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Involvement of different mesotocin (oxytocin homologue) populations in sexual and aggressive behaviours of the brown anole

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The oxytocin (OT) family of neuropeptides are known to modulate social behaviours and anxiety in mammals and birds. We investigated cell numbers and neural activity, assessed as Fos induction, within magnocellular and parvocellular populations of neurons producing the OT homologue mesotocin (MT, Ile^8-oxytocin). This was conducted within the male brown anole lizard, *Anolis sagrei*, following agonistic or courtship encounters with a conspecific. Both neurons colocalizing and not colocalizing corticotropin-releasing factor (CRF) were examined. Parvocellular neurons of the paraventricular nucleus exhibited a positive correlation between courtship frequency and Fos colocalization, regardless of whether they produce just MT or MT + CRF. Magnocellular populations showed only trends towards positive relationships with courtship and no cell populations showed aggression-related Fos induction. These findings are novel because they demonstrate the involvement of MT neurons in male social behaviour, especially in reptiles for whom the involvement of MT in social behaviour was previously unknown.

1. Introduction

Social behaviours are regulated by an interacting set of signalling molecules within a social behaviour neural network [1,2]. The oxytocin (OT) family of neuropeptides has been linked to the regulation of sexual and aggressive behaviours, as well as to the modulation of anxiety [3–6]. While peripheral functions of OT and mesotocin (MT, Ile^8-OT), the OT homologue in most non-mammalian amniotes, include smooth muscle contraction, their central actions are prosocial and usually anxiolytic [3,7,8]. Effects on social behaviours include facilitation of social bonding, trust and social cognition [4–6,9]. OT has been found to usually, though not always, reduce anxiety, probably via the dampening of amygdala-related fear and stress circuitry [6,10]. The dampening of anxiety and OT's prosocial effects may be linked [9]. Interestingly, a portion of OT/MT neurons in the paraventricular (PVN), supraoptic (SON) and accessory nuclei (AN) of the hypothalamus colocalize the unrelated neuropeptide corticotropin-releasing factor (CRF). This is surprising because OT is known to downregulate CRF, which in turn usually acts as an anxiogenic agent that activates the hypothalamic–pituitary–adrenal (HPA) axis [11]; however, HPA axis activation has also been linked to social bond formation in male prairie voles (*Microtus ochrogaster*) [5].

The goal of this study was to determine the differential involvement of hypothalamic magnocellular and parvocellular neurons, either producing solely MT (MT-only) or MT and CRF (MT + CRF), in social behaviour regulation of the brown anole, *Anolis sagrei*. Activation of neurons of different phenotypes was assessed as fluorescent colocalization with Fos, a transcription factor whose expression is an indirect marker of neural activity [12]. This work

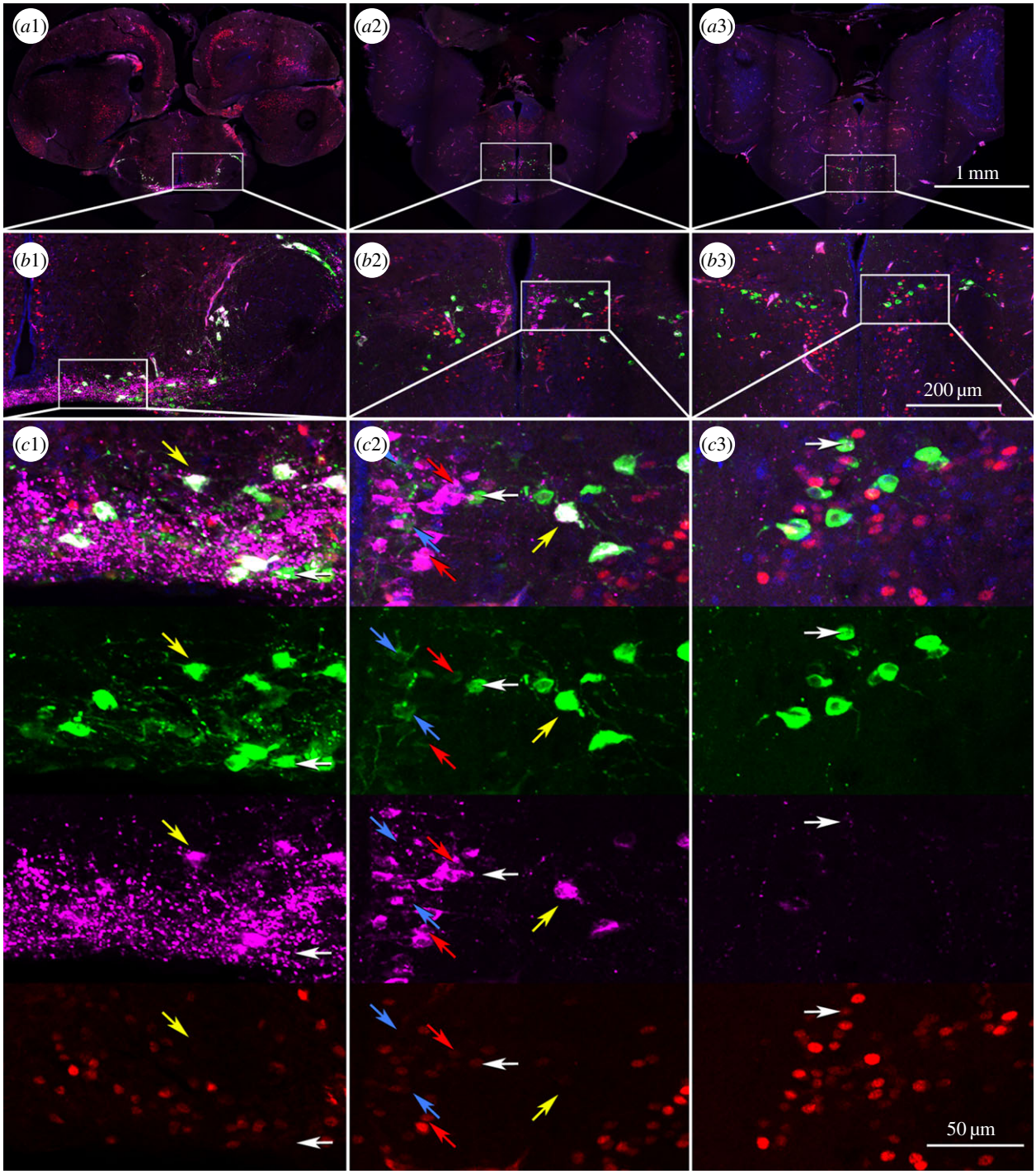

Figure 1. Photomicrographs of immunofluorescent colocalization of MT (green), CRF (purple), Fos (red) and DAPI (a general nuclear stain, blue) in 50 µm coronal brain sections of the male brown anole brain. Present are whole brain sections (*a*1–*a*3), with insets of the hypothalamus (*b*1–*b*3). Further insets (*c*1–*c*3) demonstrate (at top) neurons with all four colours displayed, and then separate displays of MT, CRF and Fos, respectively. Three rostro-caudal points are represented: (*a*1–*c*1) SON, (*a*2–*c*2) rostral PVN and (*a*3–*c*3) caudal PVN. Yellow diagonal arrows indicate example magnocellular MT + CRF neurons. White horizontal arrows indicate magnocellular MT-only neurons. Red diagonal arrows indicate the smaller and less immunoreactive parvocellular MT + CRF neurons. Blue diagonal arrows indicate parvocellular MT-only neurons.

is part of a larger study examining the social behaviour neural network throughout the brown anole brain [13,14].

2. Material and methods

(a) Subjects, treatment groups, behaviour and tissue processing

Fifty-seven adult male brown anoles (*A. sagrei*) were divided into treatment groups of control (no conspecific present, $N = 12$), agonistic encounter (male conspecific present, $N = 23$) and courtship encounter (female conspecific present, $N = 22$). Details about housing, behavioural trials and tissue processing have been reported previously [13,14].

Briefly, resident males were housed on one side of a divided terrarium. After an opaque partition was removed, 14 min behavioural tests were conducted, and aggressive and sexual behaviours were recorded. Only one trial was conducted per focal male; stimulus animals were sometimes used repeatedly, although with at least a week-long interval between trials. Behavioural frequency was defined as the sum of engagement behaviours

during a 14 min trial, and included head-bob and push-up displays (with and without extension of the dewlap), chases and the consummatory behaviours of either copulation (courtship trials) or biting (aggression trials). See the electronic supplementary material, table S1, for descriptions of measured behaviours. At 90 min, the subjects were sacrificed. This time point allows for both Fos induction and degradation [12]. Brains from these subjects were submersion-fixed with 4% paraformaldehyde, cryoprotected with 30% sucrose in 0.1 M phosphate-buffered saline (PBS), frozen and sectioned at 50-µm thickness into two coronal series.

(b) Immunohistochemistry

Forebrain sections from one tissue series were rinsed twice for 30 min in PBS, then blocked for 30 min in a solution of 2.5% donkey serum (Sigma-Aldrich), 0.3% Triton-X-100 (Fisher Scientific) and 0.05% sodium azide (Fluka) in PBS. Next, the sections were placed for 40 h at 4°C in blocking solution containing a 1 : 2000 dilution of rabbit polyclonal anti-OT antibody (VA 10, generously provided by Dr Harold Gainer, NIH, Bethesda, MD, USA), 1 : 10 dilution of mouse monoclonal anti-Fos antibody (Santa Cruz Biotechnology) and 1 : 10 000 dilution of guinea pig polyclonal anti-CRF antibody (Bachem). Following two 30 min PBS rinses, the sections were incubated for 3 h at room temperature in blocking solution containing a 1 : 500 dilution of donkey anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Life Technologies), a 1 : 200 dilution of donkey anti-mouse secondary antibody conjugated to Alexa Fluor 555 (Life Technologies) and a 1 : 125 dilution of donkey anti-guinea pig Alexa Fluor 647 (Jackson ImmunoResearch). Following two 30 min PBS rinses, tissues were mounted onto gelatin-coated slides, cleared with Xylene Substitute (Sigma-Aldrich) for 5 min and coverslipped using Prolong Gold mounting medium with DAPI (Life Technologies).

The specificity of the OT antibody has been previously determined [15]. Our own tests demonstrated that preadsorption with 10× and 100× OT peptide (Sigma-Aldrich) resulted in loss of MT signal and that MT signal was separate from vasotocin signal (guinea pig anti-vasopressin antibody, Bachem). The specificity of the Fos antibody was demonstrated by preadsorption with 5× or 10× Fos blocking peptide (Santa Cruz Biotechnology). The specificity of the CRF antibody was demonstrated by preadsorption with 10× and 100× CRF peptide (Sigma-Aldrich). See the electronic supplementary material, figures S1–S3, for preadsorption photomicrographs.

(c) Image and statistical analyses

An LSM 700 Confocal microscope and Zen 2010 software (Carl Zeiss), using a 20× objective, were used to capture 10-level *z*-stacks of photomicrographs, each 5 µm apart, in a grid that was later stitched together. A maximum intensity projection created a two-dimensional image. Individual colours were exported as separate layers using AxioVision v. 4.8 (Carl Zeiss), and these were stacked as overlayed monochromatic layers in Photoshop (Adobe Systems). Layers in the stack could thus be toggled on and off to determine colocalization of MT, CRF and Fos. Analyses were conducted by individuals blind to treatment groups.

All MT-immunoreactive (-ir) cells present within the SON and PVN were examined across all sections where present (figure 1). Parvocellular neurons were distinguished by size and relative faintness of immunoreactivity. Analyses of Fos colocalization were only conducted for cell populations containing five or more neurons (which reduced sample sizes for parvocellular cell analyses). Analyses were performed using Pearson's correlations and one-way analysis of variance, with logarithmic transformations applied to variables not conforming to parametric assumptions. Non-parametric Mann–Whitney *U*-tests were applied for analyses of consummatory behaviours, which occurred with low frequency

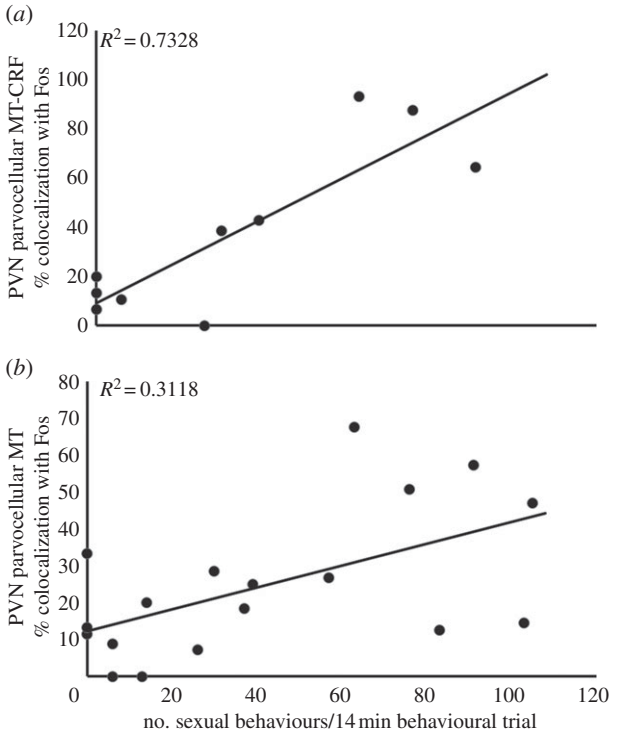

Figure 2. Correlations between social behaviours and neural measures. The frequency of sexual behaviours during a courtship trial correlates positively with Fos colocalization within both (*a*) MT-CRF neurons and (*b*) MT-only neurons in the PVN. See text for statistical results.

($N = 5$ for both copulation and biting). Benjamini–Hochberg adjustments were made for multiple comparisons.

3. Results

Courtship frequency was positively correlated with Fos colocalization in both parvocellular MT-CRF ($r = 0.86$, $N = 10$, $p = 0.002$) and MT-only ($r = 0.56$, $N = 18$, $p = 0.016$) neurons of the PVN (figure 2). No relationships were observed between Fos colocalization in these neurons and aggression ($p > 0.05$ for all, although the trend was in the opposite direction for MT-CRF neurons: $r = -0.54$, $N = 8$, $p = 0.17$), nor between Fos colocalization in magnocellular populations of the PVN and SON and any social behaviours ($p > 0.20$ for all except magnocellular PVN MT-only ($r = 0.40$, $N = 22$, $p = 0.07$) and SON MT-only ($r = 0.42$, $N = 22$, $p = 0.051$) neurons which showed trends towards positive correlations with courtship that were similar to but weaker than those observed in parvocellular PVN neurons). Follow-up analyses revealed that animals who exhibited consummatory behaviours (copulation/biting) showed no difference in Fos colocalization from animals that merely displayed to conspecifics ($p > 0.05$ for both). Additionally, the behaviours of stimulus animals were unrelated to neural measures in focal males ($p > 0.05$ for all except SON MT + CRF neurons which showed a positive correlation with female courtship behaviours at $p = 0.04$, although this did not meet the criterion for significance following adjustment for multiple comparisons). Finally, no differences in Fos colocalization were found between animals assigned (regardless of displayed behaviour) to courtship, agonistic and control treatment groups ($p > 0.30$ for all).

4. Discussion

Our analysis divided neurons based upon location and phenotype. Clear subgroup delineations, as found in rodents, were not observed; however, most neural clusters matched previously described groups [7]: most PVN MT + CRF-ir magnocellular neurons were found in locations similar to described extrahypothalamic, fornical, and anterior commissural AN, the dorsal portions of the PVN proper and lateral to the PVN. A few MT-only magnocellular PVN neurons were located in what may be the circular AN, while most were found caudally in what may be the dorsolateral AN. The parvocellular neurons were all found in the PVN proper, primarily at its ventral aspect. Magnocellular SON neurons stretched from the SON proper through the nucleus of the medial forebrain bundle. These locations are consistent with those in other amniotes [7]. Neural phenotype and location were not strictly congruent and stray neurons of an atypical phenotype for that region were sometimes found in a given nucleus.

Our study demonstrates that parvocellular PVN neurons producing MT-only or MT + CRF are both activated (co-express Fos) in proportion to displayed courtship behaviour. This is the first study to demonstrate a relationship between MT and social behaviours in reptiles; MT had been known to solely affect nesting behaviour timing in turtles [16]. Furthermore, we know of no study demonstrating a role for parvocellular MT-producing neurons in social behaviour regulation. These findings also support a view of OT/MT as being important in male courtship [17,18], despite this neuropeptide's more prevalent involvement in female social behaviour [3,4].

Although trends mirroring the results in PVN parvocellular neurons were also present between courtship frequency and Fos colocalization within MT-only magnocellular neurons of the PVN and SON, these did not reach significance. In fact, we did not observe any significant behaviour-related Fos colocalization in magnocellular neurons of the PVN or SON, although local neuropeptide release at axon terminals may be possible without somal activity and thus Fos production. The lack of behavioural correlations with SON measures in this study is consistent with findings in rats, which demonstrate limited forebrain innervation from SON OT neurons relative to PVN and AN populations [19,20]. The lack of differential activation of MT-only and MT + CRF neurons suggests that both of these cell types work in a coordinated manner, probably an MT-specific manner; a number of CRF-only populations exist both within and outside of the hypothalamus and it may be solely these CRF-only neurons that exert CRF's anxiogenic effects.

In conclusion, our results demonstrate an involvement of MT in the regulation of courtship behaviours in reptiles—a role in line with the prosocial involvements of MT/OT in mammals and birds. However, we find no specific function for neurons colocalizing MT + CRF relative to those producing solely MT.

All animal procedures were approved by the Institutional Animal Care and Use Committee.

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Data accessibility. http://dx.doi.org/10.6084/m9.figshare.1126313.

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