receptor agonist BW723C86 (2 and 8 mg kg⁻¹) and the 5-HT_{2C} receptor agonist WAY161503 (2 and 4 mg kg⁻¹) induced decreases in the levels of brain glycogen (Fig. 13). With the agonist 5-HT_{2B} (BW723C86), the decreases were significant ($P < 0.05$) 90 min after administration of both doses and 30 min after administration of the highest dose of 8 mg kg⁻¹ (Fig. 13A). In the case of the agonist 5-HT_{2C} (WAY161503), significant differences were observed 90 min after administration of the highest dose of 4 mg kg⁻¹ (Fig. 13B).

DISCUSSION

Serotonin modulates brain glycogenolysis

In the mammalian brain, glycogen content is under dynamic control by neurotransmitters, hormones and local glucose concentration (Brown and Ransom, 2007). The results reported in the present study show that the glycogen content of the fish brain is controlled by 5-HT. Nevertheless, this serotonergic control only occurs under normoglycemic and hypoglycemic conditions and not during hyperglycemia. To our knowledge, the present study is the first to provide direct experimental evidence of glycogenolytic control by an aminergic neurotransmitter in a teleost brain.

The results show that under normoglycemic conditions, central administration of 5-HT induced a significant decrease in brain glycogen levels, as observed in mammals (Quach et al., 1982; Poblete and Azmitia, 1995; Darvesh and Gudelsky, 2003), allowing us to suggest that activation of glycogenolysis had occurred. This was confirmed by testing whether 5-HT increased the activity of GPase, the key enzyme controlling the rate of glycogenolysis. This result is also fully consistent with that observed in mammals, in which it has been observed that 5-HT stimulates GPase activity in astroglia-rich primary cultures (Poblete and Azmitia, 1995). It is

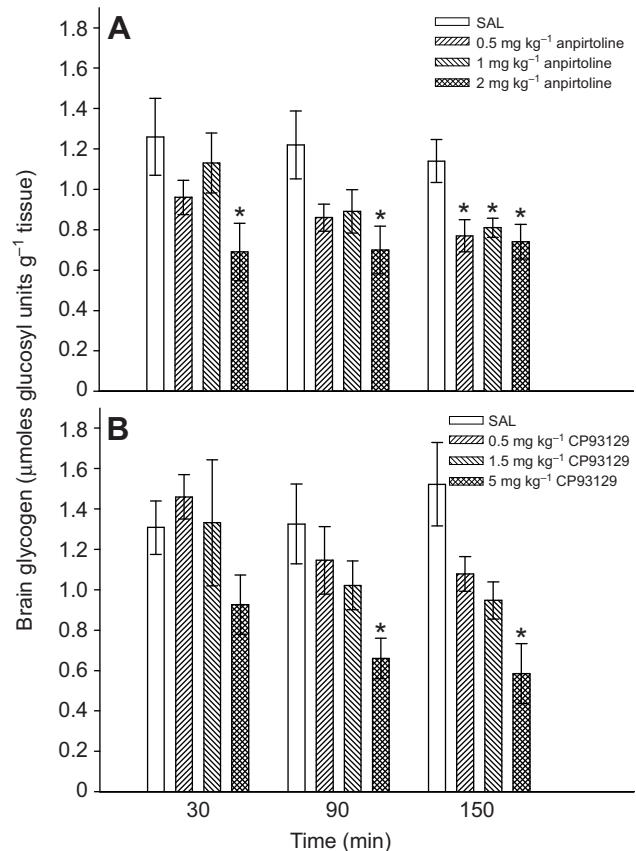


Fig. 8. Effects of intraperitoneal (i.p.) administration of different doses of anipirtoline and CP93129 (5-HT_{1B} receptor agonists) on brain glycogen levels. (A) Effect of anipirtoline at 0.5, 1 and 2 mg kg⁻¹; (B) effect of CP93129 at 0.5, 1.5 and 5 mg kg⁻¹. Brain glycogen levels were measured 30, 90 and 150 min after 5-HT_{1B} agonists or saline. Each value is the mean \pm s.e.m. of 9–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective saline control group. CP93129, CP93129 dihydrochloride; SAL, saline.

clear that in the brain of rainbow trout the decrease in brain glycogen content by 5-HT is mainly due to its action on GPase activity [some effect on the glycogen synthetase activity cannot be ruled out]. The fact that the central 5-HT does not alter plasma glucose levels (Tubio et al., 2010) rules out the possibility that the decrease in cerebral glycogen may be associated with changes in cerebral glucose availability, resulting in direct action of 5-HT on brain cells that produce glycogen.

Taking into consideration that cerebral hypoglycemia is one of the main causes of mobilization of brain glycogen deposits (Choi et al., 2003), some experiments were previously performed in salmonids subjected to a period of fasting, which is known to cause marked hypoglycemia and to induce a mobilization of brain glycogen stores (Soengas et al., 1996; Soengas et al., 1998b; Ruibal et al., 2002). We first observed a decrease in the level of brain glycogen storage of the order of 36% and 70% after 5 and 10 days of fasting, respectively. These data show that in trout, as in mammals, peripheral hypoglycemia will lead to a decrease in brain glycogen levels (Herzog et al., 2008) and the subsequent activation of brain glycogenolysis (Choi et al., 2003). We then observed that, under conditions of hypoglycemia and low levels of brain glycogen, administration of 5-HT also induced glycogenolysis, although the level was not significant at 10 days. The

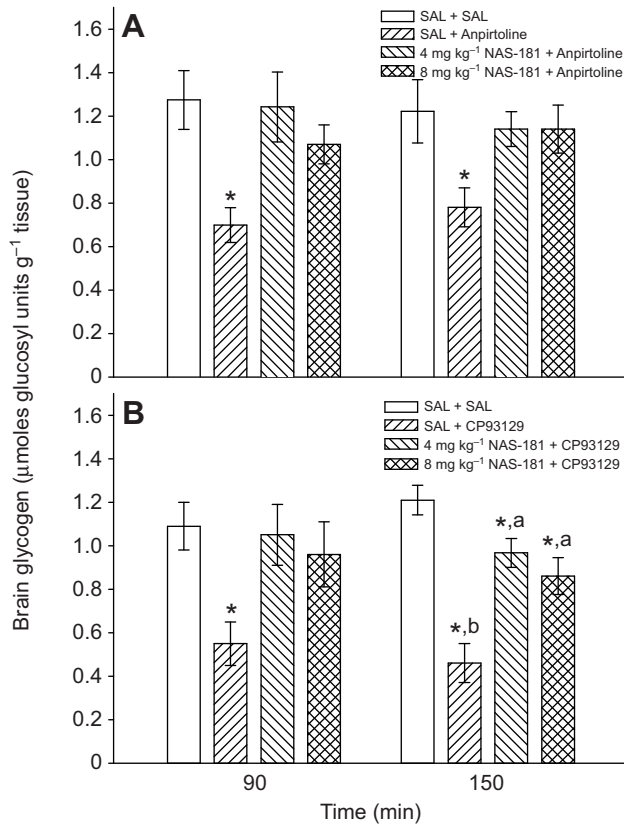


Fig. 9. Effect of NAS-181 (5-HT_{1B} receptor antagonist) on the glycogenolytic action of the 5-HT_{1B} receptor agonists anipirtoline (2 mg kg⁻¹) and CP93129 (5 mg kg⁻¹). (A) Effect of pretreatment with NAS-181 (4 and 8 mg kg⁻¹) on the action of anipirtoline; (B) effect of pretreatment with NAS-181 (4 and 8 mg kg⁻¹) on the action of CP93129. The antagonist NAS-181 was administered intraperitoneally (i.p.) 30 min before the 5-HT_{1B} agonists, and the brain glycogen content was measured 90 and 150 min after i.p. administration of the agonist. Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective saline control group. Different letters on the bars indicate significant differences ($P < 0.05$). CP93129, CP93129 dihydrochloride; SAL, saline.

latter was probably because, under these circumstances, the depletion of brain glycogen stores prior to the administration of 5-HT was sufficiently large (a decrease of 70%) so that the glycogenolytic action was not detected. The results of this experiment were confirmed in another series of experiments with normoglycemic fish, in which local cerebral glycemia was induced by i.c.v. administration of 2-DG (Soengas and Aldegunde, 2004). Under these circumstances, a decrease in 2-DG-dependent brain glycogen levels was observed, and the low levels were again reduced by the action of 5-HT. These results clearly indicate that the glycogenolytic action of 5-HT also takes place under those conditions in which glycogen plays its most important role in providing energy substrates, i.e. when there is a decrease in cerebral glucose as a result of peripheral inputs or a local deficit. It is known that different brain areas are targets of stress and stress hormones (McEwen, 2007), causing changes in levels of brain glycogen (Cruz and Dinieel, 2002; Herzog et al., 2008). Because different types of stress lead to changes in the serotonergic activity in both mammals (Morgan and Rudeen, 1976; Joseph and Kennett, 1981; Singh et al., 1994; Filipenko et al., 2002; Khanbabian et al., 2002) and fish (Winberg and Nilsson, 1993; Winberg and Lepage, 1998), the results allow us to hypothesize that in rainbow trout this

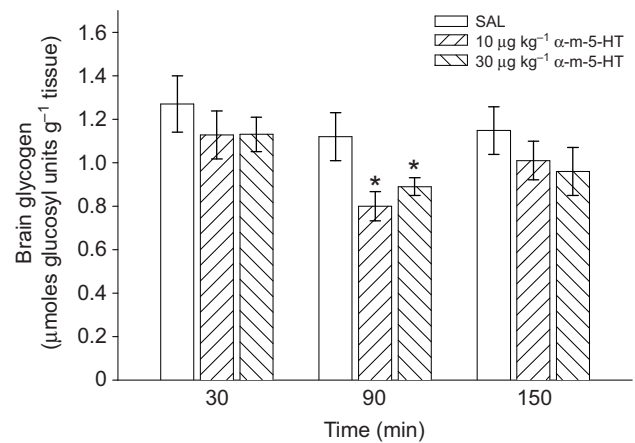


Fig. 10. Effect of the intracerebroventricular (i.c.v.) administration of different doses (10 and 30 $\mu\text{g kg}^{-1}$) of $\alpha\text{-m-5-HT}$ (5-HT₂ generic receptor agonist) on brain glycogen levels. Brain glycogen levels were measured 30, 90 and 150 min after administration of 5-HT₂ agonist or saline. Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective saline control group. SAL, saline; $\alpha\text{-m-5-HT}$, $\alpha\text{-methyl-5-hydroxytryptamine maleate}$.

neurotransmitter may be involved in the mobilization of brain glycogen deposits during situations of stress.

In the rainbow trout, ingestion of a carbohydrate-rich meal or administration of glucose results in prolonged hyperglycemia (Cowey et al., 1977; Blasco et al., 1996; Baños et al., 1998), in a similar way to that observed in mammals with non-insulin-dependent diabetes (Moon, 2001). In light of this, we were interested in studying the action of 5-HT under conditions of acute hyperglycemia induced by the administration of glucose (Ruibal et al., 2002). The results were clear-cut: (i) the administration of glucose did not induce an increase in the cerebral levels of glycogen and (ii) the administration of 5-HT

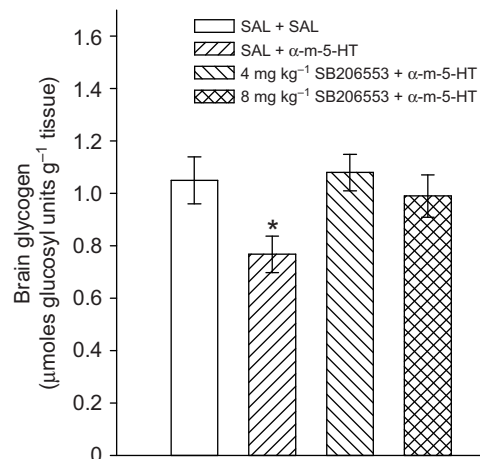


Fig. 11. Effect of intraperitoneal (i.p.) administration of different doses (4 and 8 mg kg⁻¹) of SB206553 (5-HT_{2B/2C} receptor antagonist) on the glycogenolytic action of the generic 5HT₂ receptor agonist $\alpha\text{-m-5-HT}$ (10 $\mu\text{g kg}^{-1}$ i.c.v.). The antagonist SB206553 was administered 30 min before the 5-HT₂ agonist, and the brain glycogen content was measured 90 min after administration of the agonist. Each value is the mean \pm s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the saline control group. SAL, saline; $\alpha\text{-m-5-HT}$, $\alpha\text{-methyl-5-hydroxytryptamine maleate}$.

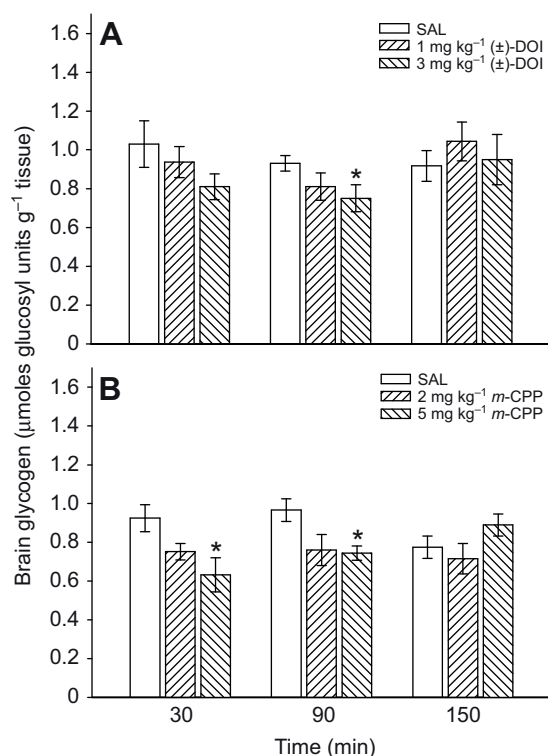


Fig. 12. Effects of the intraperitoneal (i.p.) administration of different doses of (±)-DOI (5-HT_{2A/2C} receptor agonist) or *m*-CPP (5-HT_{2B/2C} receptor agonist) on brain glycogen levels. (A) Effect of 1 and 3 mg kg⁻¹ (±)-DOI; (B) effect of 2 and 5 mg kg⁻¹ *m*-CPP. Brain glycogen levels were measured 30, 90 and 150 min after administration of 5-HT₂ agonists or saline. Each value is the mean ± s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective saline control group. (±)-DOI, (±)-DOI hydrochloride; SAL, saline.

did not induce glycogenolysis. The first observation is not consistent with findings in the mammalian brain, in which hyperglycemia may induce glycogenesis (Morgenthaler et al., 2006; Brown and Ransom, 2007). It is possible that in hyperglycemic rainbow trout the absence of any glycogenic or glycogenolytic response to 5-HT may form part of a metabolic adaptation related to the intolerance of the species to glucose. In relation to this, it has been observed in this species that under short-term hyperglycemic conditions the brain does not appear to be sensitive enough to produce changes in food intake (Soengas and Aldegunde, 2004) and that prolonged hyperglycemia is required before a slight decrease in intake occurs (Polakoff et al., 2008). It can be speculated that an inhibition of GPase brought about by an excess of glucose (Brown, 2004) may counteract the action of 5-HT on this enzyme. Nonetheless, the latter results clearly indicate that in the rainbow trout the role of glycogen as a supply of energy substrates will only take place under conditions of a deficit of cerebral glucose.

In conclusion, these results demonstrate that in fish, as in mammals, serotonin is involved in the modulation of cerebral metabolic homeostasis by activating brain glycogenolysis but that this modulation only occurs in situations of normoglycemia and hypoglycemia and not in hyperglycemia.

Brain 5-HT receptors and glycogenolysis

The cellular responses to 5-HT are mediated by several families of serotonin receptors. In mammals, the pharmacology of 5-HT modulation of brain glycogenolysis appears to involve different 5HT₂

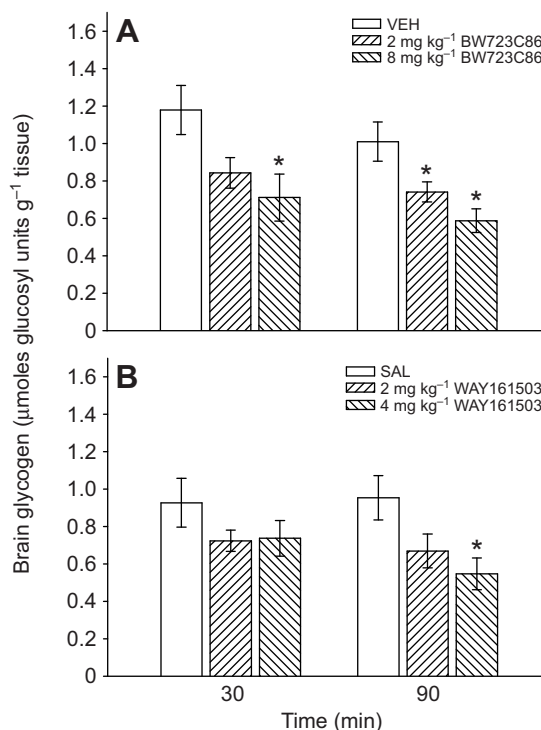


Fig. 13. Effects of the intraperitoneal (i.p.) administration of different doses of BW723C86 (5-HT_{2B} receptor agonist) or WAY161503 (5-HT_{2C} receptor agonist) on brain glycogen levels. (A) Effect of 2 and 8 mg kg⁻¹ BW723C86; (B) effect of 2 and 4 mg kg⁻¹ WAY161503. Brain glycogen levels were measured 30 and 90 min after administration of 5-HT₂ agonists or saline. Each value is the mean ± s.e.m. of 8–10 fish per group. Asterisks indicate significant differences ($P < 0.05$) relative to the respective vehicle or saline control group. VEH, vehicle; BW723C86, BW723C86 hydrochloride; WAY161503, WAY161503 hydrochloride; SAL, saline.

receptor subtypes (Xiaohu et al., 1993; Chen et al., 1995; Poblete and Azmitia, 1995; Darvesh et al., 2002; Darvesh and Gudelsky, 2003). It is known that, in the mammalian brain, glycogen is mainly stored in the astrocytes (Brown and Ransom, 2007) and that these cells express most of the 5-HT subtype receptors (Hirst et al., 1997; Porter and McCarthy, 1997; Hirst et al., 1998; Hansson and Ronnback, 2004; Li et al., 2008; Osredkar and Krzan, 2009), with the 5-HT_{1B} and 5-HT_{2A} subtypes being most widely distributed in the brain (Osredkar and Krzan, 2009). In the non-mammalian brain, glycogen is also present in astrocytes (Kruger and Maxwell, 1967; Roots, 1986; Monzón-Mayor et al., 1990), but much less is known about the possible metabolic glial interactions than in the case of mammals (Bullock, 2004; Samosudova et al., 2010; Verkhatsky, 2010). Organization of the serotonergic system is highly conserved among vertebrates (Parent, 1981; Parent et al., 1984; Jacobs and Azmitia, 1992), although very little is actually known about the presence and/or distribution of the different 5-HT receptor subtypes in the fish brain. Nevertheless, it has been reported that the neurotransmitter receptor pattern in cultured rainbow trout astrocytes appears to be quite similar to that reported for cultured rat astrocytes (Frojo et al., 2002).

The results obtained in this part of the study clearly suggest that 5-HT_{1A} and 5-HT_{1B} receptor-like subtypes mediate the glycogenolytic action of 5-HT in fish brain. The 5-HT_{1A} (8-OH-DPAT) and 5-HT_{1B} (anpirtoline and CP93129) receptor agonists exhibited a clear, dose- and time-dependent stimulation of

glycogenolysis. Moreover, in both cases, selective 5-HT1A (WAY100135 and NAN190) and 5-HT1B (NAS-181) receptor antagonists were able to counteract the stimulatory action of the agonists. There is evidence for the presence of 5-HT1A-like receptors in the nervous system of fish (Dietl and Palacios, 1988; Hansley and Cohen, 1992; Winberg and Nilson, 1996; Winberg et al., 1997; Summers and Winberg, 2006; Allee et al., 2008; Kreke and Dietrich, 2008). By contrast, there is little evidence for the presence of the 5-HT1B receptor subtype. In an autoradiographic study in fish and other lower vertebrates, Palacios and Dietl did not observe any clearly identifiable binding of [³H]5-HT to the putative 5-HT1B site (Palacios and Dietl, 1988). However, more recently, the presence of a 5-HT1B-like receptor has been reported in reptiles (Baxter et al., 2001), amphibians (Butt et al., 2002; McLean and Sillar, 2004) and fish (Kreke and Dietrich, 2008; Norton et al., 2008). The existence in fish brain of a 5-HT1 receptor with pharmacological characteristics similar to those of the mammalian 5-HT1B receptor can therefore be assumed. In summary, we report the first evidence that in the fish brain, glycogenolysis can be stimulated by activation of 5-HT1 receptors with pharmacological characteristics similar to those of mammalian 5-HT1A and 5-HT1B receptors.

The present results show that 5-HT2 receptors also mediate the glycogenolytic action of 5-HT, although to a lesser extent than previously observed in the 5-HT1 receptors. In theory, these results are consistent with findings in mammals, in which it has been found that 5-HT2 receptors mediate the glycogenolytic action of 5-HT (Darvesh et al., 2002; Darvesh and Gudelsky, 2003). Administration of the generic agonist 5-HT2 (α -m-5-HT) or the agonists 5HT2A/2C [(±)-DOI] or 5-HT2B/2C (*m*-CPP) produced glycogenolytic responses, of which that induced by the agonist 5-HT2B/2C was similar to that induced by the generic agonist (α -m-5-HT) whereas that induced by the agonist 5-HT2A/2C was clearly of lower magnitude. These results allow us to rule out involvement of the 5-HT2A subtype and focus on the possible mediation by the 5-HT2B and 2C subtypes. After administration of the selective agonists 5-HT2B (BW723C86) and 5-HT2C (WAY161503) it was found that their activation also gave rise to glycogenolytic effects, although of greater intensity than found with the mixed 5-HT2 (2A/2C and 2B/2C) agonists previously studied. Finally, it was observed that the glycogenolytic effect of α -m-5-HT is blocked by the administration of a potent selective 5-HT2B/5-HT2C receptor antagonist (SB206553).

Although these results are not easy to interpret, the following conclusions can be reached. (1) The glycogenolytic action of 5-HT is partially mediated by 5-HT2-type receptors, *via* 5-HT2B and 5-HT2C-like subtypes. In the latter case, this is consistent with what has been observed in mammals (Xiaohu et al., 1993; Chen et al., 1995). We believe that the greater intensity of glycogenolysis induced by each agonist individually, relative to that observed after administration of the agonist 5-HT2B/2C excludes the possible involvement of a possible 5-HT2-like receptor with mixed 5-HT2B/2C pharmacological characteristics. (2) The glycogenolytic action mediated by the agonist 5-HT2A/2C is clearly explained by activation of the 5-HT2C receptor, which implies that 5-HT2A-like receptors are not involved in the glycogenolytic action of 5-HT, unlike in mammals (Poblete and Azmitia, 1995). (3) The glycogenolytic action mediated by 5-HT2-like receptors is apparently of a smaller magnitude than that mediated by 5-HT1-like receptors. As in the case of 5-HT1 receptors, studies on the possible presence of 5-HT2-type receptors in fish brains are scarce. However, there is some evidence for the presence of 5-HT2 receptors in the fish brain similar to those found in the brains of

mammals (Allee et al., 2008), as well as the presence of receptor subtypes similar to 5-HT2A and 5-HT2B in zebrafish and globe fish (Kroeze and Roth, 2006; Kreke and Dietrich, 2008) and to 5-HT2C in zebrafish and salmonids (Winberg and Nilsson, 1996; Kreke and Dietrich, 2008).

In conclusion, the results demonstrate that, in normoglycemic conditions, 5-HT induces brain glycogenolysis through activation of 5-HT1 (5-HT1A and 5-HT1B-like) and 5-HT2 (5-HT2B and 5-HT2C-like) receptors by a mechanism that involves an increase in the activity of GPase.

LIST OF ABBREVIATIONS

(±)-DOI	(±)-DOI hydrochloride
2-DG	2-deoxy-D-glucose
5-HT	5-hydroxytryptamine or serotonin
8-OH-DPAT	8-hydroxy-DPAT hydrobromide
anpirtoline	anpirtoline hydrochloride
BW723C86	BW723C86 hydrochloride
CP93129	CP93129 dihydrochloride
DMSO	dimethylsulphoxide
GLU	glucose
GPase	glycogen phosphorylase
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
<i>m</i> -CPP	<i>m</i> -CPP hydrochloride
NAN190	NAN190 hydrobromide
SAL	saline
SB206553	SB206553 hydrochloride
VEH	vehicle
WAY100135	WAY100135 dihydrochloride
WAY161503	WAY161503 hydrochloride
α -m-5-HT	α -methyl-5-hydroxytryptamine maleate

FUNDING

This study was supported by a Research Grant from the Xunta de Galicia (INCITE08PXIB2000053PR) to M.A. J.J.P.M. was the recipient of a predoctoral fellowship from the Xunta de Galicia (programa Maria Barbeito).

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