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Arginine Vasotocin Neuronal Phenotype and Interspecific Cooperative Behaviour

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Key Words
Arginine vasotocin · Cleaner wrasse · Mutualism · Preoptic area · Social behaviour

Abstract

The nonapeptide arginine vasotocin (AVT) and its mammalian homologue arginine vasopressin are well known for their role in the modulation of several intraspecific social behaviours, such as social approach/withdrawal and aggression. Recently, we suggested that AVT might also be important in the regulation of interspecific social behaviours as it modulates interspecific cooperative behaviours in the Indo-Pacific bluestreak cleaner wrasse *Labroides dimidiatus.* AVT injections decreased cleaners' propensity to engage in cleaning interactions with their clients, suggesting that high levels of this peptide compromise the cleaners' cooperative motivation. Therefore, we hypothesise that low endogenous levels of AVT are a prerequisite for the expression of interspecific cleaning behaviour in cleaner wrasses, since it allows them to approach and interact with interspecific individuals, and that this should be reflected in their AVT neuronal phenotype. Here we test this hypothesis by comparing the AVT neuronal phenotypes of two phylogenetically closely related species that live in similar environments but diverge in the expression of interspecific cooperative behaviour: an obligate cleaner wrasse *(L. dimidiatus)* and a non-cleaner corallivore wrasse *(Labrichthys unilineatus)*. The two species are predicted to differ in their AVT neuronal phenotypes as a reflection of their ability/inability to approach and interact with interspecific individuals, with cleaners presenting smaller and/or less numerous AVT-immunoreactive (ir) neurons. A sex difference in AVT neuronal phenotypes was also predicted because males of both species appear to be more aggressive than females. As described for most of the other teleost species, AVT-ir neurons were restricted to the preoptic area, and in agreement with our first prediction cleaners presented smaller and less numerous AVT-ir neurons in the gigantocellular preoptic area (gPOA) compared to non-cleaners. Contrary to our second prediction, AVT neuronal phenotypes did not differ between sexes in either species, but differences in other features of the AVT system cannot be ruled out. In summary, the results presented here suggest a putative role for AVT gPOA neurons in the ability of a cleaner wrasse to approach and interact with a client, through their projections to extrahypothalamic brain areas.

Introduction

Cooperation can be defined as social interactions between two or more individuals with a net positive outcome (direct fitness effects) for all of them, i.e. the benefits

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of interacting should outweigh its costs (investments) [Bshary and Bergmüller, 2008]. When this type of interaction occurs amongst individuals of different species, the term mutualistic interaction is used [West et al., 2007; Bshary and Bergmüller, 2008]. Although much is known about the functional aspects and the behavioural mechanisms responsible for the evolution and maintenance of mutualisms [e.g. Sachs et al., 2004; West et al., 2007; Leimar and Hammerstein, 2010], little work has been conducted to unravel its underlying proximate mechanisms [Lenke, 1982; Soares et al., 2010, 2012].

At the proximate level, a brain social behaviour network capable of regulating the expression of social behaviours has been proposed [Newman, 1999; Goodson, 2005]. This network, initially described in mammals but phylogenetically conserved across vertebrates (including teleost fish), comprises 6 major interconnected nodes (the extended medial amygdala, the bed nucleus of stria terminalis, the lateral septum, the preoptic area, the anterior hypothalamus, the ventromedial hypothalamus, and the midbrain in mammals, or their homologue areas in other vertebrates [for recent reviews, see O'Connell and Hofmann, 2011, 2012]), which are able to modulate several social behaviours, including sexual, parental and aggressive behaviour [Newman, 1999; Goodson, 2005]. Together with the mesolimbic reward system, whose functional homologues have not been described in fish yet [Tay et al., 2011], the social behaviour network forms a social decision-making network which allows the animal to integrate the perceived value of external stimuli with its internal state and to produce adaptive responses to a variable social environment [O'Connell and Hofmann, 2011; Oliveira, 2012; Taborsky and Oliveira, 2012]. Neuropeptide receptors, such as those belonging to the vasopressin (AVP)/oxytocin peptide family, are extensively expressed in the social behaviour network, enabling these neuropeptides to modulate the network's response to stimuli and consequently the behavioural output and hence making them great candidates to play a key role in the modulation of social behaviour [Goodson, 2005].

AVP and its non-mammalian homologue AVT (arginine vasotocin) have been implicated in a variety of physiological [Warne and Balment, 1997; Strand, 1999; Balment et al., 2006] and behavioural functions in several vertebrate taxa, including fish, amphibians, reptiles, birds and mammals [see reviews by Goodson and Bass, 2001; Goodson and Thompson, 2010]. In teleosts, AVT-immunoreactive (ir) neurons of the preoptic area (POA) are the main source of AVT. These neurons project both to the pituitary, through which the neuropeptide is released into the systemic circulation, and to extrahypothalamic brain regions [including telencephalon, thalamus, optic tectum and hindbrain; Holmgvist and Ekström, 1995; Saito et al., 2004]. The AVT-ir neurons in the POA are commonly divided into three major neuronal groups: parvocellular (pPOA), magnocellular (mPOA) and gigantocellular (gPOA). The most rostral one (pPOA) mainly projects to the pituitary [Holmgvist and Ekström, 1995], by which AVT affects peripheral mechanisms such as osmoregulation, metabolism and stress response [Balment et al., 2006]. For example, in rainbow trout *(Oncorhynchus mykiss),* acute confinement stress events increase both plasma cortisol levels and AVT mRNA expression in the pPOA, without affecting mPOA or gPOA neurons [Gilchriest et al., 2000]. It has been suggested the pPOA AVT neurons have an important role in stress responses as they are involved in the modulation of cortisol levels through activation of the hypothalamic-pituitary-interrenal axis [Goodson and Bass, 2001; Balment et al., 2006]. In a medial position of the POA lie the magnocellular neurons with projections towards both the pituitary and the extrahypothalamic brain areas [Saito et al., 2004], which implicate them in the variation of both physiological processes and social behaviour. Finally, the gPOA AVT neurons mainly project to extrahypothalamic brain centres [Holmgvist and Ekström, 1995; Saito et al., 2004] including those belonging to the social decision-making network, therefore having important implications in the modulation of social behaviour [Dewan and Tricas, 2011]. Such extrahypothalamic projections include those to the ventral telencephalon, thalamus, periventricular pretectal nuclei and optic tectum [Holmgvist and Ekström, 1995; Saito et al., 2004]. Additional neuronal projections from the teleost POA cannot be ruled out as only few studies have addressed this subject.

In teleosts, a great deal of work on the role of AVT in social behaviours, such as acoustic communication [Goodson and Bass, 2000, 2002], aggression [Dewan and Tricas, 2011], social dominance [Larson et al., 2006], sexual behaviour [Grober et al., 2002], and approach/withdrawal [Thompson and Walton, 2004], has been conducted. In goldfish, AVT infusions into the brain inhibit social approach towards conspecific males, while the Manning compound (AVT V1 receptor antagonist) produces opposite effects [Thompson and Walton, 2004]. This inhibition is mediated by neural projections to the hindbrain [Thompson et al., 2008; Thompson and Walton, 2009] which induce peripheral physiological changes through autonomic processes that feed back to the brain, inhibiting social approach [Thompson et al., 2008]. Another important focus has been on the role of AVT/AVP on aggressive behaviour, and at least in most territorial species AVT seems to cause an increase in aggression levels [reviewed by Godwin and Thompson, 2012]. Moreover, territorial species/phenotypes often present higher AVT production or more numerous or larger AVT-ir neurons in the gPOA [Miranda et al., 2003; Dewan et al., 2008, 2011; Greenwood et al., 2008; Maruska, 2009]. Finally, Dewan and Tricas [2011] found that gPOA cell number is positively correlated with aggression level in a territorial butterflyfish.

So far, studies disclosing the role of the AVT/AVP system in social behaviour have exclusively focused on intraspecific social interactions [Goodson and Bass, 2001]. However, recent work shows that AVT also plays an important role in the regulation of interspecific cooperative behaviour in cleaner wrasses *Labroides dimidiatus* [Soares et al., 2012]. Intramuscular AVT injections significantly decreased both the cleaners' likelihood of engaging in mutualistic cleaning interactions and the quality of the service provided. On the other side, cleaners treated with the Manning compound were more likely to interact with clients [Soares et al., 2012]. Additionally, conspecific social behaviours increased in fish injected with AVT, suggesting that the mechanisms already in place for the regulation of conspecific social behaviours might have been recruited for the modulation of interspecific behaviours in this species [Soares et al., 2012]. These results imply that high levels of endogenous AVT decrease a cleaner's ability to cooperate with its clients, and they lead us to hypothesise that low AVT levels are a prerequisite for the expression of interspecific cooperative behaviour in cleaner wrasses, thus enabling them to approach and interact with interspecific individuals, and that this should be reflected in their AVT neuronal phenotype.

To test this hypothesis, the AVT neuronal phenotypes of two phylogenetically closely related species that live in similar environments but diverge in the expression of interspecific cooperative behaviour were compared. For this purpose, an immunocytochemical characterization of the AVT system was performed in an obligate cleaner wrasse *(L. dimidiatus)* and in a non-cleaner corallivore wrasse *(Labrichthys unilineatus)*. Both *L. dimidiatus* and *L. unilineatus* belong to the Labrichthynes lineage and diverged from a common ancestor in the early Miocene (~20 Ma) [Cowman et al., 2009]. Also, both are territorial protogynous species living in harems, where the sole male owns the territory [Potts, 1973; Colin and Bell, 1991]. *L. dimidiatus* is well known for its interspecific social system, where cleaners feed exclusively on what they remove from clients [Bshary and Cote, 2008]. Male *L. dimidiatus* often exhibit aggressive behaviour directed towards females [Raihani et al., 2012] or other conspecific intruders [Potts, 1973]. *L. unilineatus* is a generalist obligate corallivore species which feeds on the mucus produced by the coral polyps [McIlwain and Jones, 1997; Cole et al., 2009, 2010] and does not engage in mutualistic cleaning interactions at any time of its life cycle [Cowman et al., 2009]. Male *L. unilineatus* aggressively defend their feeding territories from other male competitors, whereas females stay associated with coral patches [McIlwain and Jones, 1997]. Therefore, males from both species are more often aggressive than females [McIlwain and Jones, 1997; Raihani et al., 2012]. Given the above information, the following specific predictions will be tested: (1) it is predicted that cleaners would present smaller and/or less numerous AVT-ir neurons than non-cleaners as a reflection of their ability to approach and interact with interspecific individuals, and (2) it is predicted that males of both species would present larger and/or more AVT-ir neurons than females as a reflection of their higher aggressiveness. These differences are expected to appear in AVT cell groups that have been implicated in the regulation of social behaviours.

Materials and Methods

Subjects

The fish used in this study were purchased from a local distributor of tropical marine fishes (Tropical Marine Centre, Lisbon, Portugal). *L. dimidiatus* specimens were collected in the Maldives, and *L. unilineatus* were collected in Indonesia and subsequently freighted to Portugal by air. All of the animals arrived in Portugal between July 2011 and January 2012 and were kept in quarantine for about 1 week. A total of 9 *L. dimidiatus* [4 males, standard length (SL; mean ± SEM) = 6.275 ± 0.253 cm, and 5 females, SL = 6.080 ± 0.252 cm] and 8 *L. unilineatus* (3 males, SL = 11.333 ± 1.225 cm, and 5 females, SL = 8.620 ± 0.907 cm) were used in this study. There were no sex differences in SL for either species (one-way ANOVA, *L. dimidiatus*: $F_{1, 7} = 0.29$, $p = 0.61$; *L. unilineatus*: $F_{1, 6} = 3.25$, $p = 0.12$). *L. unilineatus* were significantly larger than *L. dimidiatus* (one-way ANOVA, $F_{1, 15} = 18.49$, $p < 0.001$). All data presented were corrected for SL as described in Statistical Analysis. The sex of all individuals was confirmed by direct inspection of the gonads and with the help of an acetocarmine stain [Guerrero and Shelton, 1974] whenever needed. All animals, with the exception of one *L. dimidiatus* male, were collected outside their breeding season. The *L. dimidiatus* male collected during the breeding season was nonetheless included in the analysis because, although it represented the minimum value of the mean pPOA cell area/SL, a mean mPOA cell area/SL below the 25% value and a mean mPOA cell number/SL above the 75% value, all other measures for this individual were within the 25–75% interval and pPOA and mPOA cells did not differ between species or sexes (see Results).

Tissue Preparation

Fish were deeply anesthetised with tricaine methanesulfonate (MS-222; PHARMAQ), measured for SL, and perfused transcardially with 0.9% heparinised 0.1 M phosphate-buffered saline solution (PBS, pH 7.4) for 1–2 min, followed by 4% paraformaldehyde (PFA) in 0.1 M PBS for 30 min. Brains were then removed, postfixed in 4% PFA for 1–2 h and transferred to 0.1 M PBS until further processing, which never took longer than 5 days. One day prior to sectioning, brains were cryoprotected in 30% sucrose in 0.1 M PBS and kept at 4°C.

Immunocytochemistry

Cryoprotected brains were embedded in Tissue-Tek® (optimal cutting temperature compound), frozen at –80°C and then placed in a cryostat chamber at –25°C for 1 h, allowing the tissue to reach the optimal cutting temperature. Brains were sectioned with the cryostat at 25 μm on the coronal plane. Alternate brain sections were collected onto two series of Silane-prep glass slides (Sigma) and stored at –80°C until reacted. All brains were completely processed within 6 days following the perfusion.

One of the alternate series was immunoreacted as follows: on day 1, slides were defrosted at room temperature and the tissue sections were surrounded with a PAP pen for immunostaining (Sigma), washed twice and blocked with pre-soak solution [0.4% Triton® X-100 SigmaUltra (Sigma) in 0.1 M PB with 2.8% goat serum (Sigma)] for 20 min. Slides were then drained and covered with 3% H_2O_2 in 0.1 M PB for 20 min for endogenous peroxidase blockage. After two additional washes the primary AVT antibody was applied in a final dilution of 1:7,500 (the antibody was diluted with pre-soak solution) and slides were incubated overnight in a sealed humidified chamber at 4°C. The antibody used was generously donated by Dr. Matthew Grober (Georgia State University, USA) and has been successfully used in similar studies done with different teleost species [e.g. Maruska et al., 2007; Dewan et al., 2008; Maruska, 2009]. Day 2 started with two washes followed by incubation with the biotinylated goat anti-rabbit secondary antibody (KPL; Kirkegaard & Perry Labs, Inc.) for 30 min, two additional washes and incubation with peroxidase-labelled streptavidin (KPL) for another 30 min. After two more washes, the slides were reacted with a diaminobenzidine (DAB) chromogen peroxidase substrate kit (Vector Laboratories, Inc.) according to the manufacturer's instructions, for 3–6 min or until golden-brown coloration was achieved. Slides were then immersed in distilled water to stop the reaction, dehydrated in an ethanol series, cleared in xylol (Sigma) and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific). Each wash was done with 0.1 M PB for 7 min.

Due to space constraints, only one subject was included per ICC run. Nonetheless, the same protocol and the same timings were used for all of the subjects.

To control for a possible non-specific labelling of the neurons, a second alternate series was immunoreacted after pre-adsorbing the antibody with 8 μM AVT peptide (catalogue No. V0130; Sigma) overnight at 4°C. All of the other steps were maintained. The control series did not show any stained neurons, thus demonstrating the specificity of the antibody used. Other controls have been conducted to ensure the specificity of this antibody [Maruska et al., 2007; Dewan et al., 2008; Maruska, 2009].

Quantification

Slides were coded so that the specimen sex or species was not known during the quantification process. Complete brain series were inspected for localization of AVT immunoreactivity in the brain. AVT-ir neuron cell bodies were only detected in the POA and therefore all quantifications for cell number and soma size were done in this region. Each AVT-ir cell was assigned to the pPOA, mPOA or gPOA cell group based on descriptions of cells' neuroanatomical location, size and morphology made for the teleost brain by Bradford and Northcutt [1983]. No AVT-ir cells have been noticed in the nucleus preopticus parvocellularis posterioris (PPp) and therefore all pPOA cells were part of the nucleus preopticus parvocellularis anterioris (PPa) and the pars parvocellularis of the nucleus preopticus magnocellularis (PMp). All AVT-ir mPOA cells are located in the nucleus preopticus magnocellularis pars magnocellularis (PMm), and all of the AVT-ir gPOA cells belong to the nucleus preopticus magnocellularis pars gigantocellularis (PMg) [sensu Bradford and Northcutt, 1983]. This nomenclature and grouping of cells has been widely used in similar studies [e.g. Semsar and Godwin, 2003; Lema, 2006; Maruska, 2009].

Cell size was determined from digital images captured at a magnification of ×400 with a digital camera (Olympus C-2020 Z) attached to a microscope (Olympus BX50). After calibration for magnification, the cross-sectional area of 4–20 randomly selected cells from each cell group was calculated using ImageJ software by tracing the cell profile. Cell number was determined by visually counting the cells belonging to each cell group, with the microscope. Whenever an accurate attribution of a cell to a cell group was not possible, the cell diameter was measured (using the microscope ruler) and recorded as 'unidentified cell'. A regression between cell area (measured with ImageJ) and cell diameter (measured with the microscope ruler) was used in order to correctly classify each unidentified cells a posteriori. This took into account the individual distribution of cell area in the three cell groups. The larger cell measured had a profile area of 747.66 μm^2 which corresponds to a cell diameter of 38.2 μm. As the distance between two alternate tissue sections equals 50 μm and given that the antibody is only likely to penetrate a few micrometers of the tissue sections [Foran and Bass, 1998], it is not expected that the same cell was counted twice in alternate brain sections. Only cells presenting a discernible perimeter from the background and at least one neurite were measured and counted. For pPOA cells only the discernible perimeter criterion was used as neurites were not always visible. One *L. unilineatus* male was not included in the cell number analysis due to dubious staining. Nonetheless, cells from this individual with defined limits from the three cell groups were measured and used in the analysis.

Statistical Analysis

All descriptive statistics given in the text are means ± standard error of the mean (SEM) unless stated differently. In order to determine the effect of body size on cell number and cell size, linear regressions tests were used. Several significant relationships between cell characteristics and fish SL were only found for *L. unilineatus* (table 1). The lack of significant allometric relationships between body size and cell measures in cleaners is most probably due to the small variation in body size found in this species, which contrasts with the large body size variation present in non-cleaners. Because this study aims to make comparisons between the two species, all data were corrected for fish body size by dividing cell size and cell number by fish SL (cm). Differences between species and sexes for AVT-ir cell profile area and number were assessed using two-way repeated measures ANOVA with species *(L. dimidiatus* vs. *L. unilineatus)* and sex (male vs. female) as independent factors and cell group (pPOA vs. mPOA vs. gPOA) as a within-subjects factor. Repeated measures ANOVA were followed by planned comparisons for least squares means in order to compare species and sexes in each cell group.

Results

Qualitative Characterization and Distribution of AVT-ir Neurons

All AVT-ir neurons were found within the preoptic area and no qualitative differences in the pattern of cell distribution were found between the two species. The parvocellular neuronal group was the most rostral and ventral, consisting of small (*L. dimidiatus:* 45.4 ± 8.4 μm^2; *L. unilineatus:* 69.7 ± 5.3 μm^2) round cells which were usually monopolar or without obvious neurites (fig. 1a, b). This cell group started above the optic chiasm in the ventral surface of the forebrain and extended dorsocaudally until the region where magnocellular neurons started to appear. The pPOA extended more laterally than any of the other cell groups. The mPOA neurons appeared dorsally to the last pPOA cells and occupied a medial location in the preoptic area lying against the third ventricle. The mPOA cells were approximately twice the size of pPOA neurons (*L. dimidiatus:* 94.6 ± 6.7 μm^2; *L. unilineatus:* 141.6 ± 8.4 μm^2) and usually had one prominent axon extending towards the preopticohypophyseal tract (PHT) (fig. 1c, d). The gigantocellular neuronal group began dorsal to the mPOA cells and extended caudally, presenting fewer cells per tissue section. These were the largest AVT-ir cell bodies (*L. dimidiatus:* 128 ± 5.7 μm^2; *L. unilineatus:* 539.1 ± 64.3 μm^2; fig. 1e, f).

Qualitative differences in gPOA cell characteristics were found between the two species. In *L. dimidiatus,* gPOA neurons were typically solitary cells, bipolar or multipolar but with very few processes surrounding the cell bodies (fig. 2a). Gigantocellular neurons in *L. unilineatus* were multipolar, usually having many surrounding fibres extending in/from different directions (fig. 2b).

Quantitative Differences of Preoptic AVT-ir Cells

There was a main effect of species (*L. unilineatus* > *L. dimidiatus,* $F_{1,12} = 79.41$, $p < 0.001$) and cell type (pPOA < mPOA < gPOA, $F_{2,24} = 107.71$, $p < 0.001$, all planned comparisons $p < 0.001$) on the size of AVT-ir neurons. We also found a significant interaction between cell type and species ($F_{2,24} = 40.60$, $p < 0.001$). Planned comparisons showed that cell size differences between the two species were restricted to the gPOA cell group ($F_{1,12} = 40.27$, $p < 0.001$; all other planned comparisons $p > 0.05$; fig. 3). Additionally, only in *L. dimidiatus* were gPOA neurons not significantly larger than mPOA neurons ($F_{1,12} = 2.41$, $p = 0.146$; fig. 3). Thus, the reported main effect of cell type on AVT-ir neuron size is essentially due to *L. unilineatus.* Sex had no effect on the size of AVT-ir cells ($F_{1,12} = 1.23$, $p = 0.289$).

There was a main effect of cell type on the number of AVT-ir cells ($F_{2,24} = 3.96$, $p = 0.033$). Planned comparisons showed that gigantocellular neurons were significantly more numerous than parvocellular neurons

Table 1. Linear regression tests for AVT-ir cell profile areas and numbers vs. body length

	r^2	p
Cell size		
pPOA	0.588	0.001
mPOA	0.719	<0.001
gPOA	0.793	<0.001
Cell number		
pPOA	0.263	0.051
mPOA	0.266	0.049
gPOA	0.859	<0.001
L. dimidiatus		
Cell size		
pPOA	0.170	0.311
mPOA	0.005	0.871
gPOA	0.095	0.421
Cell number		
pPOA	0.099	0.448
mPOA	0.041	0.631
gPOA	0.318	0.114
L. unilineatus		
Cell size		
pPOA	0.586	0.027
mPOA	0.772	0.004
gPOA	0.570	0.030
Cell number		
pPOA	0.148	0.395
mPOA	0.190	0.328
gPOA	0.730	0.014

Results are presented for both species together and separately, showing that the regressions only remain significant for *L. unilineatus.*

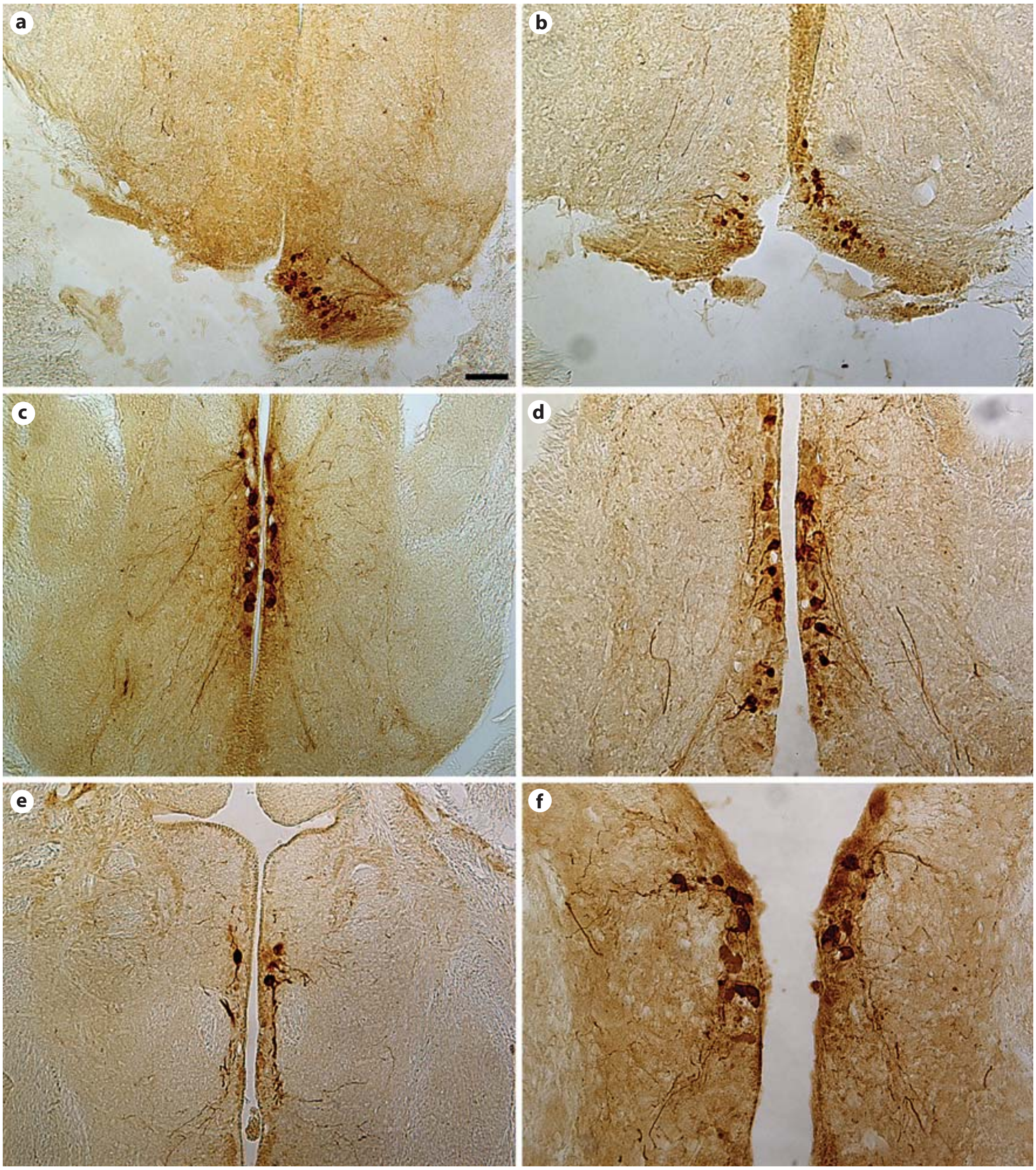
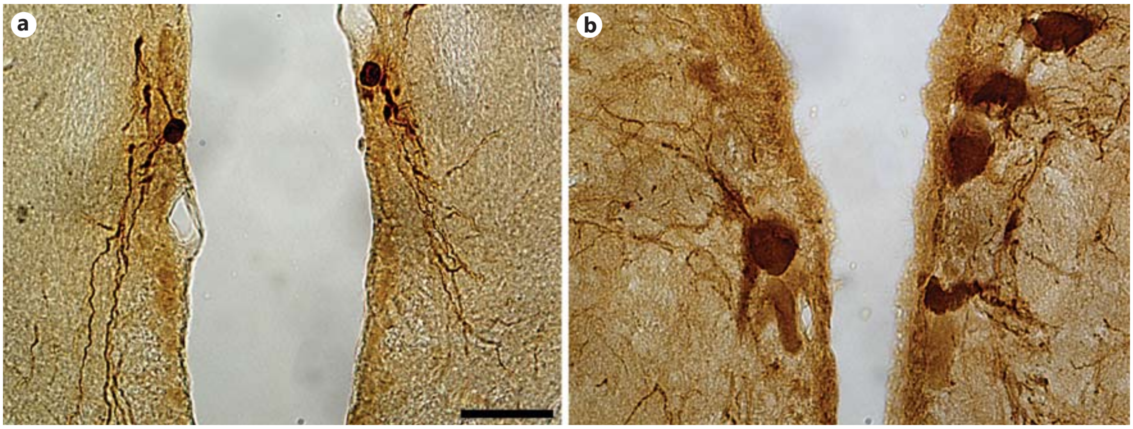


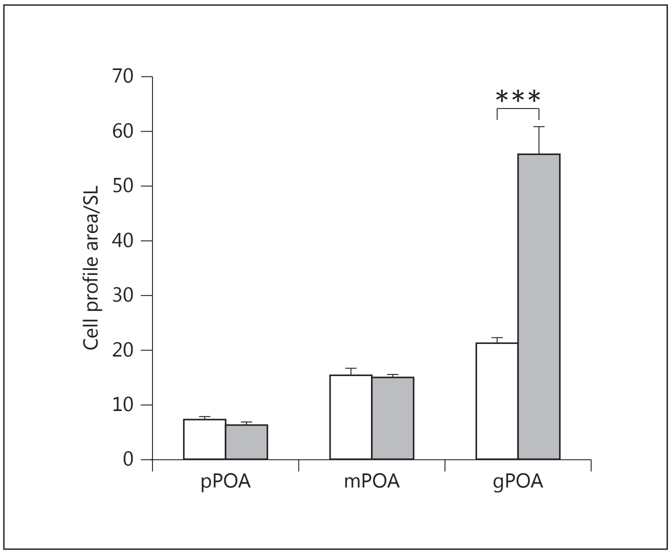
Fig. 1. Photomicrographs of AVT-ir neurons in the brain of *L. dimidiatus* (**a**, **c**, **e**) and *L. unilineatus* (**b**, **d**, **f**). Cross-sections through the preoptic area illustrate the relative position and appearance of the three cell types: parvocellular (**a**, **b**), magnocellular (**c**, **d**) and gigantocellular (**e**, **f**). The scale bar is shown in **a** and represents 50 μm.

($F_{1, 12} = 5.90$, $p = 0.032$) and that there was a marginally non-significant trend for mPOA cells to be more numerous than pPOA cells ($F_{1, 12} = 4.51$, $p = 0.055$), whereas no significant differences were observed between the number of mPOA and gPOA cells ($F_{1, 12} = 0.17$, $p = 0.691$). There was a significant interaction between cell type and species ($F_{2, 24} = 4.23$, $p = 0.027$). Planned comparisons showed that species differences were restricted to the number of gPOA cells ($F_{1, 12} = 22.21$, $p < 0.001$; fig. 4). In addition, only in *L. dimidiatus* were mPOA neurons more numerous than gPOA neurons ($F_{1, 12} = 9.28$, $p = 0.010$), and the number of pPOA neurons was



Color version available online

Fig. 2. Photomicrographs of AVT-ir neurons in the gPOA of *L. dimidiatus* (**a**) and *L. unilineatus* (**b**) showing qualitative differences between the species in the density of AVT-ir fibres surrounding this type of cells. The difference in cell size between species is also visible. The scale bar is shown in **a** and represents 50 μm.



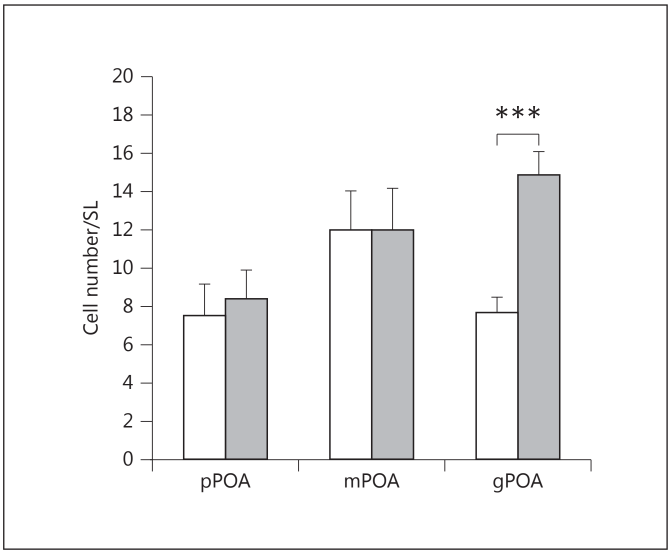


Fig. 3. Mean cell profile area (μm^2) of AVT-ir neurons corrected for fish SL (cm) within the preoptic area of *L. dimidiatus* (light bars) and *L. unilineatus* (dark bars). Data are presented as means ± SEM. Asterisks indicate significant differences between species (planned comparisons; *** $p < 0.001$).

Fig. 4. Mean cell number of AVT-ir neurons corrected for fish SL (cm) within the preoptic area of *L. dimidiatus* (light bars) and *L. unilineatus* (dark bars). Data are presented as means ± SEM. Asterisks indicate significant differences between species (planned comparisons; *** $p < 0.001$).

not different from that of mPOA ($F_{1,12} = 4.26$, $p = 0.061$) or gPOA neurons ($F_{1,12} = 0.003$, $p = 0.958$). Thus, the main effect of cell type on the number of neurons was mainly due to *L. unilineatus*. Species and sex had no main effect on the number of AVT-ir cells (species: $F_{1,12} = 1.95$, $p = 0.187$; sex: $F_{1,12} = 0.50$, $p = 0.491$).

Discussion

The two species examined herein had AVT-ir neurons limited to the three cellular groups of the POA, which is in accordance with what has been reported for most of the other teleost species [e.g. Goodson and Bass, 2000; Grober et al., 2002; Miranda et al., 2003; Lema and Nevitt, 2004; Dewan et al., 2008; Maruska, 2009; Santangelo and Bass, 2010]. Although in *L. dimidiatus* the size of mPOA neurons did not differ from gPOA AVT-ir, we are highly confident that these are distinct cell populations based on cytoarchitectural and topological criteria. Most studies report a relationship in terms of cell numbers between the three cell groups [pPOA > mPOA > gPOA; Maruska et al., 2007; Dewan et al., 2008; Maruska, 2009], which we did not find. In fact, in *L. unilineatus* the relationship was exactly the opposite, with the gPOA being the more numerous cell group. Nonetheless, it is not yet known what the functional meaning of these relative differences in cell numbers among the three cell groups is.

Preoptic AVT neurons are well known for their physiological, endocrine and behavioural roles [Goodson and Bass, 2001], and there is a general consensus that the POA represents the main (and usually the only) site of production for this neuropeptide in fish [Bradford and Northcutt, 1983; but see Foran and Bass, 1998; Goodson and Bass, 2000; Greenwood et al., 2008]. Thus, the distribution pattern of AVT-ir neurons in the two species used in this study is in line with the suggested conserved evolution of the neuroanatomical features of the teleost AVT system [Goodson and Bass, 2001]. Nonetheless, some studies in teleosts have reported AVT-ir neurons in the lateral tuberal nucleus of the hypothalamus [Goodson and Bass, 2000; Greenwood et al., 2008]. We could not identify such immunoreactive cells, although such AVT neuronal populations cannot be excluded as their expression is apparently much lower than that in the POA [Greenwood et al., 2008] and the method used here might not be sensitive enough to detect it. Independent evolution of this hypothalamic neuronal group in teleosts should also be considered [see discussion in Thompson and Walton, 2009].

Quantitative analysis of the cell number and size of AVT-ir neurons confirms the first but not the second prediction of our hypothesis. The cleaner *L. dimidiatus* had smaller and less numerous AVT-ir neurons than the non-cleaner *L. unilineatus,* and such differences were restricted to the gPOA cell group. Contrary to what was predicted, no significant sex differences in AVT neuronal phenotypes were found in either species.

We will discuss below how differences in gPOA neurons might play an important role in the ability to express interspecific cooperative behaviours, and later speculate why no differences were detected in the other cell groups. Finally, the absence of sex differences will also be discussed.

The gPOA Cell Group and Cleaning Behaviour

In teleosts, AVT mediates social approach/withdrawal responses to social stimuli [Thompson and Walton, 2004]. In the goldfish *(Carassius auratus),* higher levels of endogenous AVT induce social withdrawal, through neural AVT-ir projections to the hindbrain [Thompson and Walton, 2009]. Although it is not yet clear which teleost preoptic neurons project to the hindbrain, the gPOA neurons are good candidates [but see discussion in Thompson and Walton, 2009; Walton et al., 2010]. In fact, the density of AVT-ir fibres in the vagal motor nucleus (VMN; a hindbrain area with proposed participation in approach/withdrawal behaviour) is positively correlated with the number of gPOA in the Hawaiian sergeant damselfish *Abudefduf abdominalis* [Maruska, 2009]. Taking the size and number of AVT-ir neurons as a proxy for peptide production, the cleaner wrasse *L. dimidiatus* might have less AVT in the gPOA compared to the non-cleaner *L. unilineatus.* Such lower production levels might be important to the cleaner fish's ability to approach interspecific individuals. The results of the present study are in line with the previously described causal relationship between endogenous AVT levels and a cleaner fish's behavioural response towards an interspecific visiting species (referred to as clients). When injected with AVT, cleaner fish were less prone to approach and inspect an interspecific client – and therefore to express interspecific cooperative behaviours. In contrast, treatment with an AVT receptor antagonist had the opposite effect, increasing the cleaner fish's willingness to approach clients and to engage in cleaning interactions by its own initiative [Soares et al., 2012]. Possibly similar mechanisms to those seen in goldfish [Thompson and Walton, 2009] are involved in the regulation of interspecific social approach/withdrawal in the cleaner fish, and additional studies should clarify such hypothesis.

Divergences in gPOA characteristics are often linked to differences in territoriality and its associated aggressive behaviours. Positive correlations have been commonly found between gPOA AVT-ir neuron measures (e.g. size, numbers) and aggression levels [Greenwood et al., 2008; Dewan and Tricas, 2011], suggesting that these neurons play a role in aggressive behaviour. Such an effect has been attributed to gPOA neuronal projections to brain areas where the modulation of social behaviour occurs, such as the ventral nucleus of the ventral telencephalon (Vv) [Dewan et al., 2011]. The density of AVT-ir varicosities in the Vv is in fact strongly predictive of aggressive/territorial behaviour among butterflyfish species [Dewan et al., 2011]. Moreover, administration of AVT often produces an increase in aggressive levels [e.g. Santangelo and Bass, 2006]. The cleaner fish *L. dimidiatus* does not frequently interact aggressively with its interspecific clients [Potts, 1973], as they rely on low aggressive levels to maintain mutualistic interactions. If one takes the size and number of gPOA neurons as a proxy for the amount of AVT being transported and released in brain areas responsible for the modulation of aggressive behaviours [Holmgvist and Ekström, 1995], cleaners would have lower levels of AVT in this system than the non-cleaner species. Such lower levels are possibly responsible for the low levels of interspecific aggression observed, which facilitate the expression of cooperative behaviours.

Taken together, we suggest that the smaller measures of gPOA neurons of the cleaner fish, in comparison to the non-cleaner, might have facilitated (or be a consequence of) the evolution and maintenance of cleaning behaviours and the ability to engage in mutualistic interactions. Possibly, natural selection favoured smaller and less numerous neurons projecting to extrahypothalamic brain areas to lessen the expression of potential interspecific aggressive behaviours and interspecific social withdrawal, thus enhancing mutualistic interactions. Further comparisons using additional sets of related species that differ in their ability to engage in mutualistic interactions (or even species with ontogenic transitions, e.g. cleaner juveniles and non-cleaner adults) should help to further validate this hypothesis and better understand the relationship between the AVT system and interspecific social interactions.

The reported higher fibre densities surrounding the gPOA neurons of the non-cleaner *L. unilineatus* might be related to a higher requirement of cell communication in comparison to the cleaner species. Such communication might be extremely important in *L. unilineatus* to synchronize peptide release in distant brain areas, due to the greater amount of gPOA cells in this species. Indeed, AVT neurons are able to communicate with each other through their dendrites [Saito et al., 2004] and are capable of autoregulation to modulate distant peptide releases [Ludwig, 1998]. Alternatively, the higher fibre density in *L. unilineatus* might be a mere result of the larger and more numerous gPOA cells in this species.

The pPOA and mPOA Cell Groups and Cleaning Behaviour

No differences between the two species in the number or size of pPOA and mPOA neurons were detected in this study. Both pPOA and mPOA neurons project to the pituitary [Bradford and Northcutt, 1983; Strand, 1999] and are capable of regulating other peripheral mechanisms such as osmoregulation and stress response [Balment et al., 1993, 2006; Greenwood et al., 2008]. The lack of differences in pPOA and mPOA AVT-ir neurons is not surprising, as stressors and osmoregulation were not variables in this study (water temperature and salinity were kept constant). Moreover, confinement was applied for such a long period of time (about 1 week between catch and arrival to the laboratory) that it might have represented a chronic stressor, allowing for habituation of the AVT system. In contrast to acute stress responses, AVT mRNA production in pPOA neurons in rainbow trout did not respond to chronic stress (daily stress for 5 days) despite the elevated concentrations of plasma cortisol [Gilchriest et al., 2000]. This might help to explain the lack of differences in pPOA and mPOA neural groups between the species in the present study.

Additionally to pituitary projections, mPOA AVT-ir neurons have also been reported to be involved in the expression of social behaviours, as they also project to brain centres responsible for behavioural modulation [Holmgvist and Ekström, 1995]. Cleaners and non-cleaners did not differ in the number and size of mPOA cells, which further suggests that interspecific cooperative behaviours are more likely to be modulated by central projections of the gPOA AVT neurons.

Mismatch between Sex Differences in Aggressive Behaviour and AVT Neuronal Phenotype

No intersexual variation in AVT-ir phenotypes was found in either species despite the fact that males in both species are more often aggressive than females. *L. dimidiatus* males show higher levels of intraspecific aggression, directed towards females in response to their dishonest behaviours to the clients [Raihani et al., 2010, 2012], and *L. unilineatus* males actively patrol and aggressively defend their territory from competitor males [McIlwain and Jones, 1997]. Other studies have also failed to find sex differences in the AVT system. For example, in goldfish no sex differences in neuronal AVT phenotypes were found and sex steroids did not influence measures of AVT-ir neurons [Parhar et al., 2001]. In a recent study involving two butterflyfish species (one territorial monogamous and other shoaling polygamous), no sex and only limited seasonal differences were detected [Dewan et al., 2008]. Additional studies are therefore required to further investigate whether season and/or sex steroids have an effect on AVT-ir neurons in *L. dimidiatus* and *L. unilineatus.* The lack of sex differences in AVT-ir neurons does not rule out differences in other features of the

AVT system (AVT receptor distribution and expression, mRNA production) and future works should investigate this issue. Furthermore, given the sample size used in this study (*L. dimidiatus*: 4 males and 5 females; *L. unilineatus*: 3 males and 5 females), the lack of significant sex differences should be taken with caution and requires further confirmation in future studies.

In summary, this is the first study to demonstrate differences in AVT-ir neuronal phenotype between two closely related teleost species that differ in the expression of interspecific cooperative behaviours. The cooperative cleaner wrasse *L. dimidiatus* showed fewer and smaller gPOA neurons in comparison to the non-cleaner tubelip wrasse *L. unilineatus*. Although our results only provide correlative evidence between AVT phenotype and the ability to cooperate, we have previously showed that AVT is involved in the expression of interspecific cooperative behaviour in the cleaner wrasse. As such expression was due, at least in part, to central levels of AVT [Soares et al., 2012], the present study further supports the hypothesis that central AVT is involved in the expression of interspecific cooperative behaviours. As suggested in our previous study, the AVT system already in place for the modulation of intraspecific social behaviours might have been recruited for the modulation of interspecific cooperative behaviours. Additionally, the gPOA cellular group and its extrahypothalamic projections appear to play an important role in such levels of interspecific pro-sociality. Further studies are therefore needed to fully understand the contribution of the AVT system to the cleaner wrasse's social system and other mutualistic/cooperative systems.

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