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Hypothalamic control of prolactin release in the rainbow trout, *Salmo gairdneri: in vitro* studies

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Abstract

Hypothalamic control of prolactin (PRL) release in immature rainbow trout *Salmo gairdneri* was investigated using an *in vitro* perifusion system of the rostral pars distalis. Hypothalamic extract of trout induced a dose-dependent stimulation of PRL release. A similar effect was observed when infusing the medium from a 24h static incubation of the hypothalamus. Extracts from different control tissues (muscle, liver, gut) did not change *in vitro* release, thus confirming the specificity of this stimulatory effect. Hypothalamic extract from adult male rat, known to contain PRL release inhibiting factors, stimulated *in vitro* PRL secretion in rainbow trout. This suggests that PRL cells are predominantly influenced by PRL releasing factors. Measurement of TRH and serotonin content in trout hypothalamus indicated consistent physiological levels of these two factors. HPLC studies of hypothalamic extract showed that immunoreactive – TRH eluted at the same place as labelled TRH standard. Moreover, pizotifen, a serotonin antagonist, partially inhibited the stimulation observed with trout hypothalamic extract. These results suggest that, in immature rainbow trout, PRL release is under stimulatory hypothalamic control and that serotonin and probably TRH play a major role in this control.

Introduction

It is generally accepted that in most teleost species prolactin (PRL) secretion is primarily under inhibitory control of the hypothalamus (Ball *et al.* 1972; Schreibman and Holtzman 1975; Ball 1981; Peter and Fryer 1983). Evidence comes from histological studies of ectopically transplanted pituitaries which showed increased activity (Ball *et al.* 1972; Holmes and Ball 1974; Leatherland 1970; Nagahama *et al.* 1974). Moreover, since the pioneer work of Sage (1966), numerous *in vitro* studies indicate that PRL cells remain active during static culture, thus confirming a hypothalamic inhibitory control by neural factors (see reviews Ball 1981; Clarke and Bern 1980). Involvement of catecholamines, probably dopamine as the PRL inhibitory factor (PIF) has been demonstrated in several teleost species (Zambrano *et al.* 1973; Wigham *et al.* 1975, 1977; Nagahama *et al.* 1975; Olivereau 1975). Histochemical studies of pituitary innervation by catecholominergic fibers support such involvement (Follenius 1972; Batten and Ball 1977; Kah *et al.* 1986). Somatostatin has also been suggested as an important inhibitory factor in other teleost species (Grau *et al.* 1982; Batten and Wigham 1984; Grau *et al.*

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1985). However hypothalamic stimulatory factors (PRF) may also act on PRL cell activity as shown in eel (Hall and Chadwick 1978, 1979) and different factors such as serotonin or TRH have been suggested to account for this PRF activity (Olivereau and Olivereau 1979; Hall and Chadwick 1983).

Hypothalamic control of PRL release in salmonids has received little attention. In rainbow trout, transplanted PRL cells appeared less active than the *in situ* gland suggesting a stimulatory control (Leatherland and Lin 1976) which is in agreement with recent studies on PRL release during long-term incubation of chum salmon pituitaries (Suzuki *et al.* 1987). The recent development and validation of an *in vitro* perifusion technique for rostral pars distalis fragments (Gonnet *et al.* 1988) led us to investigate the effect of hypothalamic extract on PRL release in immature rainbow trout. Moreover, we have tried to characterize the neuroendocrine factor(s) involved in such hypothalamic control.

Materials and methods

Animals

Immature rainbow trout *Salmo gairdneri* (50–250 g) were purchased from a hatchery (Gournay-sur-Aronde) and kept in recirculating tap water (13 ± 1°C) in the laboratory under natural photoperiod. The trout were decapitated between 08.00 and 09.00h and the rostral partes distales (RPD) were dissected under a dissecting microscope and preincubated in perifusion medium. The medium consisted of Ringer's solution (NaCl 140mM, KCl 2mM, $CaCl_2$ 2mM, Hepes 15mM) gassed with 95% O_2:5% CO_2. Immediately before use the solution was supplemented with 2.5 g/l glucose and 0.3 g/l bovine serum albumin (fraction V, Sigma). The pH was adjusted to 7.4 and the osmotic pressure was 300 mOsm/kg.

Perifusion

The perifusion system employed in this study has been previously described (Gonnet *et al.* 1988a). Briefly, 3–5 rostral lobes were suspended in Bio-Gel P2 beads in siliconized glass-columns (0.9 × 12 cm) and perifused at constant flow rate (0.2 ml/min) and temperature (15 ± 1°). After a 90 minute equilibration period, test substances freshly dissolved in the perifusion medium were infused at the same flow rate. Samples were collected at 7.5 min intervals during stabilization periods or 2.5 min intervals during infusion of secretagogues. Hormone levels were measured on the day of perifusion or samples were frozen at − 20°C until assay. PRL secreted from rainbow trout RPD was determined using a salmon PRL RIA according to the techniques described by Prunet *et al.* (1985). This RIA has been demonstrated to be specific for measurement of PRL in rainbow trout (Hirano *et al.* 1985; Prunet *et al.* 1985). The sensitivity of the assay was estimated to be 0.4 ng/ml. All experiments were repeated at least 3 times. The PRL concentration was expressed as percentage of the basal value (100%) calculated as the mean of 3 samples (22.5 min) taken just before the infusion of the secretogogues. Statistical differences of PRL levels were tested by Student's t test.

Preparation of extracts and materials

Hypothalami were dissected under a dissecting microscope at 4°C and homogenized using a Polytron homogenizer in 0.1 N HCl (1 hypothalamus/ml). The homogenate was centrifuged (3,000 × g for 30 min) and the supernatant was filtered through a 0.22 μm filter (Millex, Millipore) before dilution in the incubation medium. The pH was adjusted to 7.4 and the osmotic pressure was maintained at 300 mOsm/kg. Extracts from control tissues (gut, liver, muscle) were prepared using the same protocol. The concentration of tissue extract in all experiments were expressed as tissue equivalents. One equivalent is comparable to 1 mg wet weight of tissue (the approximate weight of trout hypothalamus)/ml. In some experiments, 5 hypothalami were incubated statically in 5 ml of perifusion medium for 24h at 12°C and this medium was further tested in our perifusion system. Pizotifen was purchased from Sandoz Laboratory (Basel, Switzerland).

Measurement of serotonin and TRH content

For serotonin determination, hypothalami were extracted in 0.4 N HCl with 50mM β-mercaptoethanol using a sonicator. After centrifugation (3,000 × g for 20 min). The supernatant was neutralized using 2M potassium acetate. Serotonin concentrations were determined by HPLC according to the technique of Graffeo *et al.* (1976). Measurement of TRH in extracts of hypothalamic tissue was made according to the following protocol: 5 hypothalami were homogenized in a methanol acid solution (methanol 75%, acetic acid 6%) and further extracted using a sonicator. After centrifugation (2,000 × g for 20 min) the supernatant was evaporated and the pellet was kept for protein and TRH measurement. TRH concentrations were estimated by RIA as previously described: the radioimmunoassay was performed using an immune serum anti-TRH with high specificity at a final dilution of 1:150,000 and monoiodo ([^{125}I] His^{2})TRH purified by TLC as a tracer (Grouselle *et al.* 1982). The sensitivity of the method was around 4fmoles. Other hypothalamic extracts were analyzed by reverse-phase HPLC using a LKB liquid chromatograph. A C18 ultrasphere IP (4.6 × 150 mm, 5μ) was run isochratically using 1.5% acetonitrile, TFA 0.1% in 15mM ammonium acetate pH 4. Suitable standard (^{3}H-Pro)-TRH was run to determine the retention time of authentic TRH (Burgus *et al.* 1977).

Results

Effect of hypothalamic extracts on PRL secretion

As shown in Fig. 1, infusion of hypothalamic extract from immature rainbow trout for 5 min caused a dose-dependent stimulation of PRL release. Infusion of 0.5 hypothalamic extract equivalent induced a 86.3 ± 3.8% stimulation and the maximal effect was observed 10 min after the beginning of the infusion. Comparison between different extraction procedures of hypothalamic material (HCl 0.1N or acetic acid 1N) gave the same stimulatory effect (data not shown). Repeated administration of the same amount of hypothalamic extract did not lead to a significant reduction of the stimulatory phase (Fig. 2).

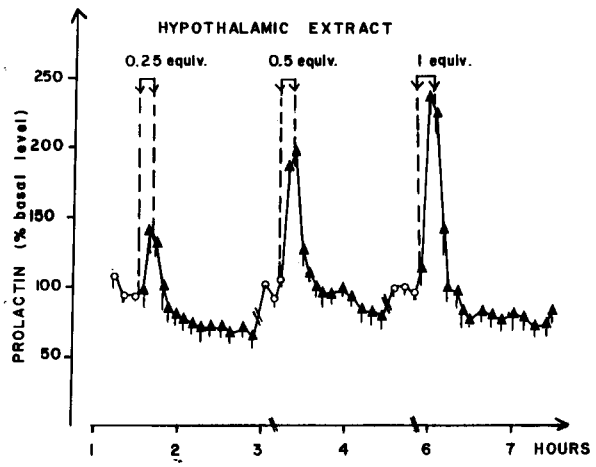

Fig. 1. Effect of increasing concentrations of trout hypothalamic extract (0.25–1 equiv.) on PRL secretion by perifused trout RPD. After a 90 minute equilibration period, the extract was infused for 5 minutes. Data represent the mean ± SEM of three independent perifusion experiments. The reference level of PRL release (100% = basal level) was calculated for each experiment as the mean PRL secretion rate during 22.5 minutes (3 consecutive fractions o----o) just preceding infusion of secretagogue.

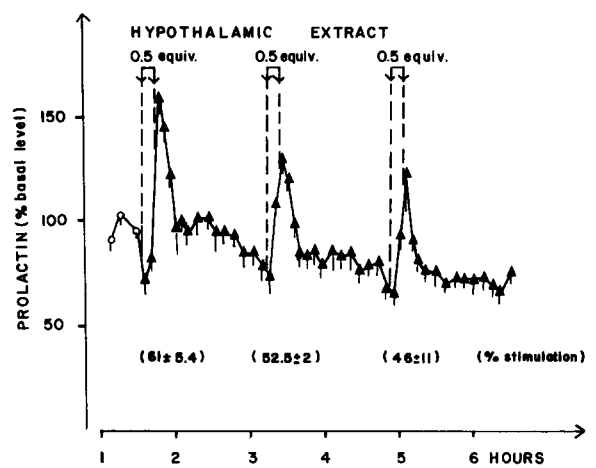

Fig. 2. Effect of repeated administration of trout hypothalamic extract (0.5 equiv.) on PRL secretion by perifused trout PRD. After a 90 minute equilibration period, the extract was infused for 5 minutes and twice subsequently with a 1h equilibration period between each infusion of extract. Data represent the mean ± SEM of 3 dependent perifusion experiments. See legend of Fig. 1 for further details.

Table 1. Effect of different tissue extracts on PRL secreted from perifused RPD

Tissue extract	s-PRL (ng/ml)
Muscle (0.5 equiv.)	18.1 ± 3.1[a]
Control (medium only)	18.4 ± 3.4
Liver (0.5 equiv.)	16.1 ± 4.4[a]
Control (medium only)	14.2 ± 3.1
Gut (0.5 equiv.)	17.7 ± 2.7[a]
Control (medium only)	19.2 ± 3.5

Data are shown as mean ± SEM, n = 3; [a] non significantly different in comparison with respective control (p = 0.05).

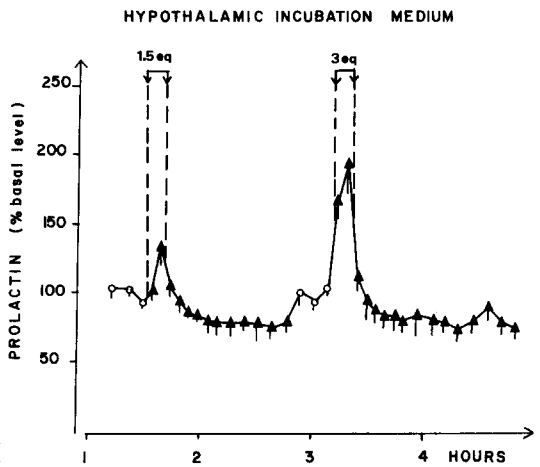

Fig. 3. Effect of trout hypothalamic extract (1 equiv.) and rat hypothalamic extract (1 equiv.) on PRL secretion by perifused trout RPD. After a 90 minute equilibration period, the rat extract was infused for 5 minutes. PRL secretion was allowed to stabilize for 1h and the trout extract was then infused for 5 minutes. Data represent the mean ± SEM of 3 dependent perifusion experiments. See legend to Fig. 1 for further details.

In order to confirm the specificity of this stimulatory effect we measured the amount of PRL in the infused hypothalamic extract and found no detectable level (data not shown). Moreover infusion of different tissue extracts (liver, gut, muscle) did not significantly stimulate PRL release (Table 1). On the contrary, infusion of medium from incubation of 1.5 and 3 hypothalami showed a stimulation of respectively 35.5 ± 4.6% and 94 ± 9.2% (Fig. 3). As shown in Fig. 4, comparison of the effect observed with hypothalamic extract (1 equivalent) from adult male rat and immature rainbow trout

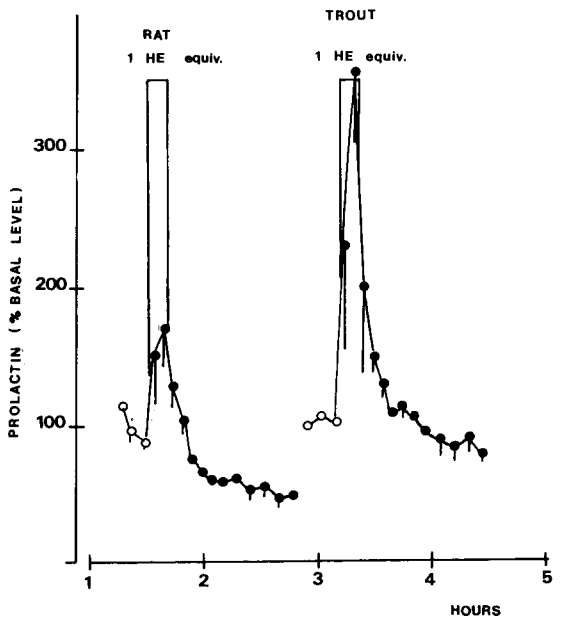

Fig. 4. Effect of medium from trout hypothalami incubations (1.5 and 3 hypothalami/ml) on PRL secretion by perifused trout RPD. After a 90 minute equilibration period, the hypothalami medium, (collected after a 24h static incubation at 12°C) was infused for 5 minutes. A 1h stabilization period was allowed between the two infusions of medium. Data represent the mean ± SEM of 3 dependent perifusion experiments. See legend to Fig. 1 for further details.

showed a 67% stimulation with the rat tissue, whereas a 254 ± 53% stimulation was observed with the fish material.

Characterization of the hypothalamic stimulatory factors

Measurement of TRH content in rainbow trout hypothalami by RIA gave a mean value (n = 5) of 4.7 ± 2.5 ng TRH/hypothalamus which corresponds to an extract concentration of 1.64×10^{-8}M. Furthermore, HPLC analysis of hypothalamic extracts indicated that immunoreactive TRH activity eluted as a unique peak at the same place as standard labelled TRH (Fig. 5).

Serotonin content was also estimated in rainbow trout hypothalamus. A mean value (n = 3) of 13 ± 5 ng (5-HT)/hypothalamus was obtained which corresponds to a concentration of 6.12×10^{-8}M,

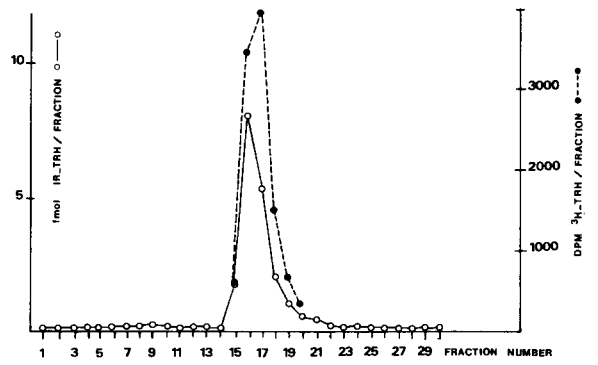

Fig. 5. HPLC of immunoreactive – TRH from trout hypothalamus extract. Elution profile of standard (^{3}H) – TRH (●----●) elution profile of immunoreactive – TRH from trout hypothalamus (o——o)

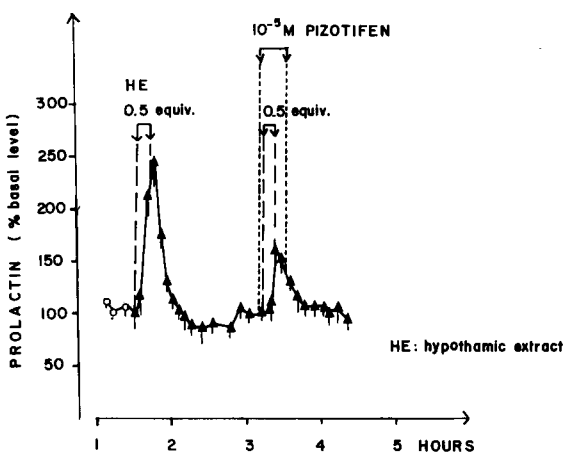

Fig. 6. Effect of trout hypothalamic extract (0.5 equiv.) in the presence or absence of pizotifen (10^{-6}M) on PRL secretion by perifused trout RPD. After a 90 minute equilibration period, the trout extract was infused for 5 minutes. PRL secretion was then allowed to stabilize for 1h and pizotifen was further infused for 25 minutes. After 20 minutes hypothalamic extract was infused for 5 minutes in the presence of pizotifen. Data represent the mean ± SEM of 3 dependent perifusion experiments. See legend to Fig. 1 for further details.

in the extract. Moreover, results presented in Fig. 6 indicated that the stimulatory effect observed after infusion of hypothalamic extract (0.5 equivalent) was partially blocked by pizotifen (10^{-6}M), a specific serotonin antagonist.

Discussion

In the present study we provide evidence using homologous perifusion (hypothalamus and pituitaries were collected from the same group of fish) that hypothalamic extracts stimulate PRL release in immature rainbow trout (Fig. 1). Moreover, the PRL secretion was proportional to the amount of hypothalamic extract infused. Adequate control experiments (Table 1) and observation of a similar stimulatory effect with hypothalamic incubation medium (Fig. 3) further supported the specificity of this effect. The observation of such stimulatory activity of fish hypothalamic extracts has also been reported in eel by Hall and Chadwick (1978, 1983). These authors also showed that this tissue extract stimulated PRL secretion from chicken pituitary *in vitro*, confirming that eel hypothalamus contained predominantly PRL releasing factors (Hall and Chadwick 1979). Using the same extraction procedure, these authors also showed that hypothalamic extract from cod (*Gadus gadus*) and flounder (*Pleuronectes flesus*) inhibited PRL secretion when tested in the same *in vitro* system (Hall and Chadwick 1979). Therefore, it is unlikely that the PRL release stimulatory activity observed in eel was due to the non-extraction of inhibitory factors. The tissue preparation procedure used in our study was the same as the one used in the above studies (Hall and Chadwick 1978, 1979, 1983). When we tested another tissue preparation protocol (1N acetic acid extraction) known to extract dopamine more efficiently (Hyde *et al.* 1987), we still observed a stimulatory effect. Rat hypothalamic extracts also stimulated PRL secretion from rainbow trout pituitaries (Fig. 4), whereas such tissue extract is known to have predominantly prolactin inhibiting capacity (Kragt and Meites 1967; Hall and Chadwick 1979; Hyde *et al.* 1987; Khorram and McCann 1986). These studies support the hypothesis that prolactin release inhibiting factors from rainbow trout hypothalami are not predominantly active in rainbow trout. This is in agreement with the absence of any effect of two of the principal candidates as PRL release inhibiting factors in fish (see reviews by Clarke and Bern 1980; Ball 1981; Bern 1983) when infusing dopamine or somatostatin in our perifusion system (Gonnet and Prunet, unpublished data).

The presence of a predominant PRL release stimulatory activity in hypothalamus from immature

rainbow trout suggests that PRL release might be under stimulatory hypothalamic control in this species. This is in agreement with the observation in rainbow trout of decreasing activity of homotransplanted PRL cells (Leatherland and Lin 1976). In addition, recent studies on PRL secretion from chum salmon pituitaries during long-term incubation experiments also led to the suggestion of a stimulatory hypothalamic control of PRL release in this species (Suzuki *et al.* 1987). Thus, in terms of neural control of PRL release, salmonid species appear to be related to avian and reptilian species, where a similar stimulatory hypothalamic control was shown (Nicoll *et al.* 1970; Tixier Vidal and Gourdji 1972; Hall and Chadwick 1979; Fiorindo 1980).

The recent demonstration of a dose dependant stimulatory effect of TRH on PRL secretion from perifused rainbow trout RPD (authors', unpublished data) led us to investigate the presence of this neuropeptide in hypothalamic extracts. Using a specific RIA, a physiological amount of TRH was found in trout hypothalamus, in agreement with a similar analysis done in salmon hypothalamus (Jackson and Reichlin 1974). We observed that a dose as low as 10^{-10}M TRH stimulated PRL secretion indicating that TRH is a very potent PRL releasing factor in immature rainbow trout (unpublished data). Furthermore, chromatographic studies of trout hypothalamus confirmed that immunoreactive trout TRH is similar to synthetic mammalian TRH (Fig. 5). This is in accordance with previous studies on fish TRH indicating TRH-like immunoreactivity and bioactivity in salmon hypothalamus and high affinity TRH receptors in goldfish pituitary gland (Jackson and Reichlin 1974; Burt and Ajam 1984). Thus, owing to the TRH concentration measured in hypothalamic extracts (1.64×10^{-8}M; in this study) and the stimulation obtained with similar doses of mammalian TRH (10^{-8}M TRH induced 200% stimulation of PRL release (author's, unpublished data), it is conceivable that the stimulatory effect observed in this study with hypothalamic extract may be accounted for by TRH, at least partially. Involvement of TRH as an important prolactin releasing factor was also suggested in other teleost species (Wigham and Batten 1984; Batten and Wigham 1984; Hall and Chadwick 1983; Barry and Grau 1986).

We have recently observed that serotonin stimulated *in vitro* PRL release from perifused RPD and that serotonin was acting directly on PRL cells as the neuroamine was still active on perifused isolated cells. In the present study physiological levels of serotonin were measured in hypothalamic extract. The stimulatory effect observed when infusing hypothalamic extract is partially inhibited by pizotifen, a specific serotonin antogonist (Fig. 6; authors', unpublished data). These results support the involvement of serotonin as another important PRL releasing factor in rainbow trout. Previous studies have demonstrated that serotonin stimulates PRL secretion in fish (Olivereau and Olivereau 1979; Olcese *et al.* 1979; James and Wigham 1984). Moreover, immunoreactive serotonin-containing cells together with fibers were localized in different regions of fish pituitary gland and brain (Goves and Batten 1985; Margolis-Kazan *et al.* 1985; Kah and Chambolle 1983). Although labelled cells or fibers were not observed in the PRL cell region, Groves and Batten (1985) suggested a neurovascular neural route of action for serotonin in the proximal pars distalis. Thus, our results are the first to demonstrate that serotonin is a major contributor of PRL releasing activity of rainbow trout hypothalamus.

In conclusion, this study suggests that PRL secretion in immature rainbow trout is under stimulatory control by the hypothalamus, and that serotonin and probably TRH are two important PRL releasing factors involved in this hypothalamic control. In salmonid species (this study; Suzuki *et al.* 1987) control of PRL secretion appears to be different to what has been reported in other teleost species where a primary inhibitory control by the hypothalamus has been shown (reviewed by Clarke and Bern 1980; Ball 1981; Bern 1983). It seems, however, more likely that a dual control of PRL exists in immature rainbow trout with stimulatory factors predominanting over inhibitory factors and it is possible that this proportion may reverse under different physiological conditions. Thus, in this species characterization of PRL release inhibiting factors requires further investigation.

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