RESEARCH ARTICLE

Cortisol Stimulates Arginine Vasotocin and Isotocin Release From the Hypothalamo-Pituitary Complex of Round Goby (*Neogobius melanostomus*): Probable Mechanisms of Action

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ABSTRACT

There were two aims of this in vitro perfusion study. Firstly, to determine which class of receptors, glucocorticoid (GRs) or mineralocorticoid (MRs), are involved in cortisol regulation of arginine vasotocin (AVT) and isotocin (IT) release from the hypothalamo-pituitary (H–P) complex of round goby (*Neogobius melanostomus*). Secondly, to determine which pathways, genomic or non-genomic, are involved in the aformentioned process.The H–P explants were perfused with cortisol (1.4×10^{-7} M, 2.8×10^{-7} M, 0.4×10^{-6} M); only the highest dose significantly increased a release of both nonapeptides. In the perfusion of H–P explants, we used cortisol (0.4×10^{-6} M) in combination with GRs antagonist RU486 (0.3×10^{-6} M) or MRs antagonist C03DA01 (0.36×10^{-6} M) or transcription inhibitor Actinomycin D (1×10^{-7} M). All inhibitors were also tested seperately. The contents of AVT and IT in the perfusion media was determined by high-performance liquid chromatography (HPLC) with UV detection. This study suggested that different mechanisms were involved in the regulation of AVT and IT release from H–P complex in round goby. Apparently it was GRs but not MRs that were involved in cortisol regulation of AVT and IT release. In the case of AVT, our data points to both genomic and non-genomic pathways mediating the effect of cortisol; in the case of IT, it is only the non-genomic pathway. This study presents the first feasible mechanisms of cortisol action on AVT and IT release from the H–P complex in round goby. *J. Exp. Zool. 9999A: XX–XX, 2015.* © 2015 Wiley Periodicals, Inc.

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Fish are exposed to stress, not only in nature, but also in aquaculture and laboratories. The physiological responses to stressors include activation of the brain-sympathetic-chromaffin cell axis (BSC-axis) and the hypothalamo-pituitary-interrenal axis (HPI-axis). In the BSC-axis, chromaffin cells of the head kidney release catecholamines (adrenaline and noradrenaline) from sympathetic nerve terminals (Wendelaar Bonga, '97). During HPI-axis activation, the corticotropin-releasing factor (CRF) released from the hypothalamus, stimulates the corticotrophic cells of the anterior pituitary to secrete adrenocorticotropin (ACTH), which in turn activates the interrenal cells in the head kidney to synthesize and release cortisol into the circulatory system (Wendelaar Bonga, '97; Reid et al, '98).

Although cortisol, the principal corticosteroid in teleost, is universally recognized as a critical component of the endocrine response to stress, other hormones are also involved in the stress response e.g. arginine vasotocin (AVT), isotocin (IT), urotensins, dopamine, serotonin or β-endorphin (Fryer et al., '85; Winberg et al., '97; Mommsen et al., '99; Arends et al., '99; Kulczykowska et al., 2001; Gesto et al., 2013). Nonapeptides, such as AVT and IT, which are fish homologues of the mammalian arginine vasopressin (AVP) and oxytocin (OT) (Acher, '93) are synthesized in separate parvo- and magnocellular neurons of the preoptic area (POA), stored in axon terminals in neurohypophysis and released into the circulatory system after proper stimulation (Holmqvist and Ekström, '95; Van den Dungen et al., 1982; Saito et al., 2004). Only mature nonapeptides, after dissociation from the non-covalent complex, play an active role as peripheral hormones and neurotransmitters or neuromodulators in the central nervous system (CNS) (Ando et al., '88). There is evidence that AVT and IT are engaged in physiological stress response in fish. In many fish species, changes in the hypothalamic, pituitary and plasma AVT and IT concentrations were found after exposure to various unfavourable situations such as confinement, disturbance, high density, food deprivation or osmoregulatory stress (Kulczykowska et al., 2001; Mancera et al., 2008). In gilthead sea bream (*Sparus aurata*), there are indications that cortisol, AVT and IT release in response to stress can be connected (Kalamarz-Kubiak et al., 2014; Cádiz et al., 2015). The application of cortisol implants in this species enhanced the hypothalamic expression of pro-vasotocin mRNA and pituitary AVT content (Cádiz et al., 2015) and an in vitro study indicated that cortisol affects AVT and IT release from the *nerve terminalis* (Kalamarz-Kubiak et al., 2014). However, to the best of the authors' knowledge, the mechanism of cortisol action on AVT and IT release in teleosts still remains unknown.

It has been established that cortisol, which in fish is produced in the head kidney (equivalent of the mammalian adrenocorticoid gland), has both a corticosteroid and a mineralocorticoid function (Wendelaar Bonga, '97). In fish, an involvement of both classes of corticoid receptors (CRs): mineralocorticoid (MRs) and glucocorticoid (GRs) was widely demonstrated during adaptation to different salinities and osmoregulatory stress (Takahashi and Sakamoto, 2013; McCormick et al., 2008; Kiilerich et al., 2011; Aruna et al., 2012a), fish reproduction (Consten et al., 2002; Milla et al., 2008) and expression of social behavior (Overli et al., 2002; Di Battista et al., 2005; Schjolden et al., 2009). Both MRs and GRs were engaged in tilapia's response to handling stress (Aruna et al., 2012b) and expressed in rainbow trout organs with slow-release cortisol implants (Teles et al., 2013). In teleost, two different GR coding genes (GR1 and GR2) and one MR gene were found (Bury and Sturm, 2007; Greenwood et al., 2003). The expression of GR1, GR2 and MR genes as well as GRs immunoreactivity (GRs-ir) were observed in most of the magno- and parvocellular neurons of the preoptic nucleus (NPO), known for producing AVT, IT and CRF, in rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis mossambicus*) and common carp (*Cyprinus carpio*) (Teitsma et al., '98; Pepels et al., 2004; Stolte et al., 2008). In pituitary, GR1, GR2 and MR mRNA expression and GRs-ir were localized in *pars distalis* and *pars intermedia* where AVT-ergic fibers give their projections (Teitsma et al., '98; Pepels et al., 2004; Stolte et al., 2008).

Glucocorticoid and mineralocorticoid receptors are involved in the genomic and non-genomic mechanism of cortisol action in fish (Sloman et al., 2001; Schjolden et al., 2009; Dindia et al., 2013). Corticosteroid-intracellular receptor complex binds to the nuclear glucocorticoid response elements (GRE) to modulate transcription and protein synthesis (Mommsen et al., '99; Prunet et al., 2006; Aluru and Vijayan, 2009) (genomic pathway). The non-genomic effect is mediated through either non-specific physicochemical interaction with plasma membrane (Falkenstein et al., 2000) or specific membrane receptors such as the G protein-coupled receptor (Tasker at al., 2006) or the plasma membrane-bound form of GR (mGRs) (Bartholome et al., 2004) (non-genomic pathway).

This study determines which class of receptors, GRs or MRs, and which pathways, genomic or non-genomic, are involved in cortisol regulation of AVT and IT release from the H–P complex of round goby (*Neogobius melanostomus*). In the perfusion of the H–P complex, we used cortisol in combination with Mifepristone (RU486) and Spirolactone (C03DA01) or the transcription inhibitor Actinomycin D. Mifepristone is a glucocorticoid receptor antagonist which affects a wide range of physiological and behavioural traits (metabolism, reproduction, osmotic stress, vocalizations and aggression in fish) (Mommsen et al., '99; Ros et al., 2012 for a review). Spironolactone is a mineralocorticoid receptor antagonist which blocks the ion uptake in osmoregulation (Sloman et al., 2001; McCormick et al., 2008) and reduces aggression during social interaction (Schjolden et al., 2009; Sakamoto et al., 2011). Actinomycin D is a transcription inhibitor which binds DNA at the transcription initiation complex and prevents elongation by RNA polymerase (Roy and Rai, 2009; Sobell, '85; Sunny and Oommen, 2001; Prevoo et al., 2011).

MATERIALS AND METHODS

Experimental Fish

Adult round gobies (*Neogobius melanostomus*) of both sexes (n = 74) were caught in the Gulf of Gdańsk (Gdynia, Poland) in autumn, after spawning season. Studies were performed on this species due to its availability and adequate size for in vitro studies and collection of plasma samples. Fish were kept in the Institute of Oceanology PAS (Sopot), in tanks with water from the Gulf (8‰), at 10°C with an artificial lighting regime (12 hr of light: 12 hr of dark) for 1 month. Before conducting experiments, ten randomly selected fish were anesthetized in a 0.5% (v/v) 2-phenoxyethanol water solution (Sigma–Aldrich, St. Louis, MO, USA) and blood samples were collected by cardiac puncture. Plasma was separated by centrifugation in heparinized tubes at 3 000 *g* for 10 min and stored at −70°C prior to cortisol analysis. Cortisol was measured in plasma samples in order to establish the adequate dose for perfusion. At the time of sampling, the rest of the fish (n = 64) were anesthetized in a 0.5% (v/v) 2-phenoxyethanol water solution and, after transection of the spinal cord, their whole brains were immediately collected. The hypothalamo-pituitary (H–P) complexes were dissected under a stereomicroscope (mikroLAB, Lublin, Poland). Before perfusion, explants were washed in Ringer solution supplemented with 10^{-6} mM Bacitracin. The gonadosomic index (GSI) was calculated as gonad weight/body weight × 100. The mean GSI (%) was 1.12 ± 0.14 for females and 0.52 ± 0.07 for males.

All experiments complied with the EC Directive 2010/63/EU for animal experiments and with the guidelines of the Local Ethics Committee on Animal Experimentation.

Perfusion System

All experiments were performed using the perfusion system from MINUCELLS and MINUTISSUE Vertriebs GmbH (Bad Abbach, Germany) according to the method developed by Kalamarz-Kubiak et al. (2011). The set used in the experiments consisted of storage medium bottles, a peristaltic pump (ISMATEC, Wertheim, Germany), a gas exchange module, a gradient perfusion container and plastic vials for the sampling medium after perfusion. The specific structure of the gradient perfusion container facilitates the uniform supply of the medium to the luminal and basal sides. Tissues were put on the Nylon Net Filter 20 μm (Merck Millipore, Darmstadt, Germany) placed between the base and tension rings of the tissue carrier (MINUSHEET; diameter of 13 mm) in a gradient culture container. The set was complemented with a mixture of 95% O_2 and 5% CO_2. The flowing medium was aerated inside the gas exchange module by the gas mixture at a pressure of 127.51 mmHg. Next, a peristaltic pump transported the aerated medium (0.1 mL/min) to the gradient perfusion container and then to the sampling vials. Tissue explants were perfused with Ringer buffer (pH 7.4) modified by Metz et al. (2004); and consisted of: 134 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 25 mM HEPES, 0.5 mM $NaHCO_3$, 0.25% (w/v) glucose, and 0.03% (w/v) BSA. Storage medium bottles and sampling vials were placed on ice and the media collected after perfusion were kept at −70°C, prior to AVT and IT assay. The perfusions were carried out in a laminar air flow (NUAIRE Biological Safety Cabinet Class II, Plymouth, MN, USA). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cortisol Analysis

Plasma cortisol concentration was determined using a competitive enzyme immunoassay (EIA) kit (Cayman, Ann Arbor, MI, USA) following an extraction procedure. Plasma samples were acetated with 3 M HCl to pH 1.5–3. Extraction of plasma samples (100 μL) was performed with methylene chloride according to the method recommended in the EIA kit protocol with modifications. The samples were held at –20°C for 20 min, which allowed for the separation of layers. Then, the methylene chloride layer was decanted into a glass tube and evaporated under a gentle stream of nitrogen. This step was repeated three times. Dried extracts were stored at –20°C for further analysis. The recovery of extraction was in the range of 90 to 110%. Extracts were dissolved in 2 mL of EIA buffer and 50 μL of the diluted samples were used for the EIA. The standard curve consisted of the following concentrations: 20, 10, 4, 1.6, 0.64, 0.256, 0.102, 0.041, 0.0164, and 0.0066 ng/mL. The cortisol-acetylcholinesterase (AChE) conjugate was used as a tracer. The assay was conducted according to the EIA kit manufacturer's instructions with slight modifications. After 18 hr of incubation at 4°C, the plate was rinsed with a wash buffer using a HydroFlex strip-washer (Tecan, Grödig, Austria). Next, the plate was developed with Ellman's reagent in the dark for 90 min. The absorbance was read at 412 nm using a Sunrise Absorbance Reader (Tecan). All samples were assayed in duplicate. The detection limit of the assay was 0.012 ng/mL. The intra-assay coefficient of variation was 0.89%. The inter-assay variation was not determined, because all samples were measured in the same assay. The mean cortisol concentration in the plasma of round gobies was 1.74 ± 0.3 ng/mL; mean ± SEM.

Experimental Design

During 200 min of perfusion, 10 fractions of 2 mL each were collected every 20 min. Perfusions were carried out in four periods. The first period (pre-incubation; 40 min), to stabilize incubation conditions, the second period (control; 20 min), to establish the initial release of AVT and IT from explants, the third period (20 min), for incubation of explants in a medium supplemented with different treatments [cortisol, Mifepristone (RU486), Spirolactone (C03DA01) and Actinomycin D], and the fourth period, for incubation of explants in the medium without any treatments (120 min). Cortisol was tested at three doses (1.4×10^{-7} M, 2.8×10^{-7} M, 0.4×10^{-6} M). Cortisol doses were

selected on the basis of our previous experiments and literature (Barton, 2002; Rotllant et al., 2001; Van der Salm et al., 2004; Martínez-Porchas et al., 2009; Marentette et al., 2013; Kalamarz-Kubiak et al., 2014). The doses of inhibitors were selected on the basis of available data (Aluru and Vijayan, 2007; Sathiyaa and Vijayan, 2003; Mazon et al., 2004; Kiilerich et al., 2007; Shaw et al., 2007). Moreover, before experiments, different doses of RU486 (1.04×10^{-5} M, 0.3×10^{-6} M), C03DA01 (1.08×10^{-5} M, 0.36×10^{-6} M) and Actinomycin D (1×10^{-6} M, 1×10^{-7} M) were tested. Finally, cortisol at 0.4×10^{-6} M dose in combination with RU486 (0.3×10^{-6} M) or C03DA01 (0.36×10^{-6} M) or Actinomycin D (1×10^{-7} M) were used in experiments.

AVT and IT Analysis

Concentrations of AVT and IT in the media collected after perfusion (2 mL) were determined by high-performance liquid chromatography (HPLC) with fluorescence and UV detection, preceded by solid-phase extraction (SPE), according to the modified procedure by Gozdowska et al. (2006). The media after perfusion were acidified with 1M HCl to pH 3–4 and loaded on SPE columns. SPE extraction was carried out on Strata^{TM}-X (30 mg/mL) columns (Phenomenex, Torrance, CA, USA). The extraction procedure for perfusion media was as follows: samples loaded on conditioned columns [1 mL of 100% (v/v) methanol and next 1 mL H_2O], then 2×300 μL H_2O and 2×300 μL of 0,1% (v/v) trifluoroacetic acid (TFA) in 5% (v/v) acetonitrile were passed through the columns to wash away impurities. Hormones were eluted with 2×600 μL of 80% (v/v) acetonitrile. Eluates were evaporated to dryness using TurboVap LV^{TM} (Caliper Life Sciences, Perkin Elmer, Waltham, M, USA). Afterwards, derivatization of peptides was performed using 4-fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F). Dried samples were reconstituted with 40 μL of 0.1% (v/v) TFA in H_2O. For the derivatization reaction, 20 μL of the sample, 20 μL of the 0.2 M phosphate buffer (pH 9) and 20 μL of acetonitrile were mixed and later 3 μL NBD-F (30 mg/mL acetonitrile) was added. The mixture was heated at 60°C for 3 min, cooled on ice, acidified with 4 μL of 1 M HCl and subjected onto HPLC column. Quantitative analyses were performed on the 1200 series Quatemary HPLC system (Agilent Technology, Santa Clara, CA, USA) with a fluorescence detector and with a diode array detector. The chromatographic separation of peptides was carried out on ZORBAX Eclipse XDB-C18 (4.6×150 mm, 5 μm) (Agilent Technology). The following optimized chromatographic conditions were used: mobile phase A [0.1% TFA (v/v) in H_2O]; mobile phase B [0.1% TFA in acetonitrile: H_2O (3:1)]; linear gradient system: 45–70% phase B in 12 min. The column temperature was 20°C and the flow rate was 1 mL/min. Fluorescence detection was performed at 470 nm with emission at 530 nm, UV detection at 215 nm. There were no sex differences in AVT and IT concentrations in any media.

Statistical Analysis

Statistical analysis was performed using STATISTICA 7.1. The concentrations of AVT and IT are presented as % of control. A one-way ANOVA followed by Duncan's multiple range test were used to compare the different treatments within each time point of incubation and the same treatment across the time of perfusion. A two-way ANOVA followed by the Newman–Keuls *Post hoc* test were used to compare two different treatments across the time of perfusion. Significance was taken at $P < 0.05$.

RESULTS

The Influence of Cortisol on AVT and IT Release From H–P Explants

In all doses cortisol (1.4×10^{-7} M, 2.8×10^{-7} M, 0.4×10^{-6} M) significantly increased AVT release ($P < 0.05$–0.001) compared to that after 60 min of perfusion (control) (Fig.1A). The AVT response was biphasic: an initial peak occurred after 80 min and a second one after 140 min of perfusion (Fig.1A). The effect of cortisol was dose-dependent (Fig.1A). Isotocin secretion was also stimulated by cortisol ($P < 0.05$), but the response did not have a biphasic character (Fig.1B). Only the highest dose, which significantly increased a release of both nonapeptides, was used further.

The Influence of Mifepristone (RU486) on AVT and IT Release From H-P Explants

A higher dose of RU486 (1.04×10^{-5} M) caused a 50% inhibition of AVT and a 40% inhibition of IT release and therefore the dose was not used in further experiments (data not shown). A lower dose of RU486 (0.3×10^{-6} M) did not influence AVT and IT release from H–P explants (Fig. 2 A and B), and this dose was used further. Mifepristone (0.3×10^{-6} M) significantly inhibited the biphasic response of AVT ($P < 0.01$; $P < 0.001$, respectively) (Fig. 2A) and the response of IT to cortisol ($P < 0.05$) (Fig. 2B).

The Influence of Actinomycin D on AVT and IT Release From H–P Explants

A higher dose of Actinomycin D (1×10^{-6} M) caused a 43% inhibition of AVT and a 33% inhibition of IT release and those doses were not used in further experiments (data not shown). A lower dose of Actinomycin D (1×10^{-7} M) did not affect the AVT and IT release from H–P explants (Fig. 3A and B), and this dose was used further. Actinomycin D (1×10^{-7} M) inhibited a biphasic AVT response to cortisol ($P < 0.01$; $P < 0.001$, respectively) (Fig. 3A). In contrast to AVT, Actinomycin D (1×10^{-7} M) did not affect the response of IT to cortisol (0.4×10^{-6} M) (Fig. 3B).

The influence of Spirolactone (C03DA01) on AVT and IT Release From H–P Explants

A Higher dose of C03DA01 (1.08×10^{-5} M) caused a 50% inhibition of AVT release and a 25% inhibition of IT release and those doses were not used in further experiments (data not

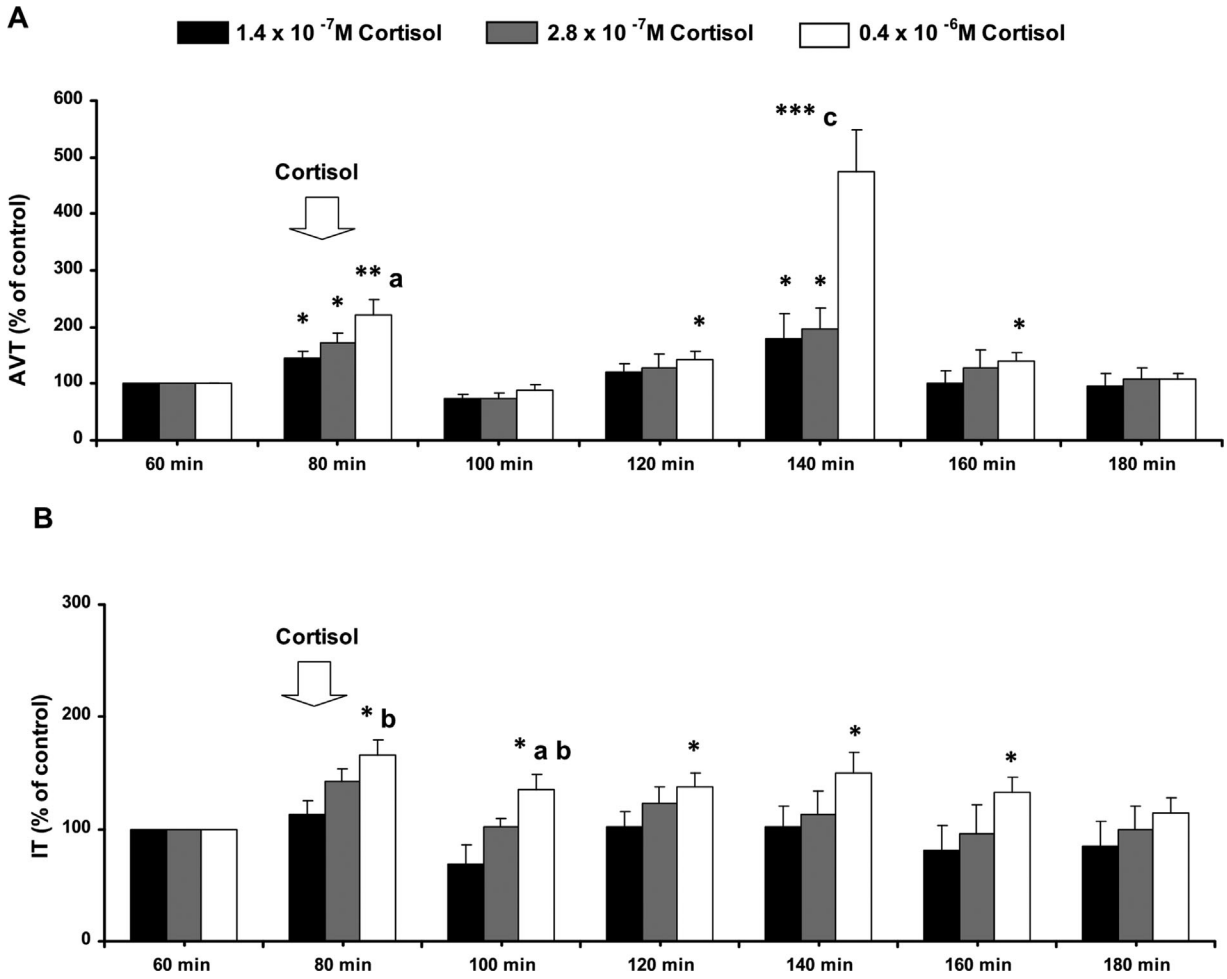

Figure 1. Arginine vasotocin (A) and isotocin (B) release from the hypothalamo-pituitary complex of round goby in response to cortisol (1.4×10^{-7} M, 2.8×10^{-7} M, 0.4×10^{-6} M). Values are the means ± SEM. Nonapeptide values are expressed as % of the control. Asterisks above bars indicate difference versus control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Letters above bars indicate difference between treatments within a time point of incubation; a ($P < 0.05$), b ($P < 0.01$), c ($P < 0.001$).

shown). A lower dose of C03DA01 (0.36×10^{-6} M) did not influence the AVT and IT release from H–P explants (Fig. 4A and B), and this dose was used at a later time. Spirolactone (0.36×10^{-6} M) did not influence AVT and IT release stimulated by cortisol (0.4×10^{-6} M) (Fig. 4A and B).

DISCUSSION

This study presents for the first probable mechanism of cortisol action on AVT and IT release from the H–P complex in round goby. The dose of cortisol used in experiments is within the range of plasma cortisol levels (10^{-7}–10^{-6} M) in fish subjected to stress [the gilthead sea bream (Rotllant et al., 2001); red porgy, *Pagrus pagrus* (Van der Salm et al., 2004); salmonids (Barton, 2002; Martínez-Porchas et al., 2009); round goby (Marentette et al., 2013)]. The results of previous studies of dispersed pituitary cells from *S. aurata* (Kalamarz-Kubiak et al., 2014) also justify the choice of the cortisol dose used in this study.

In this study, cortisol showed a dose-dependent stimulatory effect on AVT release from H–P explants similar to the one presented previously in pituitary cells of *S. aurata* (Kalamarz-Kubiak et al., 2014). Cádiz et al. (2015) showed that implants containing cortisol at the dose of 50 μg/g body weight enhanced the hypothalamic expression of AVT mRNA and hypophysial AVT content in *S. aurata*. In rats, corticosterone affected AVP release from hypothalamic slices containing paraventricular and supraoptic nuclei in a dose-dependent manner (Liu et al., '95).

The results presented here indicate that cortisol, most probably acting through GRs, stimulates AVT release. Expression of GR1 and GR2 mRNAs and GRs-ir was found in the NPO where AVT and IT are produced; and in the pituitary, where AVT-ergic fibers give

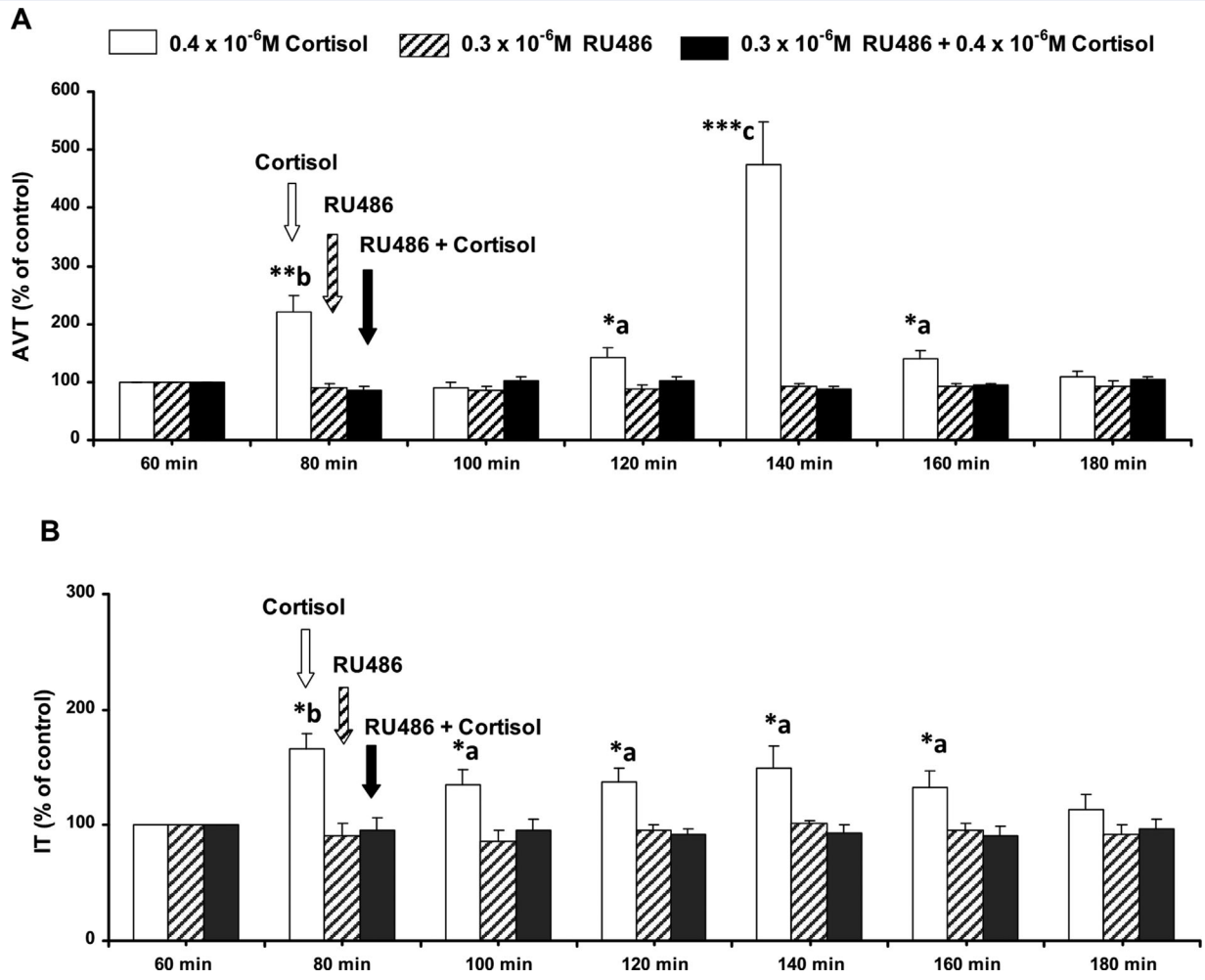

Figure 2. The effect of cortisol (0.4×10^{-6} M), RU 486 (0.3×10^{-6} M) and RU486 in combination with cortisol on AVT (**A**) and IT (**B**) release from the hypothalamo-pituitary complex of round goby. Values are the means ± SEM. AVT and IT values are expressed as % of the control. Asterisks above bars indicate difference versus control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Letters above bars indicate difference between treatments within a time point of incubation; a ($P < 0.05$), b ($P < 0.01$), c ($P < 0.001$).

their projections (Teitsma et al., '98; Pepels et al., 2004; Stolte et al., 2008). It has been suggested that cortisol preferentially binds to GR2 in teleosts, in response to low or mild stress, and to both GR2 and GR1 in response to extreme stress (Bury et al., 2003; Kim et al., 2011). It is possible that both isoforms of GRs are involved in cortisol action on AVT release from the H–P complex. However, a biphasic AVT response may reflect an initial release of mature AVT from the pool stored in the secretory granules, followed by newly matured molecules just after their dissociation from the non-covalent complex. Cortisol also has biphasic effects on the release of inflammatory mediators, such as the tumor necrosis factor-α, interleukin-6, acute phase proteins and the plasma macrophage migration inhibitory factor in vertebrates, including fish (Sapolsky et al., 2000; Yeager et al., 2008).

Our results in round goby strongly suggest that cortisol stimulates AVT release through GRs via both genomic and non-genomic pathways. The GR antagonist RU486 as well as the transcription inhibitor Actinomycin D hindered a biphasic response of AVT to cortisol. It has been demonstrated that RU486 blocks the transcriptional activity of both GR isoforms in response to cortisol in the marine medaka (*Oryzias dancena*) (Kim et al., 2011). A genomic pathway via GRs was suggested in cortisol control of immune stress in vitro in tilapia (*Oreochromis niloticus x O. aureus*) (Law et al., 2001) and handling stress in vivo in rainbow trout (Alderman et al., 2012). Moreover, it was shown that cortisol administered in vivo exerts genomic effects on gluconeogenesis and the activity of metabolic enzymes by GRs in catfish (*Clarias batrachus*) and rainbow trout

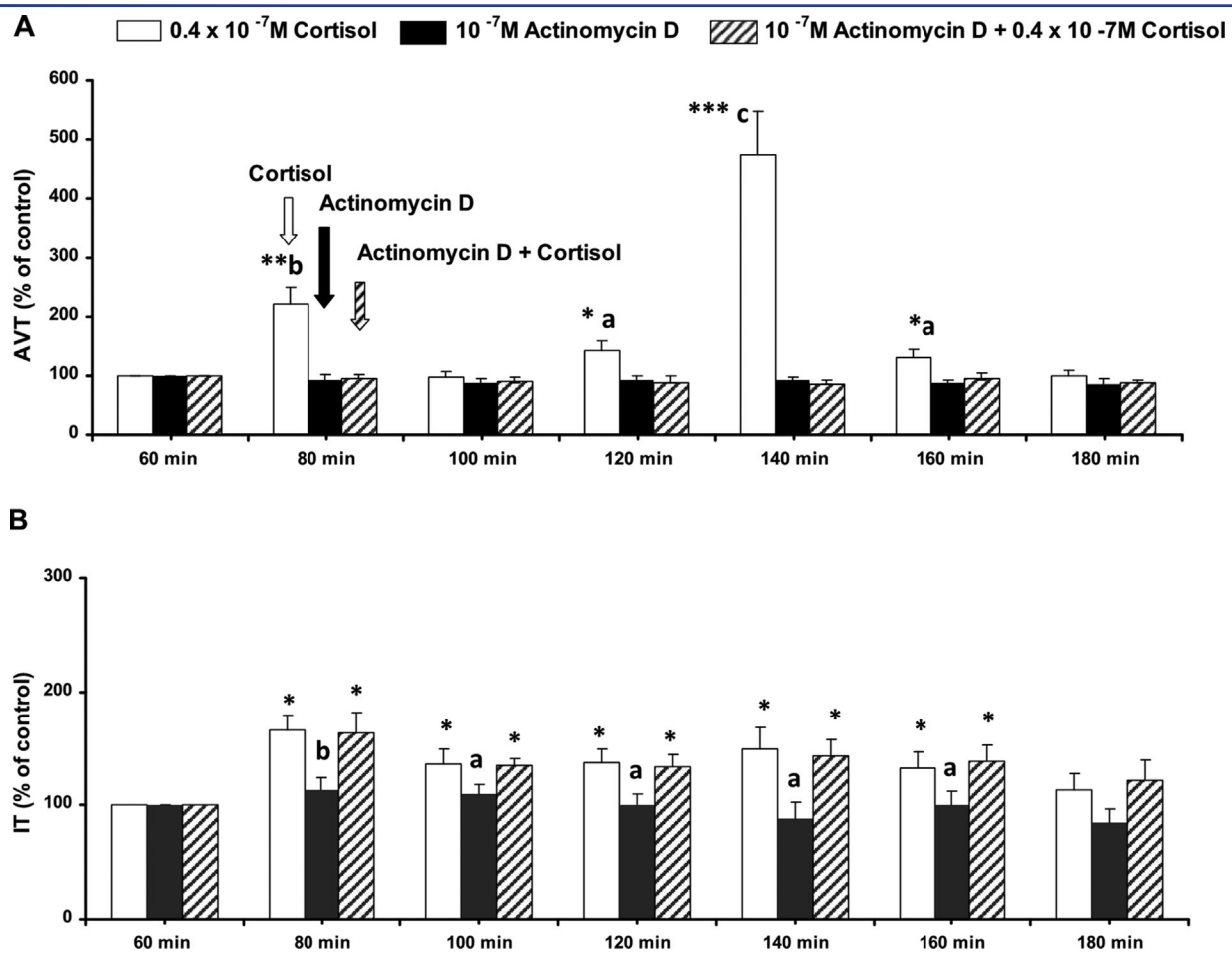

Figure 3. The influence of cortisol (0.4×10^{-6} M), Actinomycin D (1×10^{-7} M) and Actinomycin D in combination with cortisol on AVT (**A**) and IT (**B**) release from the hypothalamo-pituitary complex of round goby. Values are the means ± SEM. Nonapeptide values are expressed as % of the control. Asterisks above bars indicate difference versus control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Letters above bars indicate difference between treatments within a time point of incubation; a ($P < 0.05$), b ($P < 0.01$), c ($P < 0.001$).

(Vijayan et al., 2003; Tripathi and Verma, 2003). In fish, RU486 blocks some rapid, non-genomic effects of cortisol mediated via plasma membrane receptors (Roy and Rai, 2009; Prevoo et al., 2011; Johnstone III et al., 2013). It is possible that mGRs are involved in the first phase of the biphasic AVT response to cortisol in round goby. Alternatively, cortisol may exert non-genomic action through specific membrane receptors such as the G protein-coupled receptor or without receptor involvement via physicochemical interaction with plasma membrane, as it has been shown in mammals (Falkenstein et al., 2000) and fish (Sunny et al., 2001; Dindia et al., 2013). Moreover, it has been postulated that in higher vertebrates and fish, the mechanism of corticosteroid action may integrate non-genomic and genomic pathways (Tasker and Di, 2006; Aluru and Vijayan, 2009; Groeneweg et al., 2011). For instance, in rodents such integration between non-genomic and genomic mechanisms has been shown in the neurons of the amygdala, hippocampus and cortex in response to stress and the administration of corticosterone (Groeneweg et al., 2011 for a review).

The secretion of IT stimulated by cortisol occurred within 20 min and persisted for the next 100 min, similarly to that of AVT, but did not reveal a biphasic character. In tilapia, the non-genomic, stimulatory effect of cortisol on branchial activity of Na^+–K^- and Ca^{2+} ATPases in vivo, appeared after 30 min and persisted for 120 min (Sunny at al., 2001). Similar observations, i.e., fast and long lasting effects of corticosteroids in vitro on the excitability of different brain areas were noted in rodents (Groeneweg et al., 2011 for a review). In round goby, cortisol probably influenced IT release by GRs via the non-genomic pathway without transcription activation because cortisol action

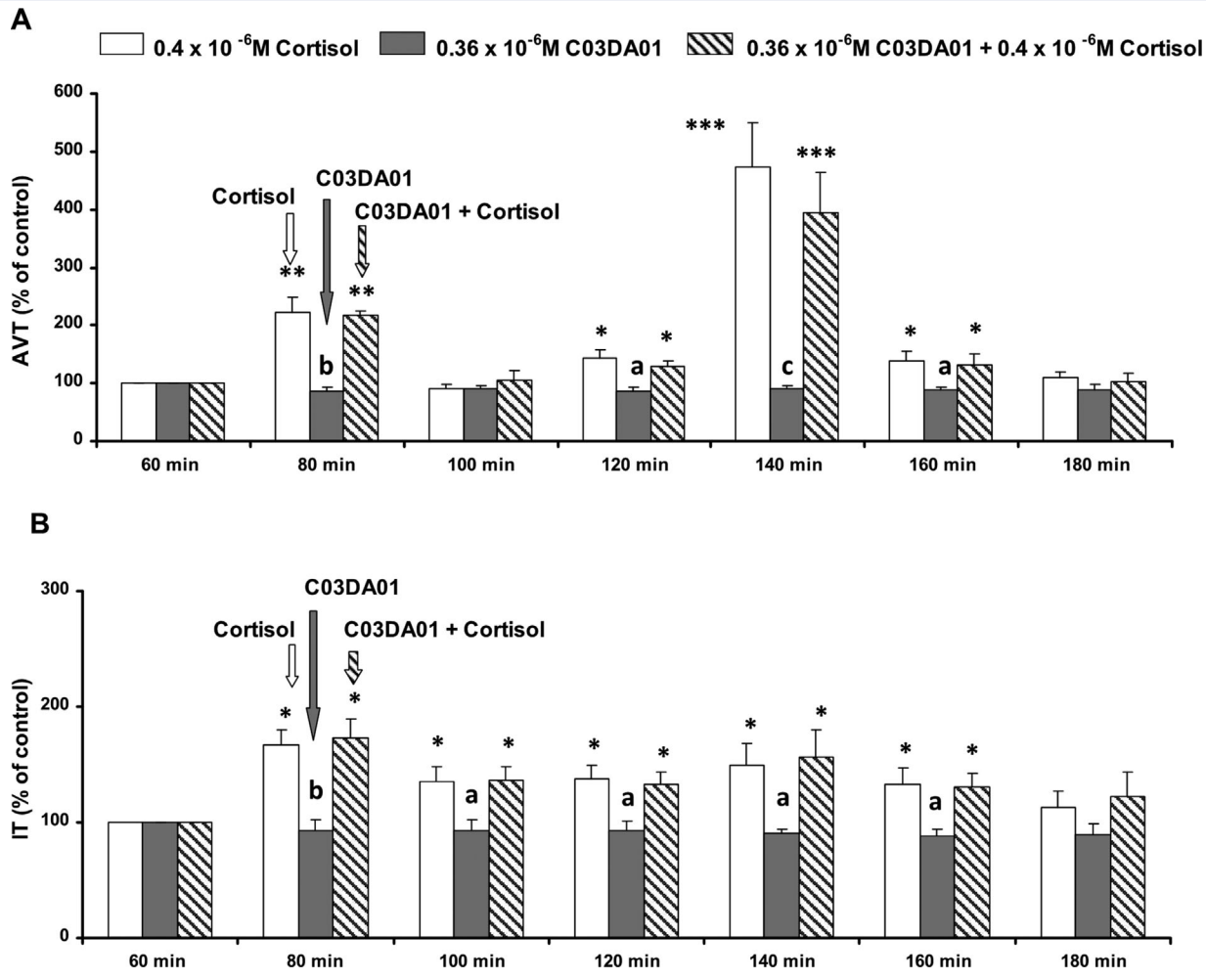

Figure 4. The effect of cortisol (0.4×10^{-6} M), C03DA01 (0.36×10^{-6} M) and C03DA01 in combination with cortisol on AVT (**A**) and IT (**B**) release from the hypothalamo-pituitary complex of round goby. Values are the means ± SEM. AVT and IT values are expressed as % of the control. Asterisks above bars indicate difference versus control; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Letters above bars indicate difference between treatments within time point of incubation; a ($P < 0.05$), b ($P < 0.01$), c ($P < 0.001$).

was inhibited by RU486, but not by Actinomycin D. In contrast to the data in round goby, a former in vitro study of pituitary cells in *S. aurata* showed that cortisol decreased the IT release from nerve endings (Kalamarz-Kubiak et al., 2014). However, round gobies were out of spawning season while gilthead sea breams approached the reproductive season. Therefore the IT responses to cortisol may be different in various species and/or dependent on their physiological status.

It is known that in fish the cortisol effects are mediated not only through the GRs but also through MRs (Wendelaar Bonga, '97). However, our study suggests that MRs do not mediate cortisol effect on AVT and IT release from the H-P complex in round goby, because the MRs' antagonist, C03DA01, does not inhibit AVT and IT release affected by cortisol. Although outside this study, an opposite effect, i.e. the stimulation of cortisol release by AVT, should be also considered. There is evidence that AVT neurons innervate pituitary corticotrophic cells in green molly (*Poecilia latipinna*) (Batten et al., '90) and that AVT synergizes with CRH/CRF (corticotrophin-releasing hormone/factor) to promote ACTH secretion from the pituitary in rainbow trout (Baker et al., '96). Consequently AVT can stimulate cortisol release as well and thus relationships between AVT and cortisol may be more complicated.

Our study presents the first feasible mechanisms of cortisol action on AVT and IT release from the H–P complex in round goby. We suggest different mechanisms where GRs are involved, whereas MRs are not. In the case of AVT, our data points to both

genomic and non-genomic pathways mediating the effect of cortisol. In the case of IT, it is a non-genomic pathway only.

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