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Do circulating plasma AVT and/or cortisol levels control pulsatile urea excretion in the gulf toadfish (*Opsanus beta*)?

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Abstract

Previous work has shown that pulsatile urea excretion at the gills of the gulf toadfish is due to periodic activation of a facilitated diffusion transport system with molecular and pharmacological similarity to the UT-A transport system of the mammalian kidney. In mammals, AVP and glucocorticoids are two important endocrine regulators of this system. The present study focused on the potential role of circulating AVT (the teleost homologue of AVP) and cortisol levels as possible triggers for urea pulses. Long-term (34–84 h) monitoring of plasma levels by repetitive sampling at 2-h intervals from chronic cannulae in individual toadfish demonstrated that circulating AVT concentrations are low (10^{-12}–10^{-11} M), and show no relationship to the occurrence of natural urea pulses. In contrast, plasma cortisol levels decline greatly prior to natural pulses and rise rapidly thereafter. AVT injections into the caudal artery or ventral aorta elicited pulse events, but these were extremely small (1–10%) relative to natural pulses, and occurred only at unphysiological dose levels (10^{-9} M in the plasma). AVP was a partial agonist, but isotocin, insulin-like growth factor-1, and atrial natriuretic peptide were without effect at the same concentration. Artificially raising plasma cortisol levels by cortisol injection tended to reduce responsiveness to AVT. Pharmacological reduction of plasma cortisol levels by metyrapone injection elicited small pulses similar to those caused by AVT. Following such pulse events, AVT was ineffective in inducing pulses. We conclude that *decreases* in circulating cortisol play an important permissive role in urea pulsing, but that circulating AVT levels are not involved.

Keywords: Gulf toadfish; *Opsanus beta*; AVT; AVP; Isotocin; Cortisol; Urea; UT-A urea transporter; Gills

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1. Introduction

Nitrogenous waste excretion in the gulf toadfish is most unusual. This is one of the very few adult teleosts known to express the Krebs ornithine urea cycle in its liver (Mommsen and Walsh, 1989; Anderson and Walsh, 1995). Moreover, while normally ammoniotelic, the toadfish exhibits facultative ureotelism in response to various environmental stresses such as high external ammonia, air exposure, crowding, or even simple confinement (reviewed by Walsh, 1997). Even more unusual is the fact that urea excretion is not continuous, but pulsatile. A fully ureotelic toadfish will excrete almost its entire daily urea production in a single 1–3-h pulse (Wood et al., 1995).

Over the past few years, we have learned that the phenomenon represents pulsatile excretion from the blood plasma, rather than pulsatile production from the liver (Wood et al., 1997) and that the site of excretion is the gills (Gilmour et al., 1998; Pärt et al., 1999). Transmission electron microscopy has demonstrated an intense trafficking of osmiophilic vesicles to the apical membrane of the gill pavement cells (and not chloride cells) occurring only at the time of a pulse (Laurent et al., 2001). In accord with a similar phenomenon in the inner medullary collecting duct (IMCD) of the rat kidney (Nielsen et al., 1996), this morphological event may correspond to an activation of facilitated-diffusion-type transporters for urea. Indeed, pharmacological and radiotracer urea flux experiments have shown that periodic activation of a bi-directional passive transporter with characteristics similar to the UT-A type urea carriers of the rat kidney appears to account for the pulsatile event in the toadfish gill (Wood et al., 1998; Gilmour et al., 1998; McDonald et al., 2000). Very recently, we have isolated and cloned a 1.8-kB cDNA from the toadfish gill ('tUT') which codes for a 475 amino acid protein with $>60\%$ homology to the mammalian and amphibian UT-A transporters, and which mediates facilitated urea transport when expressed in *Xenopus* oocytes (Walsh et al., 2000). The expression of tUT mRNA relative to the expression of β-actin mRNA in the gill tissue was invariant over the pulse cycle, suggesting that the activation of the transporter occurs beyond the level of mRNA.

In the present study, we address the possibility of endocrine control of the pulsatile event by circulating hormones, focusing on arginine vasotocin (AVT) and cortisol. These hormones were chosen for two reasons. Firstly, by analogy to what has been learned about the regulation of UT-A function in the mammalian kidney, we might expect their involvement. Arginine vasopressin (AVP, 'antidiuretic hormone', the mammalian neurohypophysial homologue of teleostean AVT) is known to cause a rapid (<40 s) c-AMP mediated activation of urea transport (Knepper and Star, 1990; Wall et al., 1992) by post-translational activation of UT-A transporters (specifically UT-A1) in the apical membrane of the renal IMCD (Star, 1990; Nielsen et al., 1996; Inoue et al., 1999). Glucocorticoids are known to cause down-regulation of this transport system in mammals, again without changing the level of mRNA (Marsh and Knepper, 1992; Naruse et al., 1997). Secondly, our earlier studies on toadfish provided some preliminary indications that these two hormones might be involved. Wood et al. (1997) reported that circulating plasma cortisol concentrations declined markedly prior to natural urea pulse events (typically 1000–6000 μmol N kg^{-1}) and increased thereafter, suggesting that removal of down-regulation might play a causative or at least a permissive role in activating the transport system. In intact toadfish, Perry et al. (1998) were able to elicit urea pulse events, albeit much smaller (a few hundred μmol N kg^{-1}) than natural urea pulses by intravascular injections of AVT. Circulating plasma levels of native AVT are unknown in toadfish, but AVT injections were effective in causing small urea pulses at a dose calculated to produce circulating levels of 10^{-10}–10^{-9} M. However, 10^{-10} M AVT in the perfusate was ineffective in causing urea pulses in an isolated-perfused head preparation (Pärt et al., 1999).

With this background in mind, we set out to investigate the possible role(s) and interactions of these two hormones in causing natural urea-N pulses in intact toadfish. Our first objective, using a sensitive radioimmunoassay (Warne et al., 1994), was to record for the first time the endogenous circulating levels of AVT in ureotelic toadfish, simultaneously to those of cortisol, in relation to natural pulse events. Our second objective was to use injections of AVT and analogues (arginine vasopressin, isotocin), cortisol, and metyrapone (a cortisol-lowering agent) alone and in various com-

binations to see if we could mimic or inhibit natural pulses. Overall, the results confirm the importance of decreases in circulating cortisol, probably in a permissive role, but indicate that changes in circulating AVT are not the proximate cause of natural pulse events.

2. Materials and methods

2.1. Experimental animals

Gulf toadfish (*Opsanus beta* Goode and Bean) were collected from Biscayne Bay, Florida, USA, in November–December 1998, by a commercial shrimp fisherman using a roller trawl. In the laboratory, they were prophylactically treated against potential ciliate infections, fed live shrimp several times per week, and held under natural photoperiod and 'uncrowded' conditions as described earlier (Wood et al., 1997). Temperature was 26°C and salinity was 31 ppt. Approximately 2–3 days prior to surgery, fish were subjected to a standardized crowding and confinement protocol (see Walsh et al., 1994) to induce the switch to ureotelism. Caudal artery catheters (PE 50 polyethylene tubing) for chronic blood sampling were surgically implanted under MS-222 anesthesia (methods as in Wood et al., 1997). In four fish of *series 2*, ventral aortic catheters (PE 50 tipped with 1 cm of PE 10) were implanted through the posterior wall of the bulbus arteriosus (methods as in Wood and Shelton, 1980). Thereafter, toadfish were confined to individual darkened containers (volume approx. 3 l), each containing a PVC shelter; the containers were continuously aerated and served with flowing seawater at 200 ml min^{-1}. This confinement ensured the maintenance of the ureotelic condition. Experiments (*series 1*) or pre-experimental monitoring (*series 2*) started approximately 24 h after surgery.

2.2. Experimental series 1-Natural variations in plasma AVT and cortisol in relation to urea-N pulses

The eight largest toadfish available were used (mean weight 322 ± 24 g, range 255–452 g) so as to minimize the effect of repetitive blood sampling on hematocrit. Water flow to the containers was closed off, the volume set to exactly 2.0 l, and the aeration set so as to provide good mixing. In order to detect pulsatile urea excretion, water samples (5 ml) were taken automatically at 1-h intervals over the next 2–3.5 days using a battery of peristaltic pumps and fraction collectors, as described previously (Wood et al., 1995). The water was renewed every 24 h. All water samples were analyzed colorimetrically for urea-N, and the initial and final samples of each 24-h period for ammonia-N to allow calculation of percent ureotelism.

For measurement of circulating AVT and cortisol levels, blood samples were withdrawn repetitively via the caudal artery catheter of each fish at successive 2-h intervals for at least 44 h, except in the case of one fish where the catheter failed after 34 h. For the two largest fish, sampling continued for 68 and 84 h. A blood sample of approximately 0.8 ml was withdrawn at each time, and immediately centrifuged for 1 min at 10 000 $\times g$ to separate the plasma. Two separate plasma samples, one exactly 500 μl (for AVT) and the other 25–50 μl (for cortisol) were immediately frozen in liquid N_2, and later stored at −80°C. The samples for cortisol were also used to measure plasma urea-N concentrations at some times. The red blood cells plus any remaining plasma were re-suspended in toadfish saline (Walsh, 1987) back to the volume of the original blood sample, and re-infused into the original animal within 10 min. Thanks to this procedure, the drop in hematocrit from the beginning to the end of the experiment was fairly small (from $21.0 \pm 1.1\%$ to $17.7 \pm 0.7\%$, $N = 8$) despite the large number (17–42) of blood samples taken.

2.3. Experimental series 2-Responses to injections of AVT, analogues, cortisol and metyrapone

Experiments using caudal artery injection were performed on 30 smaller (but still sexually mature) toadfish (mean weight 92 ± 6 g; range 38–166 g); experiments using ventral aortic injection employed four toadfish (149 ± 37 g; 71–250 g). The automatic system described above for hourly water sampling was used to monitor and document the pattern of urea-N excretion and the percent ureotelism over a 24–36-h period prior to the start of experiments in 14 fish. However, due to a shortage of pumps and fraction collectors, in the other 20 fish manual samples were taken only occasionally during this period. The latter were sufficient to establish that the fish were ureotelic, but not detailed enough to de-

scribe the pattern of excretion. For this initial monitoring on all fish, as well as for subsequent injection experiments, water flow to the chambers was stopped, and the starting volume was set to an exact value of 1.4–2.0 l. The water was renewed every 24 h.

After preliminary experiments demonstrated that various injected agents caused only very small urea-N pulses, the radioactive [^{14}C]urea technique developed by Wood et al. (1997) was adopted so as to detect such pulses with greater sensitivity than by simple colorimetric assay. In this method, the urea-N pulse is quantified by measuring the appearance of radioactivity in the external water and relating this to the measured specific activity of urea in a blood plasma sample taken prior to the pulse event. Earlier, it was demonstrated that more than 95% of the ^{14}C counts in plasma and water stayed as urea (i.e. no degradation), and that there was a 1:1 relationship between urea-N pulses measured colorimetrically and urea-N pulses measured radioactively (Wood et al., 1997).

Therefore, following the monitoring period, and at least 3 h prior to any injection experiments, the fish were loaded with 400 μCi kg^{-1} [^{14}C]urea (New England Nuclear, 8.60 mCi mmol^{-1}; 1 mCi $= 3 \times 10^7$ Bq) in 2 ml kg^{-1} of toadfish saline and washed with a further 2 ml kg^{-1} of saline. In longer-term experiments, the plasma radioactivity was periodically 'topped' up on an ad hoc basis in those fish where considerable radioactivity had been lost by spontaneous natural pulses. The specific activity of urea in the plasma was monitored prior to the injection of each exogenous agent, and urea-N excretion was monitored by sampling of the external seawater at 0.5-h intervals for at least 2 h prior to and for a predetermined period of 2–6 h after the injection, depending on the experiment.

All agents were injected in a volume of 1–2 ml kg^{-1} of toadfish saline, and were washed with a further 2 ml kg^{-1} of toadfish saline. In preliminary trials, routine injections of saline vehicle never elicited a urea-N pulse event and, therefore, were discontinued. Agents employed were: the neurohyphysial peptides arginine vasotocin (AVT), arginine vasopressin (AVP), isotocin (ISO); insulin-like growth factor-1 (IGF-1; human, recombinant); atrial natriuretic peptide (ANP; frog, synthetic); cortisol (hydrocortisone 21-hemisuccinate, sodium salt); and the cortisol synthesis blocker metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone). All were obtained from Sigma. For the purposes of calculating the extracellular concentration of injected agents, an extracellular fluid volume of 300 ml kg^{-1} was assumed (Perry et al., 1998).

As a general pattern, water samples were drawn manually at 0.5-h intervals for at least 2 h to establish a baseline rate of [^{14}C]urea appearance, and then a small blood sample (< 100 μl) was taken to measure plasma total urea, [^{14}C]urea radioactivity (and therefore specific activity), and in some cases cortisol. Agents were injected immediately after blood sampling, and manual water sampling continued at 0.5-h intervals for a further 2–6 h. No injections were given until at least 6 h had passed from the last natural urea-N pulse. In some cases, agents were injected in a particular time sequence, and additional blood samples were taken as described in Section 3. Blood samples were centrifuged immediately at $10\,000 \times g$ to separate plasma which was aliquotted for plasma urea-N (2×10 μl) and radioactivity (2×10 μl) measurements, while the remainder was frozen at -80°C for later cortisol assay (1×25 μl). In cases where the same fish was used to evaluate a different agent, 24 h were allowed to elapse before the next injection.

2.4. Analytical techniques

Standard colorimetric assays on a micro-plate reader (Thermomax; Molecular Devices) were employed for the analysis of urea-N (Rahmahtullah and Boyde, 1980; Price and Harrison, 1987) in seawater and blood plasma, and ammonia-N (Ivancic and Degobbis, 1984) in seawater. For analysis of [^{14}C]urea radioactivity, 5 ml of radioactive seawater or 10 μl of radioactive plasma plus 5 ml of 'cold' seawater was added to 10 ml of ICN Ecolume fluor; samples were counted on a TmAnalytic 6895 BetaTrac or a Beckman LS 1801 liquid scintillation counter.

Cortisol was measured on 25-μl plasma samples using a commercial ^{125}I radioimmunoassay kit (ICN Immuno Corporation) with standards diluted to the same protein range as toadfish plasma; a Canberra-Packard Minaxi gamma counter was used. Plasma protein changes over the time course of this repetitive sampling experiment amounted to no more than a 20% decline, and had no influence on the modified cortisol

RIA. For assay of AVT, the frozen 500-μl plasma samples of *series 1* were lyophilized for at least 16 h to obtain a concentrated dry powder and shipped to the University of Manchester for analysis by radioimmunoassay. Briefly, AVT was extracted from plasma by reverse phase chromatography using Sep Pak C18 cartridges, and duplicates of each sample were assayed for AVT using the method described by Warne et al. (1994). All cortisol concentrations were substantially above the detection limit of the cortisol RIA, but this was not the case for the AVT RIA. For those samples, the value of the detection limit in plasma for the assay regime (0.34 pmol l^{-1}) was assigned for the purposes of averaging.

2.5. Calculations

All urea (and ammonia) concentrations and fluxes have been expressed in units of nitrogen for comparative purposes and consistency with previous studies (e.g. Wood et al., 1995, 1997, 1998; Perry et al., 1998). Pulses of urea-N excretion were identified from stepwise increases in seawater urea-N concentration over 1-h intervals (*series 1*, and *series 2* pre-experimental monitoring) or [^{14}C]urea radioactivity over 0.5-h intervals (*series 2*). When colorimetric methods were employed, excretion rates (in μmol N kg^{-1} h^{-1}) of urea-N and ammonia-N were calculated from changes in concentration in the external seawater (μmol N l^{-1}) multiplied by volume (l) and factored by time (h) and mass (kg). When the [^{14}C]urea method was used, the total radioactivity (cpm) appearing in the water in each interval (cpm ml^{-1} × volume in ml) was divided by the measured specific activity of the preceding plasma sample (cpm μmol^{-1} urea-N), and then again factored by time (h) and mass (kg). Total urea-N pulse sizes were calculated in the same way, but not factored by time.

Data have been expressed as means ± 1 S.E.M. (N) where N represents either the number of fish, or the number of urea-N pulse events, or the number of samples, as specified. Time series data were analyzed by analysis of variance followed by Duncan's new multiple range test. The significance of differences between means was evaluated using Student's two-tailed t-test, paired or unpaired as appropriate. Percent occurrence data were analysed by the parametric test of proportion. For multiple comparisons, the probability values were adjusted via the Bonferroni procedure to yield an overall fiducial level of 0.05. Data which were not normally distributed were subjected to appropriate transformation (e.g. arc-sin for percent data; logarithmic for plasma AVT) prior to test.

3. Results

3.1. Ureotelic behavior

In both *series 1* and *series* 2, the toadfish were predominantly ureotelic in the typical fashion, excreting more than 75% of their nitrogenous waste as urea-N, with approximately 90% of this occurring in pulses at a frequency of approximately once per day (Table 1). There was, however, a substantial difference in the overall rates of both urea-N and ammonia-N excretion (relative to body mass), which were three to fivefold higher in the fish of *series 2* than in those of *series 1.* Indeed these rates approximated the upper and lower bounds of rates measured in previous studies on ureotelic toadfish (see Section 1 for references). For urea-N, this was mainly due to a 3.5-fold higher mean pulse size in the fish of *series 2* relative to *series 1*; pulse frequency was

Table 1
A comparison of the rates and patterns of N-waste excretion in the toadfish of *series 1* and *series 2*. Mean ± 1 S.E.M.

	Series 1 ($N = 7$)[b]	Series 2 ($N = 14$)[c]
Body weight (g)	303.5 ± 17.9	79.3[a] ± 9.3
Urea-N excretion rate (μmol N kg^{-1} h^{-1})	42.9 ± 8.4	208.7[a] ± 54.8
Amm-N excretion rate (μmol N kg^{-1} h^{-1})	16.4 ± 4.6	46.2[a] ± 9.4
% Ureotelism	75.9 ± 5.0	77.9 ± 4.2
% Urea-N excretion in pulses	89.3 ± 4.4	91.9 ± 3.4
Urea-N pulse size (μmol N kg^{-1} h^{-1})	1201.9 ± 246.0	4334.0[a] ± 1130.5
Urea-N pulse frequency (pulses day^{-1})	0.79 ± 0.12	1.13 ± 0.09

[a] $P < 0.05$.

[b] One fish (out of eight total) which showed negligible ammonia-N excretion and only one incompletely resolved urea-N pulse at the time of the final two water samples was excluded from this summary.

[c] Values from the 14 fish (out of 34 total) for which 24–36 h of pre-experimental monitoring data were obtained.

also approximately 40% higher (Table 1). While part of this difference could be allometric, reflecting the fourfold higher average mass of the fish of *series 1*, it is likely that another important factor was methodological. In *series 1*, repetitive sampling of substantial blood volumes (0.8 ml every 2 h), and replacement of the plasma with saline free of both urea-N and ammonia-N may have removed a significant portion (approx. 14 μmol N kg^{-1} h^{-1}) of the fish's endogenous N-load, thereby lessening the need for excretion. Alternately, or additionally, disturbance associated with this extensive sampling may have altered their excretion behavior. In this regard, it is notable that mean plasma urea-N concentration increased significantly in the fish of *series 1* from the first to the last samples of the experiment, from 11.88 ± 1.52 to 17.26 ± 2.28 mmol N l^{-1} ($N = 8$), suggesting a tendency for urea-N retention. By way of contrast, in the fish of *series 2* where just a few samples of low volume were taken, plasma urea-N levels were stable overall, averaging 13.70 ± 1.06 and 12.20 ± 0.92 mmol N l^{-1} ($N = 22$), respectively.

3.2. Series 1-Natural variations in plasma AVT and cortisol in relation to urea-N pulses

Circulating AVT levels in toadfish plasma were extremely low, averaging generally less than 1 pmol l^{-1} (i.e. 10^{-12} M range), though occasional surges to as high as 26 pmol l^{-1}, and lasting up to 6 h were seen. Circulating cortisol levels were much higher, generally in the order of 100–500 nmol l^{-1} (or 40–200 ng ml^{-1} in traditional units), with considerable variations over time. Based on an analysis of 12 individual urea-N pulse events in seven toadfish of *series 1*, plasma cortisol concentration consistently declined prior to a pulse (on average by approx. 60%), and rose again immediately thereafter (Fig. 1a). In contrast, there was no relationship at all between plasma AVT levels and pulse events in the present study (Fig. 1b).

Urea-N pulses events occurred randomly over the day. In accord with this, an analysis of plasma cortisol levels revealed no significant diurnal fluctuations (Fig. 2a). However, for plasma AVT, the majority of surges occurred in the late afternoon and evening; levels at 17.00–23.00 h were significantly elevated relative to levels at 15.00 and 01.00 h, suggesting the existence of a diurnal rhythm (Fig. 2b). In no case did a surge of AVT

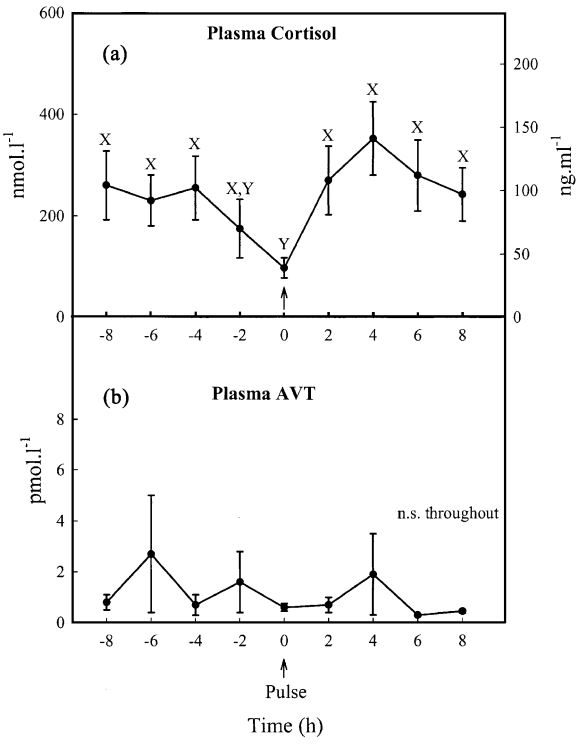

Fig. 1. Changes in (a) plasma cortisol concentrations and (b) plasma AVT concentrations over the 8-h period prior to, and the 8-h period following a natural urea-N pulse event in the toadfish of *series 1*. Means $\pm$ 1 S.E.M. ($N = 12$ pulses in seven fish). In (a) means which share a common letter are not significantly different from one another; in (b) there are no significant differences. Note the fall in cortisol levels prior to the pulse and the very rapid rise thereafter, while AVT did not change.

immediately precede or accompany a natural urea-N pulse. All pulses were preceded by a decline in cortisol, but in several cases a decline in cortisol occurred without a following urea-N pulse.

3.3. Series 2-Responses to injections of AVT, analogues, cortisol and metyrapone

An initial set of experiments compared the responses to arginine vasotocin (AVT), arginine vasopressin (AVP), and isotocin (ISO). In early trials with these fish, it became apparent that responses to AVT, injected via the caudal artery at a dose calculated to produce a circulating level of 10^{-10} M in the blood plasma, occurred only inconsistently. Thereafter, a dose of 10^{-9} M was used for all three agents. Typical records are shown in Fig. 3, and averaged responses in Fig. 4. AVT at 10^{-9} M was successful 100% of the time

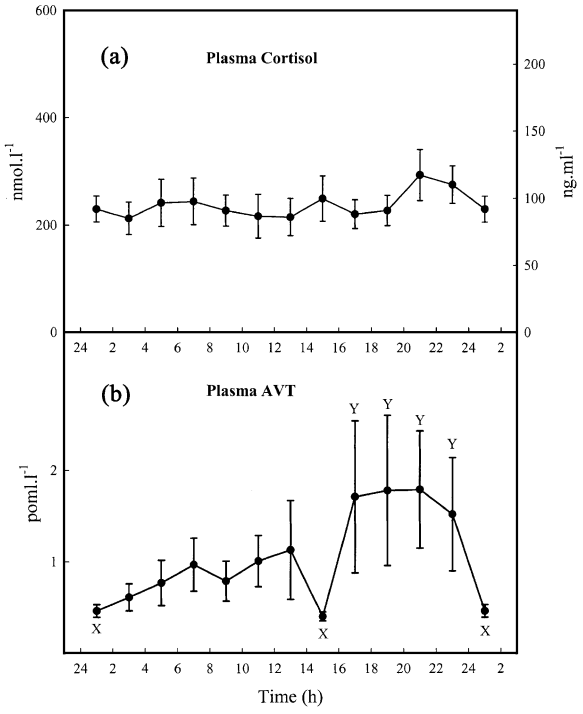

Fig. 2. Variations in (a) plasma cortisol concentrations and (b) plasma AVT concentrations at 2-h intervals over the daily cycle in the toadfish of *series 1*. Mean ± 1 S.E.M. ($N = 16–20$ samples at each time from eight fish for cortisol, and $N = 14–17$ samples at each time from seven fish for AVT). In (a), there were no significant differences. In (b), means at 17.00, 19.00, 21.00, and 23.00 h were significantly higher (analysis on log-transformed data) than those at 15.00 and 01.00 h, suggesting a diurnal rhythm. None of the other means were significantly different from one another. The 01.00 h point is repeated for clarity.

(18/18 positive responses in 14 fish; Fig. 4b) in inducing an immediate urea-N pulse event. Based on the natural pulse frequency recorded during the pre-experimental monitoring period for these same 14 fish, only a 7% response incidence (1/14 positive responses) would be predicted to occur spontaneously during the 2-h post-injection period, so the positive response to AVT was highly significant. However, despite the fact that the injected dose was approximately three orders of magnitude higher than the circulating in vivo measurements of *series 1*, these pulses were tiny (approx. 100 μmol N kg^{-1}) relative to natural pulse events in these same fish (approx. 4400 μmol N kg^{-1}). Note the logarithmic scale used to capture this difference in Fig. 4a. When the same dose of AVT was injected via the ventral aorta so as to contact the gills more directly, identical responses occurred (small pulses averaging 125 μmol N kg^{-1} in four of four fish).

Based on the findings of Babiker and Rankin (1978) and Warne (unpublished results) with respect to AVT effects on renal function and blood pressure, respectively, in teleost fish, the mammalian homologue AVP (10^{-9} M) was tested as a potential blocking agent for AVT receptors in toadfish. It, however, turned out to be an agonist, inducing small urea-N pulses comparable to those of AVT (Figs. 3 and 4a) on approximately 60% (10/17 positive responses) of the tests (Fig. 4b), perhaps reflecting a common metabolic action of the two hormones. The other teleost neurohypophysial peptide ISO (10^{-9} M) was also tested, because according to Hausmann et al. (1995), AVT can serve as a fairly potent stimulatory ligand (25% of the potency of isotocin) at the isotocin receptor in fish. However ISO was never successful in inducing pulses (Figs. 3 and 4), and did not have any blocking effectiveness against AVT (two tests-data not shown).

A second set of experiments examined possible interactions between cortisol and AVT. The cortisol synthesis blocker metyrapone, injected at a dose of 30 mg kg^{-1} (see Hopkins et al., 1995) was successful in lowering plasma cortisol (43% decrease measured at 3 h; Fig. 5a), whereas injection of cortisol (60 μg kg^{-1}) was successful in raising the plasma concentration (52% increase measured at 15 min; Fig. 5b). As illustrated by the typical records of Fig. 6 and summarized in Fig. 7, metyrapone was just as effective as AVT, eliciting small urea-N pulses 100% of the time (9/9 positive responses). However, unlike the immediate pulse events following AVT injections, metyrapone-induced pulses generally occurred only after a 30–90-min post-injection delay (Fig. 6) When AVT was injected 3 h after metyrapone, it was usually ineffective (Fig. 6). Urea-N pulses occurred in only 2/9 trials (22%, a significant decrease; Fig. 7b), and the pulses were extremely small (Fig. 7a).

Cortisol injections never elicited pulses (Figs. 6 and 7). When AVT was injected 15 min after cortisol (i.e. at the time of plasma cortisol elevation; Fig. 5b), the response was blocked in some trials (e.g. Fig. 6) and overall success rate significantly lowered to 63% (5/8 tests; Fig. 7). By 3 h post-injection, there was no significant effect on the response frequency. In both cases, urea-N pulse size remained unaltered.

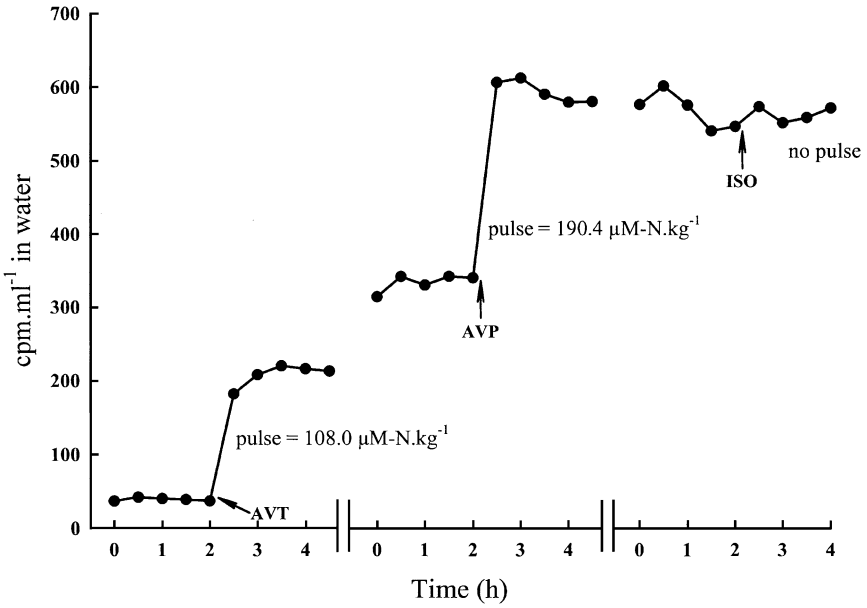

Fig. 3. Representative original records of water [^{14}C]urea radioactivity in the same toadfish injected on 3 separate days with arginine vasotocin (AVT), arginine vasopressin (AVP), and isotocin (ISO), all at doses producing 10^{-9} M concentrations in the blood plasma. The urea-N pulse sizes are shown, calculated from these data plus measurements of plasma specific activity at the time of the injection. Note the rapid pulse responses to AVT and AVP, and the lack of effect of ISO.

IGF-1 (10^{-9} M; four trials) and ANP (10^{-9} M; five trials), tested, respectively, because of known metabolic interactions with cortisol (Mommsen et al., 1999) and known ionoregulatory actions (Hazon and Balment, 1998), were both completely completely ineffective in eliciting pulse events.

4. Discussion

4.1. Overview

The present results provide strong evidence against any direct involvement of *circulating* AVT (or isotocin) as the proximate cause for initiation of natural urea-N pulses from the gills of toadfish. They do, however, reinforce the view (Wood et al., 1997) that decreases in *circulating* cortisol levels are involved, probably in a permissive role.

4.2. Evidence against plasma AVT involvement

The strongest evidence in this regard is simply that plasma AVT levels did not change in any consistent fashion prior to a natural pulse event in the toadfish of *series* 1 (Fig. 1b). Conversely, occasional surges in plasma AVT, usually in the late afternoon and evening (Fig. 2b), were not associated with urea-N pulses. Interestingly, in the rainbow trout, a similarly timed diurnal rhythm in plasma AVT levels (Kulczykowska and Stolarski, 1996) and in AVT mRNA in neurohypophysial neurons (Gilchriest et al., 1998) has been reported, although its functional significance is unknown. A second piece of evidence is that plasma AVT levels proved to be extremely low in the gulf toadfish, generally in the 10^{-12} M range, with occasional surges to 10^{-11} M (Fig. 1b, Fig. 2b). To date, circulating AVT levels have been measured in only a few teleost species, and the toadfish data seem to fall in the lower end of this range (10^{-12}–10^{-10} M; Perrot et al., 1991; Balment et al., 1993; Warne et al., 1994; Kulczykowska and Stolarski, 1996; Hazon and Balment, 1998). By way of contrast, injections of sufficient exogenous AVT to produce a circulating level of 10^{-9} M (i.e. an unphysiological dose) were required to consistently produce pulse events in the present study. Doses of 10^{-10} M were usually ineffective. Furthermore, the size of these induced urea-N pulses was only approximately 100 μmol N kg^{-1}, a small fraction of the size of typical natural pulses (1000–6000 μmol N kg^{-1}; Table 1, Figs. 4 and 7). Even when the same dose

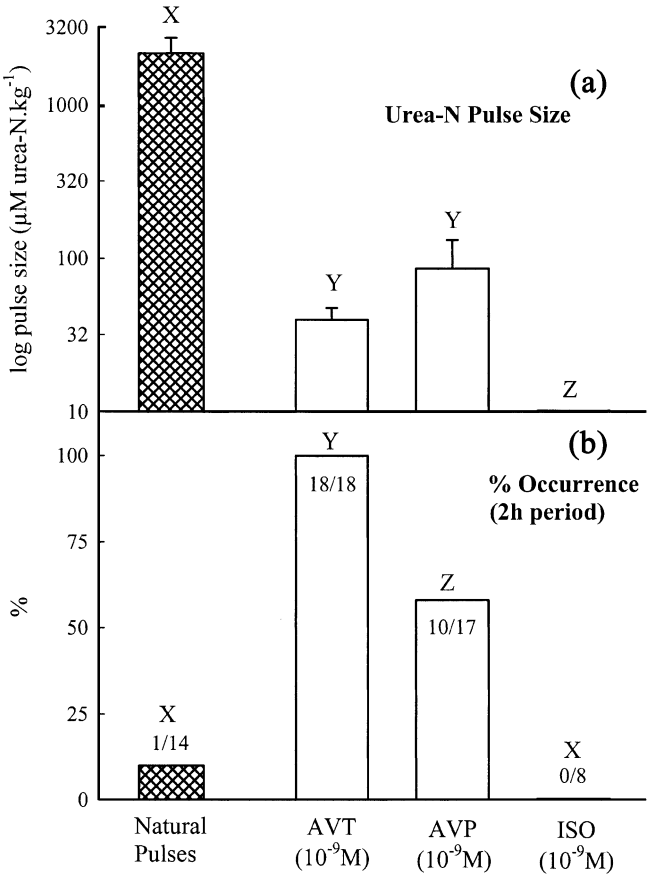

Fig. 4. A comparison of natural urea-N pulses (cross-hatched bars), and pulses induced by injection of AVT, AVP, or ISO (open bars; at doses producing 10^{-9} M concentrations in the blood plasma). (a) Urea-N pulse size (note logarithmic scale) and (b) percent occurrence of urea-N pulses in a 2-h period following injection. In (a) means ± 1 S.E.M. for up to 18 tests in 14 fish are shown; in (b) actual numbers of tests are given. Values not sharing a common letter are significantly different from each other

of AVT was injected into the ventral aorta so as to directly contact the gills with a much higher effective concentration, the size of these pulses remained very small.

The results of *series 2* (Fig. 4) confirm the earlier study of Perry et al. (1998) which showed that high doses of AVT cause small urea-N pulses. However, the present fish appeared to be somewhat less responsive than those of Perry et al. (1998) in which the threshold occurred at 10^{-10} M, and pulses reached several hundred μmol N kg^{-1} at 10^{-9} M. The difference may be methodological or seasonal. Perry et al. (1998) monitored [^{14}C]urea pulses by the Fick principle using toadfish in flow-through respirometers, and performed experiments in May (vs. Nov–Dec. in the present investigation). However, using the same batch of toadfish as Perry et al. (1998), in May, Pärt et al. (1999) were unable to find any effect of 10^{-10} M AVT on urea-N excretion in an isolated-perfused head preparation. Regardless, the more important conclusion is that in none of the studies did physiological circulating levels of AVT cause 'normal' pulse events.

What is injected AVT doing then? One possible explanation is that the small urea-N pulse response is non-specific or indirect, perhaps associated with the massive cardiovascular disturbance caused by such high doses of AVT (Warne and Balment, 1997; Perry et al., 1998) or the insertion of aquaporins (Nielsen et al., 1995; Inoue et al., 1999; Borgnia et al., 1999). Aquaporins are now known to be present in teleost gills (C. Cutler, personal communication), and some mammalian aquaporins are known to transport urea weakly (Borgnia et al., 1999). In this regard, Perry et al. (1998) reported that a small increase in branchial water permeability accompanied AVT-

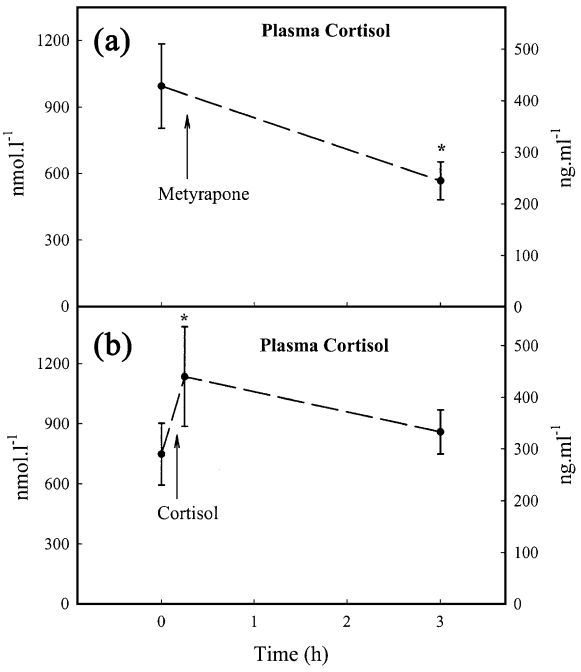

Fig. 5. Changes in plasma cortisol concentrations in toadfish in response to injections of (a) metyrapone (30 mg kg^{-1}; $N = 9$); and (b) cortisol (60 μg kg^{-1}; $N = 8$). Means ± 1 S.E.M. Asterisks indicate means significantly different from the value at the time of injection (0 h).

induced pulse events. By this line of reasoning, AVT (similarly isotocin) would not be involved in triggering the natural pulse event. Interestingly, in the mammalian IMCD, two differentially distributed UT-A transporters of very different size are present (UT-A1, 4.0 kB mRNA; UT-A2, 2.9 kB mRNA), probably as splice variants, and only the larger one (UT-A1) is sensitive to neurohypophysial peptides (Nielsen et al., 1996; Sands et al., 1997; Shayakul et al., 1997; Inoue et al., 1999). Our molecular studies have in fact identified two mRNA's in toadfish gill with homology to mammalian UT-A sequences, a predominant one of 1.8 kB and a minor one at 3.5 kB. Perhaps the small responses to injected AVT involve activation of only the latter, whereas natural pulses involve the former. Another possibility is that these non-physiological levels of AVT, especially in combination with manipulations of cortisol levels, may exert effects on urea-pulsing, which are essentially secondary metabolic consequences. These agents may potentially modify glucose and amino acid metabolism, nitrogen turnover and, therefore, the excretion of ammonia and urea.

Another possible explanation is that AVT re-

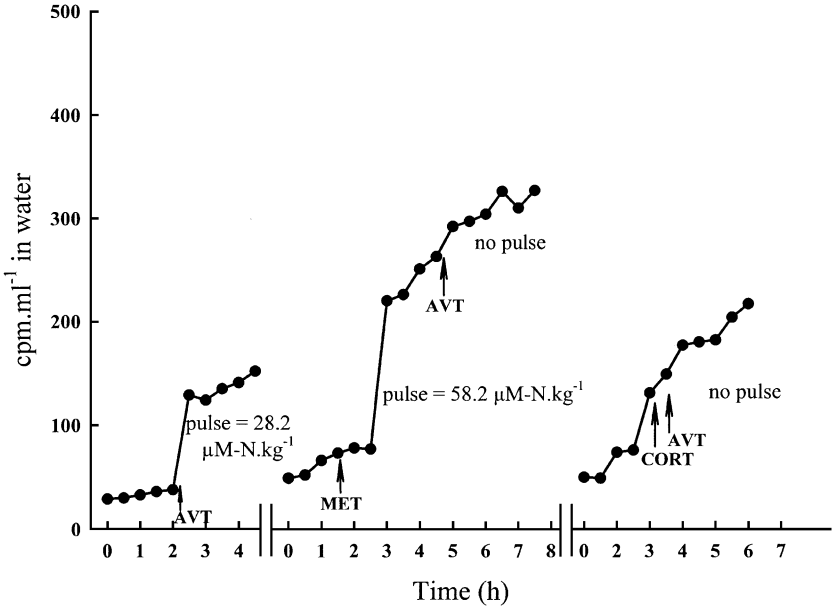

Fig. 6. Representative original records of water [^{14}C]urea radioactivity in the same toadfish injected on 3 separate days with AVT (to produce 10^{-9} M concentration in the blood plasma); metyrapone (30 mg kg^{-1}) followed 3 h later by AVT (10^{-9} M); and cortisol (60 μg kg^{-1}) followed 15 min later by AVT (10^{-9} M). The urea-N pulse sizes are shown, calculated from these data plus measurements of plasma specific activity at the time of the injections. Note the delayed pulse response to metyrapone (1 h after injection), and the absence of an AVT response thereafter. Note also the lack of response to AVT when injected shortly after cortisol.

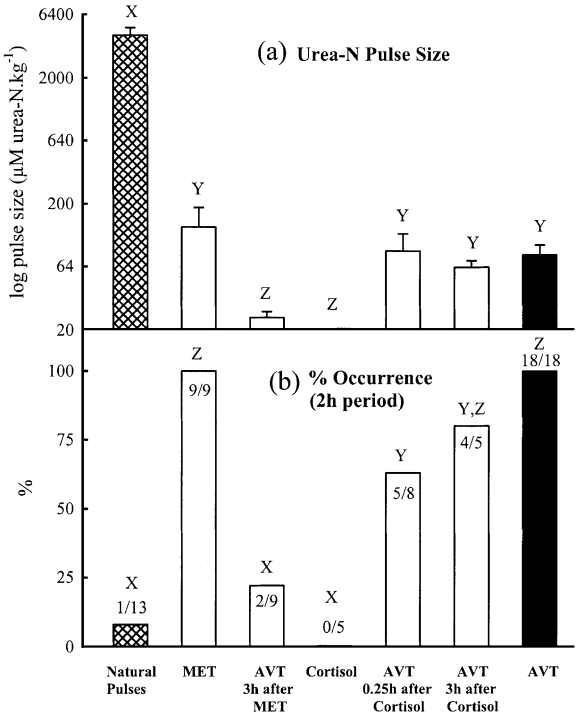

Fig. 7. A comparison of natural urea-N pulses (cross-hatched bars), and pulses (open bars) induced by injection of metyrapone (30 mg kg^{-1}), AVT (10^{-9} M in the blood plasma; administered 3 h after metyrapone), cortisol (60 μg kg^{-1}), and AVT (10^{-9} M administered at 15 min and 3 h after cortisol). For reference, the response to AVT alone (solid bars; from Fig. 4) is also shown. (a) Urea-N pulse size (note logarithmic scale) and (b) percent occurrence of urea-N pulses in a 2-h period following injection. In (a) mean ± 1 S.E.M. for up to nine tests in 13 fish are shown; in (b) actual numbers of tests are given. Values not sharing a common letter are significantly different from each other.

ally is involved in triggering the natural pulse event, but that much higher concentrations than even 10^{-9} M are required to activate transport, and are normally delivered to specific receptor sites on the gills by a route other than the circulatory system. In this regard, it must be remembered that AVT is a neurotransmitter as well as a neurohormone, and that in general, concentrations of neurotransmitters at synaptic clefts are far higher than concentrations of circulating neurohormones. AVT-containing neurons are widespread in the teleost brain (Batten et al., 1990; Kulczykowska, 1995; Gilchriest et al., 1998), so a priori, there is no reason why they could not also project to the gills themselves, or to other neurons which innervate the gills. Indeed, in another member of the family Batrachoididae, the closely related midshipman (*Porichthys notatus*), AVT-immunoreactive neurons project from the pre-optic area to other areas of the brain involved in vocalization, an important social behavior associated with courtship and reproduction (Goodson and Bass, 2000a,b). Neural activation would likely provide a much more rapid and precise control of the pulse event. In this regard, a search, using immunocytochemistry, for AVT-containing neurons in the gills of the toadfish may prove rewarding.

4.3. Evidence in favor of plasma cortisol involvement

The most compelling evidence in favor of a role for cortisol was the consistent marked decline in plasma cortisol concentration immediately preceding a pulse event, in *series 1* (Fig. 1a). This data set is in direct agreement with our earlier findings (Wood et al., 1997). We interpret the fall in cortisol as being a permissive event, rather than the direct trigger, because in several instances plasma cortisol fell without a natural pulse occurring. Nevertheless, in *series 2*, a pharmacologically-induced fall in plasma cortisol (via metyrapone injection) invariably resulted in a pulse event, albeit one no bigger than that induced by AVT. Potentially, this could indicate relief of tonic inhibition (Figs. 6 and 7). An anthropomorphic interpretation is that spontaneous cortisol declines are correlated with a fall in 'stress' levels, and the toadfish feels 'safe' in emitting a urea-N pulse at this time. In this regard, Walsh (1997) has proposed that the adaptive significance of urea-N pulsing is to avoid providing olfactory signals to potential predators most of the time.

The time course of the present cortisol effects is fairly rapid-less than 4 h for the cortisol fall preceding a large natural pulse (Fig. 1a), 30–90 min for the small metyrapone-elicited pulses (Figs. 6 and 7), and only 15 min for a significant inhibitory influence of cortisol elevation on the small AVT-elicited pulses (Figs. 6 and 7). This raises the prospect that some or all of the cortisol effects on this system are non-genomic, which at least in mammals are very rapid (seconds to minutes). Non-genomic actions of cortisol in fish are poorly understood at present (Mommsen et al., 1999) but have been documented in vitro in at least one system, prolactin release from a perifused tilapia pituitary preparation (Borski et al., 1991). Here the time course was 15–20 min, con-

sistent with effects mediated through c-AMP and intracellular calcium signaling.

At a molecular level, high levels of plasma cortisol may down-regulate urea transport in the toadfish gill by exactly the same mechanism as described in the mammalian IMCD — a post-translational reduction in UT-A type protein without alteration of m-RNA levels (Naruse et al., 1997). Since Naruse et al. (1997) showed that this effect was exerted specifically on the AVP-sensitive urea transporter (UT-A1), we tested the interaction between cortisol and the teleost homologue AVT. An interaction was present, though not of the sort that might have been predicted a priori. Thus, pharmacological reduction of plasma cortisol did not potentiate the response to injected AVT, but rather virtually eliminated it (Figs. 6 and 7). Since metyrapone itself induced a urea-N pulse after some delay (presumably reflecting the time taken for plasma cortisol to fall), it would appear that the 'AVT-sensitive' pulsing mechanism had already been discharged or exhausted by the first pulse which was instigated by cortisol-reduction.

When plasma cortisol levels were artificially raised by injection of exogenous cortisol, the incidence of positive responses to AVT injections was reduced, but not eliminated, and the urea-N pulse size remained unaltered (Fig. 7). Arguably, the exogenously-induced rise in plasma cortisol was either not large enough or of long enough duration (AVT injections were made 15 min after cortisol) to produce a consistent down-regulation, though that remains to be tested. Furthermore, while our focus here was on AVT-cortisol interactions at the urea-N transport sites, it must be remembered that there are other important potential sites of interaction — in the brain and pituitary gland. In teleosts, AVT is one of the two hypothalamic peptides (the other is CRH) which synergistically controls the release of ACTH from the pituitary, which in turn mobilizes cortisol from the inter-renal tissue (Baker et al., 1996). Given the facts that 'urea pulsing' appears to be a social behavior (Walsh, 1997), that AVT receptors occur in brain neurons and modulate social behavior (Goodson and Bass, 2000a), and that teleost social behavior is highly sensitive to cortisol (Gregory and Wood, 1999), the potential for interactions is immense. The challenge now is threefold: first to discover the true proximate trigger for pulsing (i.e. what specific hormone, neurotransmitter, or paracrine agent directly elicits the pulse event); second to discover the mechanism by which cortisol interacts with this trigger; and third to understand the true significance of the pulsatile event in a social context. The first can be addressed by broad-range pharmacological testing of potential agonists and antagonists and by neural sectioning; the second through the use of specific corticosteroid blocking agents, and by rapid time course studies to evaluate possible non-genomic actions of cortisol; and the third through behavioral studies in mesocosms or in the field. Initiatives in all three areas are currently underway.

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