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Immunoreactivity Distribution of Vasotocin and Vasoactive Intestinal Peptide in Brain Nuclei of Two Songbird Species with Different Breeding Systems

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Key Words

Bed nucleus of the stria terminalis · Lateral septal areas · Medial preoptic nucleus · Songbirds · Vasoactive intestinal peptide · Vasotocin

Abstract

Vasopressin influences social behaviour in mammals, in particular social recognition and bonding. However, much less is known about its avian analogue, vasotocin, although vasotocin appears to modulate singing behaviour and agonistic interactions together with vasoactive intestinal peptide (VIP) in some songbirds. The objectives of our study were to compare the expression of vasotocin and VIP in brain nuclei hypothetised to be part of the social behavioural network, i.e. septal areas, bed nucleus of the stria terminalis and medial preoptic nucleus (POM), in two songbird species in the wild: the blue tit *(Cyanistes caeruleus)* and European penduline tit *(Remiz pendulinus)*. These two closely related passerine birds differ in their pair bonding and mating systems: blue tits are socially monogamous with extensive pair bond lasting for several months, whereas in the European penduline tit, pair bond is short and it dissolves during or after laying of the eggs. The two species did not differ in the distribution of vasotocin in the observed brain regions; however, VIP was more abundant in all three regions of penduline tits than in blue tits. We found a sex difference in favour of males in the distribution of vasotocin- and VIP-immunoreactive neurones, fibres and terminals in all three regions in penduline tits. In blue tits, such gender differences were only observed in the POM. The limited differences between the two species suggest that the levels of vasotocin and VIP in the socially relevant brain regions are likely influenced by many other social or environmental factors than just by differences in the duration of pair bonding.

Abbreviations used in this paper

AVT	vasotocin
AVT-LI	vasotocin-like immunoreactive/immunoreactivity
BNST	bed nucleus of the stria terminalis
POM	medial preoptic nucleus
SL	lateral septal area
VIP	vasoactive intestinal peptide
VIP-LI	vasoactive intestinal peptide-like immunoreactive/immunoreactivity

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Introduction

In birds, the two neuropeptides arginine vasotocin (AVT) and vasoactive intestinal polypeptide (VIP) are involved in several social aspects of behaviour, therefore they can be particularly good candidates for the analysis of changes reflecting pair bond length, an important factor that shapes the mating system.

In rodents, the involvement of vasopressin, the homologue of AVT, in social behaviours, in particular social recognition, is well known [for review, see Donaldson and Young, 2008]. Indeed, social recognition is impaired in genetically vasopressin-deficient Brattleboro rats [Feifel et al., 2009] and by vasopressin antagonists in juvenile rats [Dantzer et al., 1987]. It has been shown to be mediated through V_{1a}-receptors [Bielsky et al., 2005]. Social recognition is crucial to formation and maintenance of social bonds such as parental bonding and pair bonding. The latter has been extensively studied in voles [for review, see Young et al., 2011]. The lateral septal areas (SL), bed nucleus of the stria terminalis (BNST) and medial preoptic areas have been identified as sites of action of vasopressin involved in the formation of pair [for review, see Young et al., 2011].

However, less is known about the relationship between social bonding and AVT in birds, although its modulator roles in social behaviours, such as aggression, reproduction and parental behaviour, have been recognized. First, vasotocin stimulates mating behaviour in chicken and pigeon [Kihlström and Danninge, 1972]. It is inhibitory to male sexual behaviour in quails [Castagna et al., 1998]. Vasotocin inhibits courtship in zebra finches and enhances aggressive behaviours in both violet-eared waxbills and zebra finches [Harding and Rowe, 1997; Goodson, 1998b]. Some effects of AVT are sexually dimorphic: in a mate competition test, AVT increases aggression in both males and females, but AVT antagonist decreases aggression only in males [Goodson et al., 2004b]. Second, AVT infusion into the septum facilitates aggressive singing in male song sparrows [Goodson, 1998a]. Canaries treated with an AVT analogue sing more in the autumn and less during the winter [Voorhuis et al., 1991]. Vasotocin induces singing in estrogen-primed, photo-stimulated and non-reproductive short-day female white-crowned sparrows [Maney et al., 1997]. As in mammals, BNST, SL and the medial preoptic nucleus (POM) – all of them being sexually dimorphic – are key sites for the expression of social behaviours [Jurkevich and Grossmann, 2003]. In these nuclei, AVT immunoreactivity varies according to season and reproduction stage (breeding/non-breeding) in males of many avian species [Goodson et al., 2012b]. It should be noted that in opportunistically breeding finches like the zebra finch, the anatomy of AVT innervation does not change seasonally in SL and BNST [Kabelik et al., 2010]. The expression of AVT is under steroid regulation [Voorhuis et al., 1988; Aste et al., 1997; Kimura et al., 1999]. In courting male zebra finches, the BNST has more AVT neurones and higher levels of co-localization of AVT and cFos than the BNST of non-courting males [Goodson et al., 2009a]. Third, cFos activity increases in these AVT neurones as a response to social stimuli, such as conspecifics in gregarious species, while it decreases in non-social species [Goodson and Wang, 2006]. Nevertheless, in case of pair bonding, co-localization of cFos and AVT also increases in the BNST of asocial species. Of note, in gregarious species, conspecifics are associated with positive social stimuli, while they are aversive in asocial species [Goodson and Wang, 2006]. Anti-sense knockdown of AVT production reduces gregariousness and increases anxiety in zebra finches [Kelly et al., 2011].

Studies on vasotocin have either concentrated on the roles of the POM in the sexual behaviour in galliform birds [Xie et al., 2011] or on the differences between gregarious and territorial oscine species in relation to agonistic behaviours [Goodson, 1998a; Goodson et al., 1999, 2012a], all the latter species being monogamous and biparental. Yet the relation of the AVT system to the social bonding linked to the breeding pattern (mono- vs. polygamy) has not been examined.

In birds, agonistic behaviours are also modulated by VIP, with an effect opposite to that of AVT. Intraseptal infusion of VIP reduces aggression in male zebra finches [Goodson and Adkin-Regan, 1999] and facilitates aggression in the violet-eared waxbill [Goodson, 1998b], but has no effect on overt aggression in field sparrows [Goodson, 1998a]. VIP modulation of song depends on the song type and the species [Goodson, 1998a; Goodson and Adkin-Regan, 1999]. VIP immunoreactivity in the septopreoptic region is also sensitive to steroids; castration in males results in VIP innervation increases in the SL particularly in its caudal portion [Aste et al., 1997], suggesting that the VIP circuitry could be sexually dimorphic. Moreover, singing behaviour is modulated via the septal vasotocinergic and VIPergic innervations in an antagonistic manner [Goodson, 1998a]. VIP-immunoreactive fibres have also been demonstrated in the SL, preoptic region and BNST of a variety of avian species (estrildid finches [Goodson et al., 2006], pigeons [Péczely and Kiss, 1988; Hof et al., 1991], doves [Den Boer-Visser and Dubbel-

dam, 2002], chicken [Kuenzel and Blähser, 1994] and quails [Aste et al., 1995]).

VIP is the major stimulatory system of prolactin in birds [Mauro et al., 1992]. The concentration of VIP changes in the median eminence/infundibular complex according to the reproductive cycle and the photoperiod [Mauro et al., 1992; Kosonsiriluk et al., 2008]. Disruption of incubation is accompanied by a sharp decrease in visible immunoreactive neurones in the infundibular region [Prakobsaeng et al., 2011]. In ring doves, the size of the VIP-immunoreactive neurones in the infundibular nucleus increases during incubation until hatching and brooding [Cloues et al., 1990], similarly to pigeons in relation to 'lactation' [Péczely and Kiss, 1988]. These changes are correlated with changes in the GnRH system of the preoptic areas [Deviche et al., 2000]. Reduction in VIP immunoreactivity is also observed in the BNST of the field sparrow and song sparrow during winter, when they are less aggressive and display no territorial behaviour [Goodson et al., 2012b]. However, the role of VIP in social bonding remains unknown. As the modulatory role of VIP differs with the type of social behaviour, the question arises whether its distribution pattern may be influenced by the social bonding pattern related to breeding systems (mono-/polygamous).

In the present study, we investigated the correlation between the breeding patterns and the distribution of vasotocin and VIP immunoreactivities of the SL, BNST and POM, all known to be implicated in the social brain network [Goodson, 2005] and to be sexually dimorphic. We chose two related avian species differing in their mating system: the Eurasian penduline tit and the blue tit.

The European penduline tit *(Remiz pendulinus)* shows sequential polygamy and may have up to 6–7 mates during its long breeding season (from early April to August). Unmated males start to build their nest while singing to attract a female. A few days after the start of egg laying, one of the birds, usually the male, deserts the nest leaving the incubation and parental care to the mate [Pogány, 2009]. Biparental care has never been observed in this species [Van Dijk et al., 2012]. Rupture of the social bond occurs very quickly [Van Dijk et al., 2012].

The blue tit *(Cyanistes caeruleus)* forms stable pairs throughout the breeding season and sometimes across the breeding seasons [Valcu and Kempenaers, 2008]. Both parents care for the chicks. Although socially monogamous, it is a facultative polygynous bird with occasional extrapair mating [Kempenaers, 1994; Valcu and Kempenaers, 2008]. Nevertheless, social bond is long-lasting.

Based on previous studies, we hypothetised that (1) the species exhibiting short pair bond (penduline tit) would present a different immunoreactivity pattern of AVT and VIP than that of the species with long pair bond (blue tit) in all three observed brain regions; (2) male and female birds of both species show a sexually dimorphic AVT and VIP expression in these nuclei, and (3) circulating prolactin levels differ between males and females and between the two species with long and short pair bond.

Materials and Methods

Animals

Five adult male and two adult female blue tits *(C. caeruleus)* and five adult male and one adult female Eurasian penduline tits *(R. pendulinus)* were caught in the Dniestr Delta National Park, Ukraine, between May 21, 2009 and May 26, 2009. Birds were captured at their nests by using a mist net and by playing species-specific songs as bait. The research was approved by the Ministry of Environmental Protection of the Ukraine and the National Park of the Lower Dniestr Region. The work was carried out in accordance with the Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes.

After ketamine-xylazine anaesthesia, morphological data (weight, tarsus length and wing length as well as mask size in penduline tits – this pigmented facial patch is a sexual display [Pogány and Székely, 2007]) were assessed and blood samples were collected for prolactin measurement. Birds were then decapitated, their brains and testes were dissected and immediately fixed by immersion in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer. Samples were stored at 4°C until further processing. Fixed brains were transferred to a 20% sucrose solution before being sectioned at 60-μm thickness on a freezing microtome (Frigomobil, Zeiss). Three series of alternate sections were taken. One series was immediately mounted and stained with cresyl violet for the identification of structures. Two other series were processed for immunocytochemistry. The weight of fixed testes was also recorded.

Immunohistochemistry

Two antisera were used for this study: an anti-AVT (a kind gift from Prof. David Gray, University of Witwatersrand, Johannesburg, South Africa [Gray and Simon, 1983]) and an anti-VIP (a gift from Dr. Tamás Görcs [Gulyás et al., 1990]). Both were raised in rabbits and diluted in PBS-Tween-20 (anti-AVT: 1/60,000, anti-VIP: 1/10,000).

Sections were washed in PBS. Endogenous peroxidase activity was quenched by 0.1% H_2O_2 in PBS for 15 min. Following several washes in PBS containing 0.1% Tween-20, sections were incubated for 2 h in a solution of 1% normal goat serum in PBS-Tween-20 and then transferred overnight to the rabbit primary anti-serum. Then, sections were extensively washed in PBS-Tween-20, incubated for 2 h in a biotinylated goat anti-rabbit IgG (Vector, Burlingame, Calif., USA) at 1/100 in PBS-Tween-20, rinsed and incubated with avidin-biotin complex (Vector) diluted in PBS for 2 h. Sections were rinsed first in PBS, then in Tris buffer (pH 8) before being incubated in a solution containing 0.015% diaminobenzi-

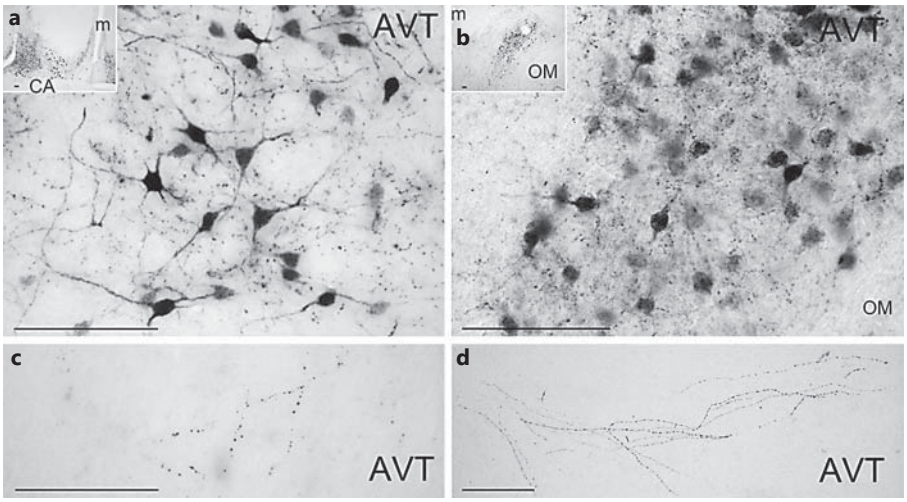

Fig. 1. Photomicrographs of vasotocin-LI in the BNST of male (**a**) and female blue tits (**c**) and male (**b**) and female penduline tits (**d**). **Insets**: low magnification of the nucleus. CA = Anterior commissure; m = midline; OM = occipitomesencephalic tract. Scale bars = 100 μm.

dine tetrahydrochloride (Sigma-Aldrich, Steinheim, Germany) and 0.25% ammonium nickel sulphate hexahydrate (Fluka Chemie, Buchs, Switzerland) in Tris buffer. After a 5-min pre-incubation, the enzymatic reaction was initiated by adding 5 μl H_2O_2 (0.1%)/5 ml diaminobenzidine tetrahydrochloride. The reaction was stopped 10 min later by rinsing with Tris buffer (pH 8) followed by PBS. Sections were then mounted on gelatinised slides and coverslipped with DPX (Sigma-Aldrich, Budapest, Hungary).

Control of specificity included omission of the primary antisera and absorption of the antiserum with the antigen.

Identification of the Brain Structures

Contour drawings of the Nissl-stained sections served as templates on which the AVT- and VIP-immunoreactive cells, fibres and terminal fields were recorded. For identification of the brain regions, we used the canary [Stokes et al., 1972] and the chicken atlases [Kuenzel and Masson, 1988; Puelles et al., 2007]. For the septal areas, we identified the different subdivisions as defined by Goodson et al. [2004a].

Statistical Analysis and Quantification

Representative photomicrographs of sections containing septum, preoptic nuclei and BNST were taken. Average pixel density (on a scale ranging from 0 to 255, as white and black, respectively) was measured in the regions of interest (BNST, SL, medial septum and POM) using ImageJ software [Schneider et al., 2012]. Intensity of labeling was calculated by subtracting the average density of neighbouring background (immunonegative) region from the densities of the regions measured for quantification. The resulting average relative pixel density values represented both the abundance of the neural elements and the intensity of their immunoreactivity. Therefore, the values give an estimate of mean optical density within a reference area regardless of its precise anatomical correlate (fibres or cells). Males of the two species were compared by Welch's t tests for means and Levene's tests for variance. For male/female comparisons, one-sample t tests were used with the female data as expected values. AVT and VIP label intensities were correlated to prolactin concentration using Pearson's correlation. We used the SPSS software package for statistical analysis.

Average testis size as percentage of body weight was 64% larger in blue tits ($t = 3.35$, d.f. = 11, $p < 0.01$). Face mask size of penduline tit was larger in males than females ($t = 3.77$, d.f. = 4, $p < 0.05$).

Radioimmunoassay

Prolactin concentrations were determined in duplicate aliquots from 50 μl of plasma sample by heterologous radioimmunoassay at the Centre d'Études Biologiques de Chizé, France, as described in detail by Cherel et al. [1994].

Results

Distribution of Vasotocin-Like Immunoreactivity

In both species, the great majority of AVT-like immunoreactive (LI) neuronal cell bodies were distributed in the hypothalamic ventral supraoptic, suprachiasmatic, periventricular and paraventricular nuclei (online suppl. fig. 1, www.karger.com/doi/10.1159/000357831). Many cells were dispersed amongst the fibres of the lateral forebrain bundle. These neurones gave rise to axons, which coursed toward the median eminence through the lateral hypothalamus and the periventricular zone, some of them also crossing the supraoptic decussation. No difference was apparent between males and females in these regions.

Bed Nucleus of the Stria Terminalis

In males of both species, numerous perikarya of the BNST are densely immunopositive for AVT, surrounded by thin varicose AVT-LI fibres (fig. 1a, b). Fibres were present in the lateral, ventromedial and, mainly, dorsolateral parts, the magnocellular part being virtually devoid of AVT immunoreactivity. In females, no cell bodies were stained. Rare thin and varicose AVT-LI fibres crossed the nucleus (fig. 1c, d). Label intensity was higher in male

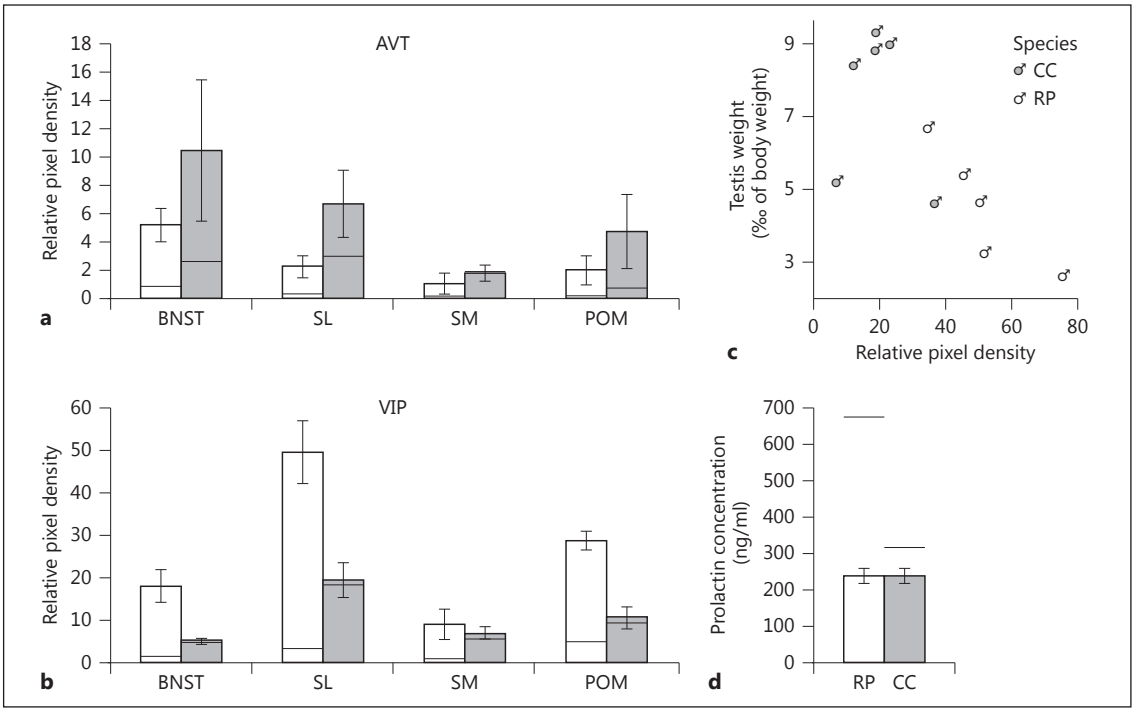

Fig. 2. a, **b** Intensity of optical density for AVT (**a**) and VIP (**b**) in the BNST, SL and medial septal area (SM) and POM in penduline tits (open columns) and blue tits (grey columns). Black bars over columns represent the value of the female (penduline tit) or the average of two females (blue tit) of the same species. See text for the results of statistical tests. **c** Correlation between VIP label density in the lateral septum and testis size in male penduline tits (RP, open symbols, $r = -0.90$, $p < 0.05$, $n = 5$) and blue tits (CC, grey symbols, $r = -0.227$, nonsignificant, $n = 6$). **d** Circulating prolactin concentration in penduline tits (open columns) and blue tits (grey columns). Black bars over columns represent the value of the female (penduline tit) or the average of two females (blue tit) of the same species. See text for the results of statistical tests.

than female penduline tits ($t = 3.35$, d.f. = 4, $p < 0.05$; fig. 2a) and appeared to be higher in male than female blue tits ($t = 1.68$, d.f. = 4, $p = 0.168$; fig. 2a) probably due to higher variance among males. No clear species differences of optical density were noted except for a tendency to less intensely stained neurones in penduline tits. However, the variance in label intensity was greater in the blue tits (Levene's $W = 6.58$, $p < 0.05$).

Septal Areas

A dense network of AVT-LI varicose fibres and terminal fields characterised the SL of male birds (fig. 3a–d). Numerous AVT-LI terminals covered the perikarya. The density of the fibres and terminal fields was greater at post-commissural levels in the ventral and caudal part of the SL. The medial septal areas were virtually devoid of AVT immunoreactivity. Very few AVT-LI varicose fibres were observed in septal areas of the female compared to male penduline tits ($t = 3.01$, d.f. = 4, $p < 0.05$; fig. 2a). No sexual difference was observed in blue tits. No clear species differences were observed either, although there was a tendency to a greater density of fibres in the blue tit (fig. 2a). Intra-specific variation was larger in blue tits (Levene's $W = 11.01$, $p < 0.01$). It should be noted that the number of AVT-LI neurones and fibres as well as the intensity of the reaction varied considerably between males, possibly in relation to their hormonal and physiological status. In one male penduline tit, the vasotocinergic fibres were rather few, contrasting with the intense VIP staining. However, there was no negative correlation between the label intensity of the two peptides.

Preoptic Nuclei

In the POM, AVT-LI fibres form a dense network in males, while they are almost absent in females of both species (fig. 4c, d). Densitometric analysis supported this observation (penduline tit: $t = 3.33$, d.f. = 4, $p < 0.05$; blue tit: $t = 3.57$, d.f. = 4, $p < 0.05$; fig. 2a). In this sexually dimorphic nucleus, some fibres are visible in the female blue tit but not in the penduline tit. In addition, some AVT-LI peri-

karya are also stained more densely in the blue tit than in the penduline tit, but no clear species difference was observed either in the mean or the variance of optical density.

Distribution of VIP-LI

Numerous perikarya were immunoreactive for VIP in the lateral septal organ, the hypothalamic inferior and infundibular/tuberal nuclei of all birds, without any difference between species or sexes. The variance in VIP label intensity did not differ significantly between species in any region observed.

Bed Nucleus of the Stria Terminalis

Immunoreactive VIP-LI fibres were coursing through and terminating in the lateral, dorsolateral, ventrolateral and magnocellular part of the BNST (fig. 4a, b). Many fibres and dense terminal fields were present in the lateral part of the rostral BNST situated along the ventral part of the lateral ventricle. The VIP-LI fibres were more numerous in the medial BNST of male rather than female penduline tits ($t = 4.09$, d.f. = 4, $p < 0.05$; fig. 2b), but this was not the case in blue tits. A small but significant difference was observed between species, with more intense staining in the penduline than in the blue tit ($t = 3.84$, d.f. = 9, $p < 0.005$; fig. 2b).

Septal Areas

A dense network of VIP-LI fibres terminated in the septal areas (fig. 3e, f), but their densities varied considerably within the structure. The number of VIP-LI fibres and terminals was small at the rostral level of the SL, but increased considerably when moving caudally from the medial septum level. They remained dense in the caudal SL in blue tits. The medial nucleus was almost devoid of VIP fibres. More intense VIP-LI label was observed in the SL of the male than the female penduline tit ($t = 6.21$, d.f. = 4, $p < 0.005$; fig. 2b). No sexual difference was observed in the blue tit. However, penduline tit males were more intensely labelled than blue tit males ($t = 4.22$, d.f. = 9, $p < 0.05$; fig. 2b). VIP showed a positive correlation with testis weight in penduline tits but not in blue tits (fig. 2c).

Preoptic Areas

Many VIP-LI fibres terminated in the preoptic nuclei and surrounding areas. The densest terminal field was observed in the POM (fig. 4e, f), and the abundance of fibres was less in the dorsolateral preoptic nucleus. More intense VIP-LI label was observed in the POM of male than female penduline tits ($t = 9.91$, d.f. = 4, $p < 0.005$; fig. 2b). No sexual difference was observed between the individuals of the blue tit. However, optical density was more intense in penduline tit males than in blue tit males ($t = 5.13$, d.f. = 9, $p < 0.001$; fig. 2b).

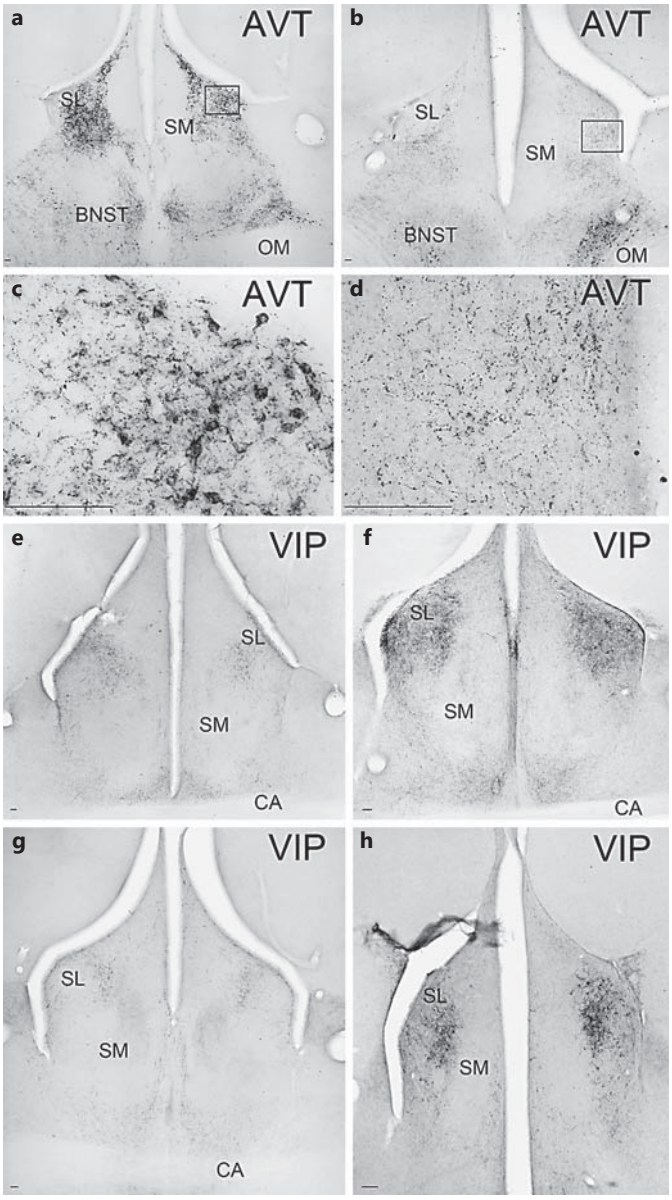

Fig. 3. Photomicrographs of AVT (**a–d**) and VIP (**e–h**) immunoreactivity in the septal areas of male (**a**, **c**, **e**) and female blue tits (**g**), and male (**b**, **d**, **f**) and female penduline tits (**h**). **c**, **d** Higher magnification of the enclosed area in **a** and **b**, respectively. CA = Anterior commissure; OM = occipitomesencephalic tract; SM = medial septal nucleus. Scale bars = 100 μm.

Prolactin Levels

Circulating prolactin levels in males were lower than those in females in both species (penduline tit: $t = 21.7$,

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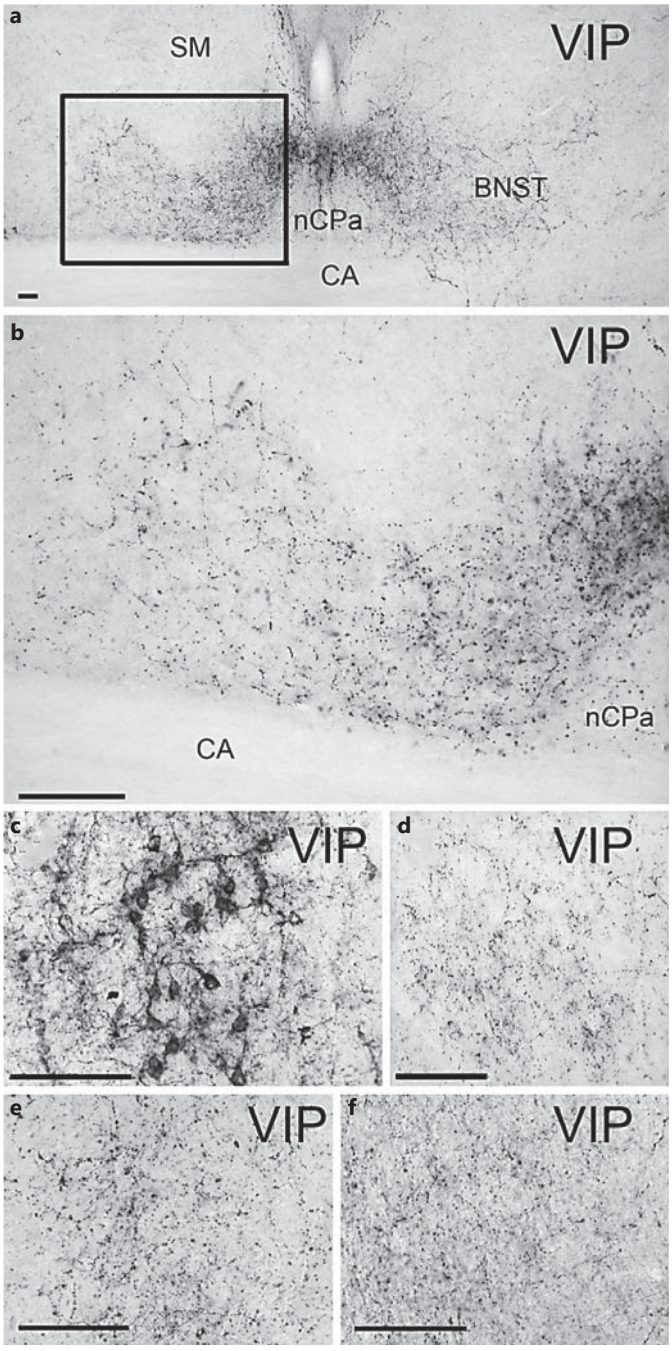

Fig. 4. a, **b** Photomicrographs of the VIP-LI in the BNST of male blue tits. **b** Higher magnification of the enclosed area in **a**. **c–f** Photomicrographs of the vasotocin-LI (**c**, **d**) and VIP-LI (**e**, **f**) in the POM of male blue tits (**c**, **e**) and male penduline tits (**d**, **f**). CA = Anterior commissure; nCPa = nucleus of the pallial commissure; SM = medial septal nucleus. Scale bars = 100 μm.

d.f. = 5, $p < 0.001$; blue tit: $t = 4.18$, d.f. = 6, $p < 0.01$; fig. 2d). There was no difference between the males of the two species.

Discussion

General Considerations and Caveats

Our work is one of a handful of studies that report on the distribution of AVT and VIP immunoreactivity in wild bird populations.

Our first hypothesis assuming differences in the distribution and intensity of immunoreactivity between species of different pair bonding systems has been confirmed in the case of VIP. Indeed, in SL, BNST and POM immunoreactivity for VIP is stronger in penduline tits – short-lasting pair bond species – than in the blue tit – long-lasting pair bond species. However, there is no clear-cut difference in the AVT distribution between the two species, although AVT immunoreactivity shows greater variability in blue tit males (long-lasting pair bond species).

As predicted by our second hypothesis, we have shown a sex difference in the distribution of AVT- and VIP-immunoreactive neurones, fibres and terminals in penduline tits. Both peptides are more abundant in males. However, in blue tits, there was no difference in VIP distribution, and only in the POM AVT was more abundant in male birds.

Our third hypothesis on a putative difference in circulating prolactin levels between the two species has not been sustained by the results: the two species display similar blood prolactin levels. However, we confirmed a sex difference: females of both species exhibited an elevated level of prolactin compared to males.

Although VIP is suggested to be associated with parental behaviour, VIP label was more intense in penduline tit males than in the caring female or caring/incubating blue tit males in all three regions (BNST, SL and POM). This might suggest that VIP is related to courtship and mating, rather than to parental care, in the observed brain regions. VIP intensity in the SL negatively correlates with testis size in male penduline tits but shows no correlation in blue tit males. Since prolactin is associated with parental behaviours in birds [Angelier and Chastel, 2009] just as in mammals, a different VIP role is apparent in the two species. It cannot be excluded that the function of VIP is rather unspecific, merely facilitating the activity of the septum probably via vasodilation [Yaksh et al., 1987]. The functional state of the septum is strongly dependent on intrinsic (hormonal) and extrinsic (environmental) stimuli, including changes over time during the mating season.

The lack of sexual dimorphism (except in the POM) in either AVT or VIP distribution in blue tits and the presence of marked sex differences in the same regions in penduline tits might represent differences in mating stages rather than mating strategies.

Due to the difficulties in catching females [Van Dijk et al., 2012], since they were less interested in the conspecific song, only one penduline tit and two blue tit females were available for the analysis. In this respect, the low sample size limits the validity of our statistical results. Despite our effort, stress during catching and handling was high and certainly affected the blood level of prolactin. With regard to the specificity of our antibodies to AVT or VIP, neither has been studied in these two species. Nevertheless, the fact that the overall distribution of immunoreactive neurones, fibres and terminals agrees with that of other avian studies allows us to be rather confident in the specificity of staining.

Since the birds were captured in their natural habitat, their age remained unknown and it was difficult to assess the exact stage of their reproductive cycle. In blue tits and great tits, as in many other songbirds, the onset of the breeding season is controlled by various environmental factors [Visser and Lambrechts, 1999]. As a result, egg-laying dates may vary as much as a month within a few square kilometres of territory. At the time and location of our study, the blue tits caught already had a mate and had been engaged in parental behaviour. Penduline tits showed greater variability in the stage of their reproduction with only the one female caring for hatchlings while other individuals were just mated or still looked for a mate. Despite such behavioural diversity, AVT intensity data showed lower variance in penduline tits than in blue tits. The finding that testis size was larger in the socially monogamous blue tits than in the polygamous penduline tits contradicted the pattern that polygamous species should have larger testis – and therefore higher peak testosterone levels – than monogamous ones [Garamszegi et al., 2005].

With all these limitations, to our knowledge, this is the first study to assess the neural correlates of reproductive behaviour in these two species.

Distribution of Vasotocinergic Structures

The overall distribution of vasotocinergic neurones, fibres and terminals is identical to that seen in other avian species. Presence of vasotocinergic neurones in BNST and fibres in the SL and POM has been demonstrated in zebra finches [Kimura et al., 1999], canaries [Voorhuis et al., 1988], quails [Viglietti-Panzica, 1986; Aste et al., 1997], domestic fowls [Viglietti-Panzica, 1986; Jurkevich and Grossmann, 2003], pigeons [Berk et al., 1982] and Peking ducks [Viglietti-Panzica, 1986]. In blue tits and penduline tits, these structures are sexually dimorphic, as in most avian species studied until now [Voorhuis et al., 1988, 1991; Aste et al., 1997; Jurkevich and Grossmann, 2003].

We did not observe clear interspecies variation in AVT distribution despite obvious differences in the reproductive strategies and in the stages of mating seasons. It is known that fluctuations in steroid levels are accompanied by changes in the synthesis of AVT [Jurkevich and Grossmann, 2003; Kabelik et al., 2010]. It is also possible that interspecies differences would be represented in other vasotocinergic systems. Viglietti-Panzica [1986] has identified species differences between fowls, quails and pigeons in the extent of the preoptico-hypothalamic distribution of AVT cell groups. In canaries, few AVT-LI neurones were identified in female BNST [Voorhuis et al., 1988], but none in the Japanese quail. In zebra finches, the sex differences in AVT are not as prominent in BNST and septal areas as in other avian species [Kimura et al., 1999]. The species differences may also be even more subtle, e.g. at the level of AVT receptors. The V_{1a}-binding sites are present in the lateral septal areas of the estrildid finches, where AVT fibres are also distributed [Goodson et al., 2006]. Socially monogamous, blue tits also engage in extrapair mating [Kempenaers, 1994]. This could partly explain the variability in AVT staining. Moreover, polygyny seems to depend on the availability of food in the habitat, this behaviour being more common in flooded area/lakeshore, such as our study site, than in dry habitat [Dunn and Hannon, 1992]. Notably, though the blue tits were already engaged in incubation/parental care, the testis/body size ratio was greater in this species (males possibly looking for an opportunity of extrapair mating) than in the obligatory polygamous penduline tit.

Distribution of VIPergic Structures

Distribution of VIP-LI neurones, fibres and terminal field is in accordance with observations in pigeons [Péczely and Kiss, 1988; Hof et al., 1991], doves [Cloues et al., 1990; Den Boer-Visser and Dubbeldam, 2002], fowls [Kuenzel and Blähser, 1994; Kosonsiriluk et al., 2008], quails [Aste et al., 1995] and juncos [Deviche et al., 2000]. As in juncos [Deviche et al., 2000], we did not observe VIP-LI neurones in the preoptic areas, although these are present in non-oscine species [Péczely and Kiss, 1988; Cloues et al., 1990; Den Boer-Visser and Dubbeldam, 2002]. VIP-LI neurons in the septal areas were not observed either, although their presence had been noticed in Thai hen [Kosonsiriluk et al., 2008]. The distribution

of VIP-LI fibres is mostly similar to that of the VIP binding sites in the SL, BNST and preoptic areas, as observed in the estrildid finches [Goodson et al., 2006], doves [Askew et al., 1997] and pigeons [Hof et al., 1991]. The distribution of VIP is sexually dimorphic, as it is in the dove [Askew et al., 1997]. In that species, no difference appears between non-breeding and breeding birds [Askew et al., 1997].

In male quails, VIP fibres, although present in the POM, are less numerous than in the surrounding preoptic regions. VIP-LI increases with castration and decreases with testosterone replacement in the SL, while it remains unaffected in the POM [Aste et al., 1997]. Notably, we found an inverse correlation between VIP-LI of the SL and testis size in penduline tit males.

In conclusion, our study is consistent with the assumption that differences in the distribution of both AVT and VIP in certain regions which are homologous to the mammalian 'social brain network' [Goodson, 2005] are associated with gender and/or pair bond duration. Although in the present study we focused on pair bonding, these two species differ by other aspects of their breeding behaviour, for instance mating system or parental care. To distinguish between the neural correlates relevant to AVT and VIP of these behaviours, extensive sampling of males and females during different reproductive stages (courtship, pair bond and parental care, for example) is needed. In addition, studying of further species that exhibit various breeding strategies might be necessary.

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