Socially-mediated activation in the snake social-decision-making network

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Abstract

Brain areas important for social perception, social reward, and social behavior – collectively referred to as the social-decision-making network (SDN) – appear to be highly conserved across taxa. These brain areas facilitate a variety of social behaviors such as conspecific approach/avoidance, aggression, mating, parental care, and recognition. Although the SDN has been investigated across taxa, little is known about its functioning in reptiles. Research on the snake SDN may provide important new insights, as snakes have a keen social perceptual system and express a relatively reduced repertoire of social behaviors. Here, we present the results of an experiment in which ball pythons (*Python regius*) interacted with a same-sex conspecific for one hour and neural activation was investigated through Fos immunoreactivity. Compared to controls, snakes that interacted socially had higher Fos counts in brain areas implicated in social behavior across taxa, such as the medial amygdala, preoptic area, nucleus accumbens, and basolateral amygdala. Additionally, we found differential Fos immunoreactivity in the ventral amygdala, which facilitates communication between social brain areas. In many of these areas, Fos counts differed by sex, which may be due to increased competition between males. Fos counts did not differ in early sensory (i.e., vomeronasal) processing structures. As ball python social systems lack parental care, cooperation, or long-term group living, these results provide valuable insight into the basal functions of the vertebrate social decision-making network.

Key words: Social decision-making network, vomeronasal system, Fos immunoreactivity, social neuroscience, ball pythons, amygdala

Introduction

The Social Behavior Network (SBN; see Table 1 for a list of all abbreviations) is a set of brain regions that are important for perceiving social cues and mediating social interactions (Newman, 1999; Bickart et al., 2014), and are highly conserved across vertebrate species (Goodson, 2005). The network consists of six highly interconnected limbic areas: the extended medial amygdala (consisting of the medial amygdala, MeA, and the medial bed nucleus of the stria terminalis, mBNST), lateral septum (LS), preoptic area (POA), anterior hypothalamus (AH), ventromedial hypothalamus (VMH), and midbrain areas including the periaqueductal gray (PAG; Newman 1999; Figure 1). Activity in the SBN has been implicated in the performance of reproductive and courtship behaviors, parental behaviors, and aggression in both sexes of several mammalian species (Newman, 1999).

The SBN has important connections and some overlap with the mesolimbic reward system, and the two together have been referred to as a Social Decision-making Network (SDN; O'Connell & Hoffmann, 2011, 2012; Figure 1). In mammals, the POA, AH, VMH, and PAG project onto the LS and MeA/mBNST, which are part of both systems and themselves project onto other reward centers such as the ventral tegmental area (VTA), nucleus accumbens (NAcc), basolateral amygdala (blAMY), and ventral pallidum (VP; O'Connell & Hoffmann, 2011).

Though the structures in the SDN, their interconnections, and their neurochemical homologies have been extensively mapped across vertebrate species (O'Connell & Hoffmann, 2012), data on the functioning of the system in mediating specific social behaviors is less widespread taxonomically. Specifically, there is not nearly as much known about how the SDN functions in reptiles compared to mammals, birds, and teleost fish (Goodson, 2005). Among reptiles, exploring the SDN in snakes is particularly likely to expand our understanding of the

network's function, for two reasons: snakes have complex chemosensory systems and form simple social structures.

Table 1: List of abbreviations

AC: Anterior commissure	OpC: Optic chiasm
AOT: Accessory olfactory tract	OpTr: optic tract
AH: Anterior hypothalamus	PAG: Periaqueductal gray
AOB: Accessory olfactory bulb	PH: Posterior hypothalamus
blAMY: Basolateral amygdala	PLC: Posterior lateral cortex
DC: Dorsal cortex	POA: Preoptic area
DLAC: Dorsal lateral anterior cortex	S: Septum
DVR: Dorsal ventricular ridge	SD: Dorsal septal nucleus
HIP: Hippocampus	Si: Nucleus septalis impar
LFB: lateral forebrain bundle	SL: Lateral septal nucleus
LH: Lateral hypothalamus	SM: Medial septal nucleus
LS: Lateral septum	SO: nucleus supraopticus
mBNST: Medial bed nucleus of the stria terminalis	Str: Striatum
	SBN: Social behavior network
mPOA: Medial preoptic area	SDN: Social decision-making network
MeA: Medial amygdala	SOC: Supraoptic commissure
MC: Medial cortex	VA: Ventral amygdala
NAcc: Nucleus accumbens	VLAC: Ventral lateral anterior cortex
nAOT: nucleus of the accessory olfactory tract	VMH: Ventromedial hypothalamus
	VNS: Vomeronasal system
NS: Nucleus sphericus	VP: Ventral pallidum
OS: Olfactostriatum	VTA: Ventral tegmental area

Snakes interact with their environments – both social and non-social – primarily via odor. Animals that rely on chemosensory information often have two processing systems – the vomeronasal system (VNS) and the olfactory system. Although these systems converge in higher-order brain structures that are highly conserved across vertebrate species (Halpern & Martinez-Marcos, 2003), early processing happens separately. In snakes, which have the most well developed VNS of any vertebrate, the VNS is more specialized than the olfactory system for processing social cues (Halpern & Martinez-Marcos, 2003). Additionally, research on a wide array of species including mice, rats, opossums, hamsters, salamanders, and snakes has revealed

that chemosensory systems play an important role in facilitating numerous social behaviours such as mating, recognition, aggression, and territory marking (see Halpern & Martinez-Marcos, 2003, for review). Supporting this finding, there is overlap between brain regions connected to the VNS processing pathway and the SBN (Figure 1). For example, the MeA, where vomeronasal and olfactory information converge, is important for male recognition of familiar females in mice, and offspring recognition in female sheep (Bielsky & Young, 2004). Additionally, information from the VNS is relayed through structures such as the dorsal ventricular ridge (DVR; Lanuza & Halpern, 1997), olfactostriatum (OS; Martinez-Marcos et al., 2005b), and possibly the ventral amygdala (VA; Bruce & Neary, 1995) to the hypothalamus (i.e, to the SBN; O'Connell & Hoffman, 2011; Halpern & Martinez-Marcos, 2003), which plays a key role in social behaviours such as mating and aggression (Veening et al., 2005).

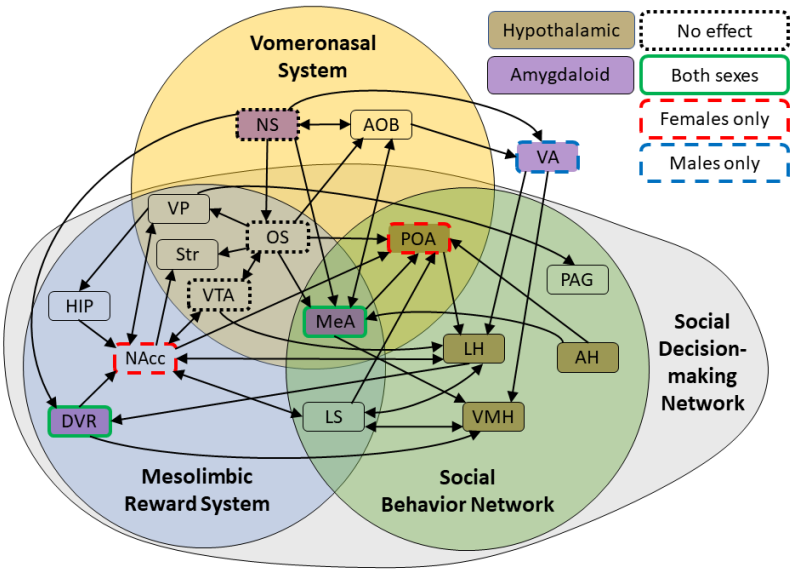

Figure 1: organization of the vomeronasal system (VNS; yellow shaded circle), mesolimbic reward system (blue shaded circle), and social behavior network (SBN; green shaded circle), and major connections of the brain regions that constitute them. Only some connections are shown, for legibility. The SBN and mesolimbic reward system together are referred to as the social decision-making network (SDN; grey shaded area). Amygdaloid brain regions are shaded purple; hypothalamic regions are shaded brown. Areas that we imaged have thick borders: black dotted borders indicate we found no effect of social interaction on cFos expression, dashed borders indicate an effect in one sex only (red for females, blue for males); and solid green borders indicate an effect in both sexes. See Table 1 for abbreviations.

Elucidating the precise functioning of the SBN is complicated both because all the participating structures appear to be engaged by most social behaviors to various degrees (as suggested by Newman, 1999), and due to the wide range of mammalian social behaviors that the network is implicated in, from mating and parental care to aggression. Snakes, however, form

simpler social structures than most mammals. With rare exceptions, snakes do not provide parental care to their young (Shine, 1988), mate seasonally, and do not engage in collective hunting. The social behaviors snakes do exhibit are generally less elaborate than those of mammals or birds, and their brains are correspondingly simpler, especially in cortical areas (Halpern, 1980). Snakes may therefore serve as an excellent model for examining the basic functions of the SDN, when activated primarily by one sensory modality (olfaction) and in aid of relatively simple social behaviors.

Here, we examined *c-fos* expression in response to social interactions in ball pythons (*Python regius*), focusing on regions of the SDN. Social interactions consisted of sharing a cage with a same-sex conspecific for one hour (control snakes spent one hour alone in a cage). Ball pythons do not hibernate – a common driver of social aggregation in other snake species (Gregory, 1984) – and are solitary ambush predators. They are not territorial (Webb et al., 2015), mate seasonally (Shine, 2003), and show little fear-based aggression (Brashears et al., 2020). They are nocturnal, so we conducted our social interactions in darkness, ensuring that the only sensory information available to activate the SDN was olfactory or somatosensory. Using exclusively same-sex pairings also controlled for the possibility of mating behavior-related activation, as opposite-sex interactions have been closely tied to VNS activity (see below). Thus, any activation of the SDN in response to social interaction in our snakes will reflect the basal activity of the system, distinguishing social from non-social events.

The snake VNS is important for processing a variety of stimuli including prey, predator, and conspecific chemical cues (Halpern 1987, Halpern & Martinez-Marcos, 2003; Graves & Duvall, 1985; Kubie & Halpern, 1979; Miller & Gutzke, 1999). The VNS has also been shown to play an important role in facilitating both sexual and non-sexual social behaviour (Halpern,

1987). For example, gartersnakes (*Thamnophis sirtalis*) that had their VNS detached had difficulty locating conspecific aggregations (Heller & Halpern, 1982) and blocking the VNS of male adders (*Vipera berus*) abolished species-typical male-male contests over access to females (Andren, 1982). Despite the importance of the VNS in facilitating social interactions, there has been no research on the recruitment of higher-order VNS structures during non-agonistic same-sex social interactions in snakes. Animals that rely heavily on chemosensory perception for processing may make excellent models for such research. The current study, by helping to locate socially-mediated activity in the snake brain, is a first step in exploring the functional role of vomeronasal social inputs to the activity of the SDN.

Despite the similarities noted above between the snake SDN and those of other vertebrates, squamate brains have several unique structures that appear to be part of both the SDN and VNS: the nucleus sphericus (NS), the olfactostriatum (OS) and the dorsal ventricular ridge (DVR; Figure 1). The NS is an amygdaloid structure present in the brains of many reptiles which is thought to be homologous to the mammalian posteromedial cortical amygdala (Martínez-García et al., 2002). The NS is a prominent structure in the squamate amygdala and the size of the NS has been hypothesized to reflect the importance of vomeronasal perception for a species (Lanuza & Halpern, 1997). The OS is thought to be a subdivision of the NAcc involved in processing the reward value of vomeronasal stimuli (Lanuza & Halpern, 1997; Martinez-Marcos et al., 2005a, 2005b). Efferent connections from the OS overlap heavily with efferent connections from the NAcc proper (Martinez-Marcos et al., 2005b) and include the VP, the medial forebrain, the POA, lateral posterior hypothalamic nucleus, various amygdaloid structures including the MeA/BNST, and the VTA (Martinez-Marcos et al., 2005b). Finally, the DVR is an

area of sensory information convergence that may be homologous to the mammalian neocortex (Aboitiz, 1999) or basolateral amygdala (Lanuza et al., 1998).

It is notable that many of the structures described above are involved in both the VNS and the putative SDN in snakes. The higher-order vomeronasal pathway in snakes has been mapped out using gartersnakes as model animals (Lanuza & Halpern, 1997; Martinez-Marcos et al., 2005a, Martinez-Marcos et al., 2005b). Vomeronasal information is first processed by the accessory olfactory bulb (AOB), followed by the NS and then the OS (Lanuza & Halpern, 1997; Martinez-Marcos et al., 2005b). Similarly, many structures commonly considered part of the mesolimbic reward system also show connectivity to the VNS pathway. In particular, the NAcc and the VTA are important in social reward (Hung et al., 2017; Dölen et al., 2013). Due to this overlap between the VNS and the reward system, it has been suggested that conspecific chemosensory cues may be rewarding to some species (Martinez-Marcos et al., 2005a). In support of this assertion, research has confirmed that chemosensory stimuli from the opposite sex can be intrinsically rewarding in both mice and male Syrian hamsters (Trezza et al., 2011), and gartersnakes display a preference for spending time near conspecific odors (Skinner & Miller, 2020). As snakes rely heavily on their VNS for the processing of social cues, and due to the interconnectivity between the VNS and the reward system, it seems likely that snakes find social stimuli rewarding, and that this motivates much snake social behavior.

To begin to understand the processing of social information in snakes, the current study explored two hypotheses. First, due to the highly developed VNS in snakes, we hypothesized that social information is processed by prominent vomeronasal structures such as the NS and OS. Additionally, we hypothesized that social information is also processed by the SDN. In particular, we expected to find increased neural activation in structures that are important for

social behavior in other species, especially structures that overlap or facilitate communication between the VNS and the SDN.

Methods

Subjects and housing

Subjects were 19 ball pythons (13 females and 6 males), purchased from local breeders. Their ages ranged from 2-3 years at the time of testing. Snakes were individually housed in a snake rack (ARS-7030, ARS Caging, Indianapolis, IN) in translucent tubs (84 cm x 44.5 cm x 14.5 cm). Snakes were kept on forest floor bedding (Zoo Med Forest Floor Reptile Bedding) and had access to belly heat (33 °C) provided by heat tape (THGTape, Cornel's World, Calgary. AB), two medium reptile shelters (23 cm x 16 cm x 6.5 cm; Cornel's world), and two water dishes (11.5 cm x 7.5 cm; placed forward in the enclosure; 15 cm x 15 cm x 6 cm; Ziplock; placed over the heat tape). Snakes were kept on a 12h reverse light cycle (lights on from 7 pm to 7 am). The housing room was kept at an ambient 28 °C with humidity ranging from 50-70%. Snakes were fed one thawed rat weekly. Meal sizes were chosen based on the body size of the snakes and feeding frequency.

Procedure

Snakes were habituated in their own empty glass terrarium (77 cm x 32 cm x 32 cm) with a metal mesh lid for one hour each day for three consecutive days prior to testing. In order to facilitate habituation, the terrariums were not cleaned between the habituation or testing trials. This is important for snakes, as they may dishabituate to clean enclosures (Chiszar et al., 1980). No snakes defecated or expelled urate in the terrarium during habituation or testing. On the fourth day, snakes in the Control condition (7 females; 3 males) were exposed to the same

conditions as habituation (spending one hour alone in the terrarium), whereas snakes in the Social condition (6 females; 3 males) were exposed to a same-sex, novel conspecific for one hour in the same terrarium. We designated one male snake and one female snake as the 'social exposure' snakes, so that all subjects in the Social condition received the identical same-sex snake as their partner. As ball pythons are nocturnal or crepuscular, habituation and testing trials started one hour after the beginning of the dark phase. Following the test trial, snakes were returned, alone, to their home cages for 1 hour, after which they were perfused and their brains removed (see below). Three snakes were tested each week over a 6-week period.

Immunohistochemistry

Ball pythons were anaesthetized with an overdose of sodium pentobarbital (100 mg/kg body weight). They were perfused transcardially with 500 ml of 0.1 M Phosphate Buffered Solution (PBS), pH 7.3, followed by 500 ml of 4% paraformaldehyde dissolved in 0.1 M PBS, pH 7.5. Brains were removed from the cranium and cryoprotected in phosphate buffer containing 20% sucrose overnight at 4℃, snap frozen on dry ice and stored at -80℃ until sectioned. Brains were sectioned coronally at 30 μm thickness using a cryostat, mounted on gelatin-coated slides, placed in order (anterior to posterior), and allowed to dry. The tissues were submerged in 0.6% H_2O_2 (600 μl H_2O_2.:30 ml PBS) for 30 minutes to block endogenous peroxidases. Following this, the sections were blocked in 10% Normal Goat Serum in PBS from the Vector ABC Rabbit Kit (MJSBioLynx, Brockville, ON) for 30 minutes. Tissues were submerged in a solution of primary antibody (rabbit polyclonal anti c-Fos antibody F7799; Sigma, Oakville, ON) in 10 ml of PBS with 1% normal goat serum (antibody concentration 1:5000) and stored in a humidity chamber for 48 hours at 4 ℃. Following the primary antibody, the tissue was treated with the secondary antibody from the Vector ABC Rabbit Kit at room temperature for 3 hours. The avidin-biotin-

peroxidase complex (Vector ABC Rabbit Kit) was applied to the brains for 2 hours at room temperature. To visualize the Fos-antibody peroxidase complex, 3,3'-diaminobenzidine (DAB) staining was applied (0.4% DAB, 0.0004% H_2O_2in PBS) for 5 minutes. After each step of the process, the sections were submerged twice in PBS with 0.1% Triton X for 5 minutes. After visualization, the sections were allowed to dry, and were coverslipped using Permount mounting medium.

Antibody characterization

The anti-cfos antibody was produced using a synthetic peptide corresponding to the N-terminal region of human c-Fos. The immunohistochemistry potential of the antibody was confirmed using formalin fixed paraffin embedded human colon carcinoma tissue at a 1:5000 concentration (manufacturer's information). Published studies have obtained similar results using this antibody (e.g., Poller et al., 2022; Umezu et al., 2021), and similar antibodies have been used to identify c-Fos in zebrafish (*Danio rerio*; Ruhl et al., 2017), salamanders (*Plethodon shermani*; Laberge et al., 2008), and a different species of snake (*Bothrops jararaca*; Zambotti-Villela et al., 2007). In addition, blasting the human, Indian cobra (*Naja naja*; Suryamohan et al., 2020), and Burmese python (*Python bivittatus*; Castoe et al., 2013) c-Fos sequence found that the antigen peptide used to make the antibody is highly conserved.

Microscopy

Sections were evaluated under a microscope. Brain regions were primarily identified using Halpern (1980) and Smeets (1988). Images of the brain regions of interest were captured using a digital microscope camera (Olympus XM10) by a single researcher blind to the conditions and sex of the snakes (DD). Pictures were taken so that each image only contained the

area of interest at 20x or 40x magnification. Pictures were all taken at the same, approximately central, area for each brain region. Pictures of a brain area were all taken at the same magnification. The best quality image per brain area per snake was used. CellProfiler v 4.2.1 (Stirling et al., 2021) was used to process the images (the CellProfiler pipeline is available for download through our data repository). The *analyze particle* function in ImageJ v 1.53k (Schneider et al., 2012) was used to count Fos immunoreactive (Fos-IR) cells. The parameters for identifying Fos-IR cells were determined by taking the area, circularity, and solidity of a subset of 100 randomly sampled cells determined to be Fos-IR. An identical processing procedure was used for all images. All Fos-IR nuclei in each image were counted.

Statistical analysis

All statistical analyses were conducted using R v 4.0.2 (R Core team, 2022) using the *MASS* package. We ran statistical analyses on each brain region separately. We used generalized linear models to model the cell counts. As Poisson distribution models were over dispersed, we used negative binomial distributions and therefore report the odds ratios (OR) for finding Fos-IR across conditions, with an odds ratio of 1 indicating that Fos-IR were equally likely in both conditions. To find the models that best fit our data, we built them progressively. We started with an intercept only model then added Condition (Control vs. Social), followed by Sex, followed by a Condition by Sex interaction as predictors. We compared models using the Akaike Information Criterion (AIC) and, in all situations, we report the model with the lowest AIC for each brain region. As the same two stimulus snakes were used for all trials, it is possible that a change in their behavior over testing days could affect the test snake. To account for this, we looked for order effects in test snakes in the social condition by running an overall mixed effect model

which included 'order of testing', brain area, and the interaction as fixed effects. Snake identity was included as a random intercept. An ANOVA was run on the model to get the overall effects.

Results

For the major secondary vomeronasal structure, the NS, the best model contained both Condition and Sex (Figure S1A). The amount of Fos-IR expression in the NS was not significantly higher in the Social condition than the Control condition (OR = 1.2, 95% CI [0.89, 1.63], p = 0.233). The amount of Fos-IR expression in male snakes was marginally lower than in female snakes (OR = 0.72, 95% CI [0.51, 1.02], p = 0.061). However, this model was not significantly better than the intercept only model. For the OS, the best model was the intercept only model (Figure S1B). The intercept only model was also the best model for the other major reward structure along the vomeronasal pathway, the VTA (Figure S1C). Differential Fos-IR expression was found in the NAcc. For the NAcc, the best model contained both Condition and Sex such that snakes in the Social condition had marginally higher amounts of Fos-IR expression than snakes in the Control condition (OR = 1.39, 95% CI[0.99, 1.97], p = 0.058) and male snakes had significantly reduced amounts of Fos-IR expression compared to female snakes (OR = 0.56, 95% CI[0.38, 0.84], p = 0.004). Although the best model for the NAcc did not contain a Sex by Condition interaction, inspection of the mean cell counts suggested that differences in Fos-IR rates were driven primarily by female snakes (Figure 2A).

Higher-order vomeronasal structures that demonstrated significant differences in the amount of Fos-IR expression included the MeA (Figure 2B) and the POA (Figure 2C). For the MeA, the best model only included Condition, with snakes in the Social condition having a significantly higher rate of Fos-IR expression than snakes in the Control condition (OR = 2.24, 95% CI[1.46, 3.47], p < 0.001). For the POA, the best model had a significant interaction

between Condition and Sex (OR = 0.34, 95% CI [0.14, 0.83], p = 0.016). The interaction was such that female snakes in the Social condition had higher rates of Fos-IR than female snakes in the Control condition but male snakes did not differ across conditions. There was also a main effect of Condition, with snakes in the Social condition having more Fos-IR expression than snakes in the Control condition (OR = 2.16, 95% CI[1.39, 3.37], p < 0.001) but no main effect of Sex (OR = 1.32, 95% CI[0.75, 2.33], p = 0.34).

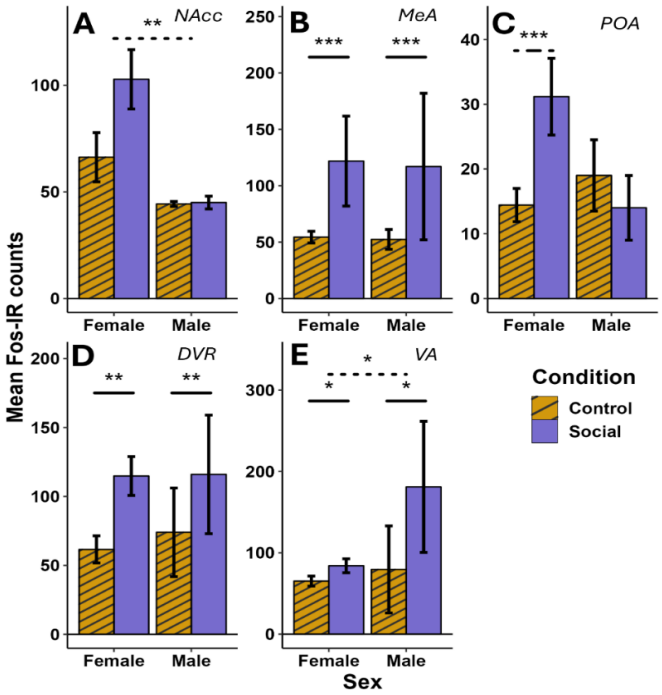

Figure 2. Mean Fos-IR counts for the Nucleus Accumbens (A), Medial Amygdala (B), Preoptic Area (C), Dorsal Ventricular Ridge (D), and Ventral Amygdala (E), displayed by Sex and Condition. Solid, dotted, and dot dashed horizontal lines represent effects of Condition, Sex, and an interaction between the two, respectively. * p < .05, ** p < .01, *** p < .001. Error bars are ± SE.

We also found significant differences in Fos-IR expression in the DVR (Figure 2D) and the VA (Figure 2E). For the DVR, the best model had a significant effect of Condition with snakes in the Social condition having a more Fos-IR than snakes in the Control condition (OR = 1.14, 95% CI[0.64, 2.05], p = 0.007). The best model for the VA had both Condition and Sex as significant effects. In the VA, snakes in the Social condition had more Fos-IR expression (OR = 1.52, 95% CI[0.99, 2.33], p = 0.048) and male snakes had higher amounts of Fos-IR than Female snakes (OR = 1.70, 95% CI[1.08, 2.73], p = 0.022). Examples of Fos-IR are shown in Figure 3.

There was no effect of testing order ($\chi^2_1 = 0.11$, $p = 0.735$) nor was there an interaction between order of testing and brain area ($\chi^2_7 = 8.24$, $p = 0.312$).

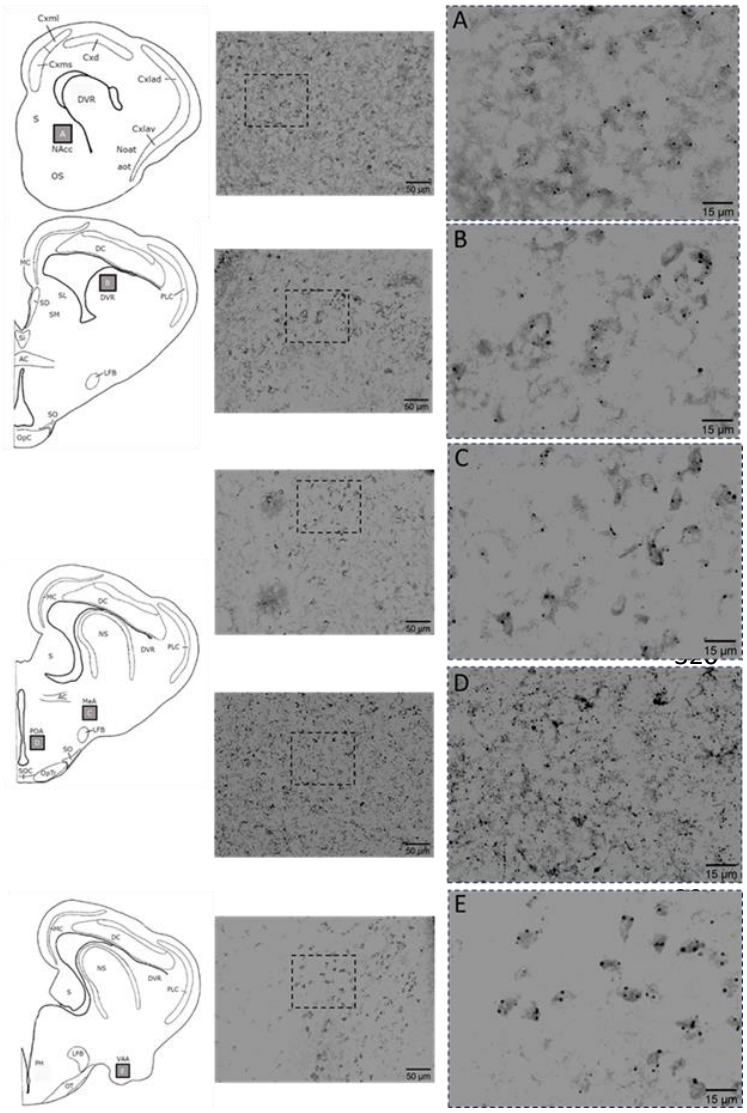

Figure 3. Representative tracings of brain slices and photomicrographs of Fos-IR from ball pythons that had a 1 hr social experience with a same-sex conspecific . Photos come from the approximate location of the labelled grey squares on the tracings to the left. The images correspond as follows: A nucleus accumbens, B dorsal ventricular ridge, C medial amygdala, D preoptic area, E ventral amygdala. Tracings are adapted from Smeets (1988). See Table 1 for all abbreviations.

Discussion

We allowed ball pythons to interact for one hour in the dark with a same-sex conspecific and examined Fos-IR rates in a range of structures along their vomeronasal pathway and in their social decision-making network (SDN). We hypothesized that social interaction – which is largely mediated by odor cues in snakes – would increase activity in prominent ophidian vomeronasal structures, SDN structures, or structures that are part of both systems. We did not

find differences between the Social and Control conditions in secondary and tertiary vomeronasal structures (the NS and OS), but did find increased Fos-IR counts in areas associated with both the VNS and SDN, such as the MeA, as well as areas that facilitate communication between the systems, such as the DVR (a locus of multisensory integration), VA, and POA. In addition, we found sex differences in response to social interaction, with female snakes tending to have more activity in the NAcc, and male snakes having marginally more activity in the VA.

P*rocessing of social interactions*

The neural structures that make up the ophidian vomeronasal pathway have been mapped using tracer injections (Lanuza & Halpern, 1997; Martinez-Marcos et al., 2005a, Martinez-Marcos et al., 2005b). These studies have shown that, after the AOB, the NS and OS are the major vomeronasal processing structures. Our data show no major differences between snakes in the Social or Control conditions in neural activation in either of these early higher-order vomeronasal structures. Instead, the largest differences we found were in later vomeronasal pathway structures that are also considered part of the SDN, including the MeA and POA (O'Connell & Hoffman, 2011). Interestingly, research using Fos-IR activity in mice has also shown patterns of neural activation in the MeA and POA in response to social stimuli (Halem et al., 1999; Paredes et al., 1998).

Our results suggest that the MeA and POA in ball pythons, rather than reptile-specific vomeronasal structures such as the NS and OS, are important for same-sex social interactions. Both the MeA and the POA are part of the social behaviour network (SBN) and, in other taxa, play an important role in regulating various social behaviours such as parental care, aggression, and mating (Goodson, 2005; Raam & Hong, 2021; Tsuneoka & Funato, 2021). Further neural-behavioural work targeting specific areas within the snake SDN will be required to fully

understand the role of these structures in snake social interactions. However, as a large body of research suggests that these structures are conserved across taxa (Goodson, 2005), we can extrapolate from data on other species to offer an interpretation of our results.

The extended MeA has been implicated in numerous affiliative and avoidant social behaviours (Newman, 1999; Goodson et al., 2005; Raam & Hong, 2021) and this area is known to react to the presentation of social stimuli (Ball & Balthazart, 2001; Newman, 1999). The MeA is also known to facilitate the encoding of a conspecific's identity during a social interaction (Ferguson et al., 2001). As ball pythons cannot share food and males are thought to compete for mating opportunities, higher rates of Fos-IR in the MeA of socially interacting snakes in our data may indicate encoding the identity of a potential competitor. In many species, the ability to identify conspecific competitors is an important aspect of social interactions (see Grether, 2011, for review) and this may require significant neural resources in species with relatively sparse social encounters, as conspecific identity information must be retained for longer and is recalled less often. Alternatively, increased MeA activation may facilitate more general conspecific approach or avoidance behavior (Goodson et al., 2005). Related research on songbirds has demonstrated that less gregarious birds have increased neural activation in the MeA compared to more gregarious species (Goodson et al., 2005). Future research should determine if less gregarious snakes show more MeA activation than more gregarious snakes.

Research on rodents has shown that the POA is recruited in the social investigation of both same- and opposite-sex conspecifics (Wei et al., 2018). This would explain the higher rates of Fos-IR we found in the POA of socially interacting female snakes. We did not find a difference across conditions in males. Sexual dimorphism in the structure and function of the POA is common across species (Wei et al., 2018). Research on gartersnakes has shown that the

POA is important for courtship behaviour in male snakes (Krohmer, 2004). Therefore, ball pythons might also display functional sexual dimorphism in the POA with male ball pythons strictly relying on the POA for opposite-sex rather than same-sex social interactions. However, these sex differences should be treated with caution as our sample of male snakes was small. Additionally, as ball pythons are sexually dimorphic, with males smaller than females, it is possible that sex differences in neural density could have influenced rates of Fos-IR.

We found that both male and female snakes in the Social condition had higher Fos-IR counts in the DVR than snakes in the Control condition. It has been suggested that the DVR in reptiles is homologous to the mammalian basolateral amygdala, as it is an important convergence point for both vomeronasal and olfactory information (Lanuza et al., 1998). Thus, differential activation of the DVR in the Social condition may be the result of the multisensory nature of our social stimuli. We found significantly higher rates of Fos-IR in the VA of male snakes compared to females. Additionally, there was a marginal difference in the rates of Fos-IR between the Social and Control conditions in this area. Although there is a paucity of research on the function of the VA in reptiles, research has shown that it is highly connected to areas of the SBN (i.e., VMH & LH; Bruce & Neary, 1995; Figure 1) that are important for social approach and avoidance behaviors (VMH; Hashikawa et al., 2017; Falkner et al., 2014; LH; Nieh et al., 2016). As such, increased activation of the VA in our Social condition may indicate a social-approach/avoidance response to a novel competitor (Nieh et al., 2016), which is more pronounced in male snakes. However, more research is needed on both the role of the VA in processing social information in reptiles and ball python social behavior generally.

Reward processing

We hypothesized that, if social interaction is rewarding to ball pythons, it would activate mesolimbic reward structures. We did not find differential Fos-IR counts in the reward structures that overlap the SBN and the VNS, such as the OS (a hypothesized substructure of the NAcc in snakes), or the VTA. Instead, we found higher Fos-IR counts in the NAcc of female snakes in the Social condition. This suggests that, as in other animals (Halpern & Martinez-Marcos, 2003), social cues may be rewarding to ball pythons, even those resulting from same-sex interactions. By this explanation, non-mating aggregations of snakes may be driven by mutual attraction to female snakes, as has been previously suggested for gartersnakes (Skinner & Miller, 2022).

Conclusion

Many animals rely on chemosensory stimuli to mediate social interactions. Of those animals, snakes have a highly specialized VNS with unique higher-order structures, such as their extensive nucleus sphericus and olfactostriatum. Here we show that these structures are unlikely to differentiate social cues. Instead, areas of the social decision-making network that are highly conserved across taxa are more likely candidates for social perception in snakes. Although our findings align strongly with other research on brain areas important for social interaction, we note that our testing paradigm cannot differentiate between the processing of novel conspecific-related cues and the processing of a novel stimulus generally. Future research should dissociate these, and the role of novelty in snake social interactions. Most of the research on the structure and function of the brain's social interaction networks has involved highly social animals. Unlike more typically studied social animals, snakes are often considered non-social and do not appear to form permanent social groups. Our research adds to a growing body of literature on socially induced fos expression in 'less-social' animals (Kollack-Walker & Newman, 1995; Goodson et al., 2005). This research suggests social brain functions may have originally evolved to solve

simple social problems such as recognition of potential competitors or mates, and whether to approach or avoid a conspecific. To what extent subtle differences in the structure and function of brain areas within the SDN correspond to differences in social behaviour across taxa will require functional explorations of the social brain in a broad sample of species with different social systems.

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Raw Data: All the data reported in this paper are archived at https://osf.io/gez38/?view_only=601b7f7dd62a43f99ae8d1c28685181e

Ethics Approval: All experimental procedures conformed with Canada Council on Animal Care guidelines and were approved by the Wilfrid Laurier University Animal Care Committee (AUP R21003).

Conflicts of interest: The authors declare no conflicts of interest.

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638 **Supplementary Information**

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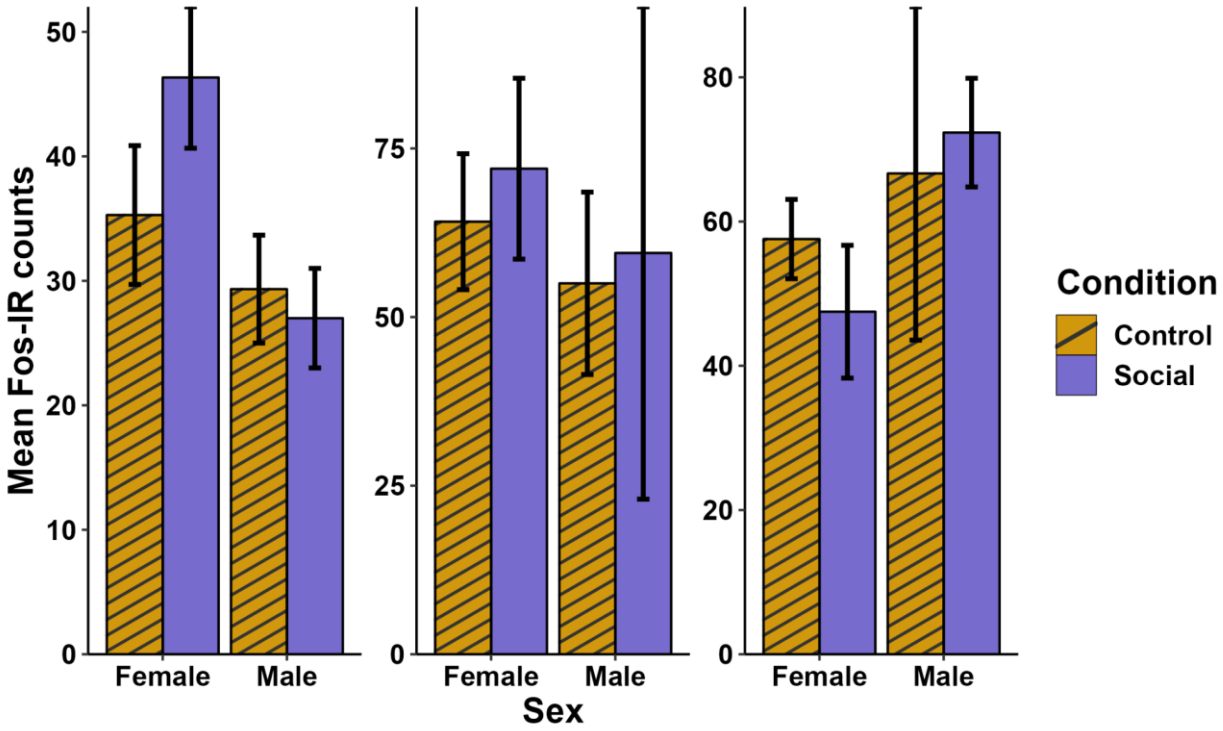

640 **Figure S1.** Mean Fos-IR counts for the NS (A), OS (B), and VTA (C) displayed by Sex and Condition. Error
641 bars are ± SE.