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Running title: Effects of mesotocin on hen preference in chicks

# Effects of oxytocin-family peptides and substance P on locomotor activity and filial preferences in visually naïve chicks

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## Abstract

Nonapeptides from the vasopressin/oxytocin family have been implicated in a wide variety of social behaviors across vertebrates. Experimental manipulations that alter nonapeptide levels or receptor function in the brain have provided evidence for understanding how nonapeptides influence responses to social stimuli in adults. While behaviors in adults have been extensively studied, much less in known about roles of nonapeptides in early life and the development of affiliative social behaviors. We examined an experience-independent preference (social predisposition) that is present at hatching and is characterized by the tendency of visually naïve chicks (*Gallus gallus)* to prefer to approach a stuffed-hen stimulus over a control stimulus in a choice test. Among chicks that show the social predisposition preference, bilateral intracranial mesotocin injections resulted in higher mean hen preference scores compared to saline-injected controls. Equimolar doses of mesotocin and vasotocin injections had different effects on locomotor activity: vasotocin, but not mesotocin, resulted in hypoactivity. We also tested whether intraperitoneal substance P had an effect on hen preference scores because previous research has proposed that vasotocin effects on social approach are mediated by peripheral release of substance P, but found no significant effect. All together, our data suggests that mesotocin signaling may be important for social predispositions and can potentially enhance the perceived salience of social stimuli soon after hatching. Specifically, mesotocin release and signaling in the brain may regulate the ability to recognize naturalistic stimuli and/or to act on the motivation to approach naturalistic stimuli.

**Introduction**

Mammalian neurohypophyseal hormones vasopressin and oxytocin and their avian homologs vasotocin and mesotocin have modulatory effects on a wide range of behaviors in adults, including courtship, partner preference, parental care, aggression, affiliative behavior and gregariousness [for reviews see (Goodson & Thompson, 2010; Albers, 2012, 2015; Kelly & Goodson, 2014; Ondrasek, 2016)]. While the effects of these nonapeptides on social behaviors have been most extensively studied in adults, recent work has highlighted the need to dedicate efforts towards understanding their roles in early life and in particular regarding the development of social behavior (Baran *et al.*, 2016, 2017; Di Giorgio *et al.*, 2016; Baran, 2017). The use of avian species has been outlined as valuable for understanding the diversity and evolvability of social systems in vertebrates due to the conserved distribution of specific vasotocin and mesotocin neuronal populations, as well as basic functional properties of these neurons (Goodson *et al.*, 2012; Goodson, 2013). Furthermore, substance P (SP) has been proposed as key for mediating the effects of vasotocin on social approach (Thompson *et al.*, 2008) and here we tested whether it had an effect during early stages of filial imprinting.

In newly hatched chicks of precocial bird species, successful filial imprinting is essential for survival: chicks imprint on their siblings and mother by forming a strong memory of them and demonstrate attachment with following behavior (Lorenz, 1937; Bateson, 1966, 1979; Bateson & Reese, 1969; Johnson & Horn, 1988; Bolhuis, 1991; Rosa-Salva *et al.*, 2015). Filial imprinting is influenced by two main factors: an

experience-independent predisposition to approach objects that resemble conspecifics and a robust ability to learn the distinguishing features of these objects [recently reviewed in (Rosa-Salva *et al.*, 2015; Versace & Vallortigara, 2015; Di Giorgio *et al.*, 2016)]. In a preference test, newly hatched, visually naïve chicks will preferentially approach a stuffed-hen (i.e. predisposed stimulus) over a less naturalistic version of a hen (i.e. a control stimulus or non-predisposed stimulus) and the existence of this innate '*social predisposition*' is thought to underlie this approach response, which does not require any previous experience with conspecifics (Fig. 1). While there has been extensive behavioral research on the topic of social predispositions in chicks (Horn & McCabe, 1984; Bolhuis & Trooster, 1988; Johnson & Horn, 1988; Bolhuis *et al.*, 1989; Hampton *et al.*, 1995; Bolhuis & Horn, 1997; Vallortigara *et al.*, 2005; Vallortigara & Regolin, 2006; Rosa-Salva *et al.*, 2010, 2015; Versace & Vallortigara, 2015; Mayer *et al.*, 2016; Mayer, Rosa-Salva, & Vallortigara, 2017; Mayer, Rosa-Salva, Morbioli, *et al.*, 2017; Versace *et al.*, 2017; Lorenzi *et al.*, 2017; Di Giorgio *et al.*, 2016; Mascalzoni *et al.*, 2010) much less is known about the neuronal and physiological mechanisms underlying this response. Nevertheless, recent studies in the domestic chick (*Gallus gallus)* have identified patterns of neural activation in visually naïve chicks during their first exposure to predisposed stimuli. For example, exposure to a live conspecific chick results in a greater number of c-Fos (an indirect marker of neuronal activation) expressing cells in the lateral septum, arcopallium and nucleus taeniae compared to controls, which were not exposed to any stimulus (Mayer, Rosa-Salva, & Vallortigara, 2017). Similarly, exposure to a live conspecific also elicits greater activation of the lateral septum and preoptic area compared to chicks that are exposed to a rotating stuffed chick (Mayer, Rosa-Salva, Morbioli, *et al.*, 2017) which demonstrates the involvement of these brain areas not

only in processing the first experience of seeing a conspecific, but also in detecting motor patterns unique to biological motion.

It is reasonable to speculate that during the sensitive period for imprinting, regulation of the hypothalamic-pituitary-adrenal (HPA) axis that facilitates or favors interactions with conspecifics is desirable. Nonapeptides have well established regulatory effects on the HPA axis, which makes them interesting candidates to understand whether they are important behavioral modulators during the very first social interactions within the sensitive period for imprinting. On the one hand, both oxytocin and mesotocin inhibit HPA axis activity (Neumann *et al.*, 2000); on the other hand, corticotropin releasing hormone combined with either vasopressin or vasotocin activate the HPA axis (Baker *et al.*, 1996; Kuenzel & Jurkevich, 2010). In fact, prenatal corticosterone treatment injected into eggs before incubation has been show strengthen the preference for predisposed stimuli upon hatching (Nordgreen *et al.*, 2006). The process through which imprinting occurs has been well-characterized with regards to behavior as well as neural changes that occur as a result of memory formation [reviewed in (Bolhuis, 1991; Horn, 1998; Solomonia & McCabe, 2015)]. The sensitive period during which imprinting occurs is highly amenable to address questions about how nonapeptides might influence the perception and response towards social stimuli soon after hatching, a research topic that is largely unexplored (Martin & van Wimersma Greidanus, 1978; Insel & Fernald, 2004; Rosa-Salva *et al.*, 2015; Baran *et al.*, 2016; Di Giorgio *et al.*, 2016).

The lateral septum, preoptic area and amygdala are part of the social behavior network (SBN) which comprises bidirectionally interconnected nodes that in adults are rich in sex steroid receptors and are known to be activated during an array

of social behaviors in vertebrates (Newman, 1999; Goodson, 2005; O'Connell & Hofmann, 2011). Four avian nonapeptide receptors are known and the nomenclature of the corresponding mammalian receptors based on homology is listed in Table 1. Notably, telencephalic brain nuclei that show greater activation with exposure to predisposed stimuli have also been documented to have enriched expression of mRNAs for nonapeptide receptors in the white-throated sparrow and zebra finch (Leung *et al.*, 2011) as well as in the rock dove, European starling and house sparrows (Ondrasek *et al.*, 2018).

In rodents, many studies have shown that oxytocin and vasotocin are important for social recognition, as well as for establishing and maintaining partner preference (i.e. monogamy vs. promiscuity (Ferguson *et al.*, 2000, 2001; Insel & Young, 2000; Bielsky & Young, 2004; Young & Wang, 2004; Bielsky *et al.*, 2005). Despite important advances in our understanding of how and where nonapeptides in the brain mediate social recognition in mice and partner preference in voles, in birds the evidence for mechanistic overlaps for the same or similar behaviors is less clear. For example, in male chickens, more vasotocin neurons in the medial part of the bed nucleus of the stria terminalis (BSTM) are activated, as indicated by colocalization with c-Fos, during appetitive (i.e. courtship) but not consummatory sexual behaviors (Xie *et al.*, 2011) suggesting these neurons may be part of a circuit for courtship and responsive to the visual stimulus of the opposite sex. On the other hand, in the zebra finch, bilateral injections of vasotocin, mesotocin, a vasopressin antagonist and an oxytocin antagonist aimed at the lateral ventricle had no effect on partner preference, intersexual affiliation or male courtship singing (Goodson, Lindberg, *et al.*, 2004). There is evidence, however, that in zebra finches, vasotocin (AVT)

neurons in the BSTM strongly promote the tendency of these birds to prefer large groups (Kelly *et al.*, 2011). Moreover, density of the VT3 receptor (see Table 1) increases in the winter in the field sparrow and dark-eyed junco, species that flock during this season (Wilson *et al.*, 2016). All together, although in male chickens BSTM AVT neurons respond to social stimuli of positive valence this appears to be restricted to courtship, and VT3 receptor density increases during seasonal flocking in songbirds and this relies on affiliative behavior, whether and how these findings relate to early affiliative social experiences is unknown.

In the domestic chick (*Gallus gallus)* both the lateral septum and the avian partial homolog of the medial amygdala, the nucleus taeniae, are selectively responsive to features of predisposed stimuli (Lorenzi *et al.*, 2017; Mayer, Rosa-Salva, & Vallortigara, 2017) and these brain nuclei are known to express nonapeptide receptors in several species, including the chicken (Goodson *et al.*, 2009; Leung *et al.*, 2011; Selvam *et al.*, 2015; Wilson *et al.*, 2016; Ondrasek *et al.*, 2018), Therefore, it is reasonable to suggest that nonapeptide signaling in these brain nuclei may be also be important for initiating the process of imprinting. To our knowledge, there are only three studies in birds that involve the manipulation of vasotocin levels early in development (Martin & van Wimersma Greidanus, 1978; Baran *et al.*, 2016, 2017). Newly hatched mallard ducklings given an intraperitoneal injection of desglycinamide lysine vasopressin (DG-LVP), a vasopressin homolog that lacks pressor and anti-diuretic effects, demonstrate a delay to approach an imprinting stimulus compared to saline injected controls (Martin & van Wimersma Greidanus, 1978). In that study however, the imprinting stimulus used was a red box,

so whether this effect could extend to the case of predisposed stimuli such as a conspecific chick or stuffed hen is unknown.

In a more recent study, zebra finch hatchlings were given daily bilateral intracranial injections from days 2-8 post hatching, of either vasotocin, Manning compound (a strong V1a receptor antagonist and weak antagonist for the avian VT3 receptor, see Table 1) or saline, and later on tested as juveniles and adults for their affiliation to parents and opposite-sex conspecifics, respectively (Baran *et al.*, 2016). Interestingly, hatchlings that received vasotocin injections showed an increased preference for their parents compared to controls, whereas those that had received Manning compound failed to show the typical expected affiliation to their parents. Furthermore, as adults, birds that had been injected with Manning compound as hatchlings did not show an increased affiliation for the opposite sex once they reached adulthood. These results are quite remarkable and suggest that the social bond between offspring and parent may also serve developmentally as a requisite that later on allows the development of species-specific mate preferences at the onset of sexual maturity. In fact, zebra finches that are raised by Bengalese finches during the first 40 days of life will prefer to mate with Bengalese finches instead of with conspecifics (Oetting *et al.*, 1995) which suggests that exposure during the early stages of life could also be part of an acquisition stage for sexual imprinting.

Lastly, there are several models of how vasotocin might affect specific social behaviors and these models vary by major animal taxa as well as among species. One model based on goldfish, proposes that vasotocin neurons form part of a primitive social circuit that regulates social approach through projections to substance P (SP) neurons in the hindbrain (Thompson & Walton, 2004; Thompson

*et al.*, 2008). Thompson et al. (2008) demonstrated that AVT effects on social approach were dependent on peripheral release of SP from hindbrain-originating neurons. This mechanistic explanation has received renewed attention wherein the need for further studies to be conducted on non-teleost species to test how widespread this phenomenon may be across vertebrates is emphasized (Ondrasek, 2016). Indeed, SP has been documented to have behavioral effects on learning and memory (Hasenöhrl *et al.*, 1994) and responses to novelty (Kalivas *et al.*, 1996).

In birds, SP has also been shown to be linked to singing, specifically, intensity of SP-immunoreactivity in song nuclei of the telencephalon correlates with singing behavior and repertoire size (Li *et al.*, 2006). The distribution of SP neurons and immunoreactivity has also been discussed in regards to song learning (Doupe *et al.*, 2005) and the evolution of food-storing in birds (Gould *et al.*, 2001). Immunohistochemical mapping of SP cells and fibers have been generated for several mammalian species [reviewed in (Anderson & Reiner, 1990)], reptiles (Brauth *et al.*, 1983) as well as for many bird species (Creubi & Jessell, 1978; Reiner *et al.*, 1983; Davis & Cabot, 1984; Nemeroff *et al.*, 1984; Ball *et al.*, 1988; Anderson & Reiner, 1991; Gould *et al.*, 2001; den Boer-Visser & Dubbeldam, 2002; Nair-Roberts *et al.*, 2006) demonstrating its wide distribution throughout the brain. Furthermore, a conserved vertebrate neuroanatomical and neurochemical feature of basal ganglia organization based on a comparative study on pigeon, rat and turtle brains is that the vast majority of striatal SP-producing neurons with striatonigral (SN) projections also co-express dynorphin (Anderson & Reiner, 1990). Thus, striatal SP neurons are a major input of dopaminergic substantia nigra (SN) neurons and SP application to the ventral tegmental area (VTA) enhances the activity of

dopaminergic cells in this area (Cador *et al.*, 1986). Although the involvement of a role for dopaminergic neurons in social predispositions has not been studied in detail, in the social predisposition test the stuffed hen is a social stimulus with positive valence and therefore a reward-associated response in chicks may underlie the preference for the stuffed hen. Therefore, given the documented interaction between SP release and vasotocin effects on social approach, as well as the conserved connectivity between SP neurons and dopaminergic neurons in the VTA and SN, we also tested whether intraperitoneal injections of SP had an effect on social predispositions in visually naïve chicks.

In this study, we had two main objectives. First, we tested whether altering endogenous levels of the nonapeptides vasotocin and mesotocin in the brain, immediately prior to being submitted to a social predisposition test, would have an effect on the preference for a stuffed hen over a control stimulus. We performed bilateral intracranial injections in newly hatched, visually naïve chicks and predicted that both nonapeptides would increase the hen preference scores compared to saline-injected controls. These predictions were based, in part, on previous research on zebra finches that showed that increasing vasotocin levels in the brain during the first days after hatching produced greater affiliative preferences for parents later in life (Baran *et al.*, 2016) and on evidence for a relationship between VT3 expression and gregariousness in estrilid finches (Goodson *et al.*, 2009). Second, we sought to test whether intraperitoneal injections would decrease hen preference in the social predisposition test, based on the concept that social approach is regulated by a so-called primitive circuit involving vasotocin and substance P interactions.

**Materials and Methods**

*Animals and hatching conditions*

One hundred ninety-eight laboratory-hatched, domestic chicks *(Gallus gallus)* of the Ross 308 strain were used. All chicks used for intracranial injections were male, and chicks used for intraperitoneal injections and in the intact condition were males and females. Fertilized eggs were obtained from a local commercial hatchery (Agricola Berica, Montegalda (VI), Italy) and kept in the dark in incubators (Marans P140TU-P210TU) at a temperature of 37.7°C, with 60% humidity, until hatching. All handling of eggs and hatchlings prior to testing was performed in complete darkness with the aid of night vision goggles.

Four days before hatching, eggs were arranged inside the incubator onto shelves, in batches of 15 eggs per shelf, such that after hatching all chicks were able to experience physical contact with one another. Within the first 24 hr after hatching the temperature was set to 33°C and chicks were submitted to acoustic stimulation for 4 hr in individual compartments inside incubators as described in Mayer et al. (2016) (Fig. 2A). This type of acoustic stimulation has been shown to promote chicks' response in the social predisposition test (Egorova & Anokhin, 2003). Chicks were tested in the running wheel on the second day of life (between 30-47 hr post-hatch) and injections were given five minutes before starting the social predisposition test (Fig. 2A). After testing, all chicks that had been given an injection of mesotocin, vasotocin, substance P or vehicle as described below (N = 160) were weighed and sacrificed according to approved protocols: chicks were given an injection of 0.05 mL of a 1:1 Ketamine/Xylazine Solution (Ketamine 10 mg/mL, Xylazine 2 mg/mL) per 10

g of body weight. Chicks that did not receive any type of injection (N = 38) were used to test the 'intact' condition of the social predisposition test and were donated to local farmers after behavioral testing concluded. The mean body weight (BW) of injection-treated chicks on the day of testing was (mean ± SEM = 39.72 ± 0.32 g).

*Ethics statement*

The experiments reported here comply with the current Italian and European Community laws for the ethical treatment of animals and the experimental procedures were licensed by the Ministero della Salute, Dipartimento Alimenti, Nutrizione e Sanità Pubblica Veterinaria (permit number 1139/15).

*Intracerebroventricular mesotocin and vasotocin injections*

Stock solutions of mesotocin (MT) (Alpha Diagnostics International, SP-100084-5, [Ile8]-Oxytocin) and vasotocin (AVT) (Sigma V130) were dissolved in water to 10 mM and 1 mM concentrations, respectively. Each drug stock solution was aliquoted into smaller volumes and stored at -20°C. Each vial was only thawed once on the day of testing to make the appropriate dose concentration using a 0.9% saline 0.1% Evans blue solution as vehicle; the dye was used to enable assessment of the location of injection and extent of diffusion. Injections were given using 10 μl Hamilton syringes, fitted with stoppers that would only allow a depth of 3 mm, aimed at the lateral ventricles using a custom built head holder device. Bilateral injections of 3 μl per hemisphere were given of either mesotocin (1 μg/3 μl; 1 nM), vasotocin (1 μg/3 μl; 1 nM) or vehicle. Dose was chosen based on (Tachibana *et al.*, 2004; Masunari *et al.*, 2013, 2016). Each syringe was only used to give a specific treatment (MT, AVT, vehicle) and syringes were always rinsed with water between chicks.

Briefly, the procedure was as follows. Lidocaine was applied to the top of the head 5 min before performing injection. The syringe was filled to 7 µl, the chick was removed from the box and its head was placed inside the device which had guide tracts for the syringe needle, the first 3 µl were administered, then 1 µl was dispensed and discarded to ensure the needle was not clogged, and then the second 3 µl were administered to the contralateral hemisphere. The whole injection procedure took approximately 30 secs or less and a previous study has showed that this delivery method does not increase corticosterone levels (intracerebroventricular saline compared to intact non-injected chicks) (Saito *et al.*, 2005). During the entire procedure, care was taken to cover the eyes of chicks to prevent them from seeing the face of the experimenter given chicks have a preference for face-like configurations (Rosa-Salva *et al.*, 2010); in this way we made sure that the first features they saw were those of the two stimuli in the preference test. Chicks were injected five minutes prior to the beginning of the preference test (Fig. 2A). On any given test day, vehicle and mesotocin or vehicle and vasotocin groups were tested to control for any possible incubator batch and/or day effects. Brains were examined to determine the success of injection placements by 48 hr post-hatch. The number of chicks that showed blue staining in both hemispheres in the posterior dorsal part of the telencephalon (which corresponds to hippocampal tissue that lies dorsal to the posterior part of the lateral ventricle) and surrounding the lateral ventricle (Fig. 2B) were considered successfully injected (44 out of 97 chicks). We report results for number of revolutions for all 44 chicks (SAL, N = 12; MT 1 nmol, N = 20; AVT 1nmol, N = 7; AVT 0.1 nmol, N = 5) and for group comparisons of hen preference scores we included chicks that ran a minimum number of 20 revolutions and that showed a hen preference score above 50%.

*Intraperitoneal Substance P injections*

A stock solution of substance P (SP) (Sigma, S6883) was made by dissolving the drug in 0.05 M acetic acid to obtain a concentration of (3 µg/µl) and aliquoted in vials of 300 µl and stored at -20°C. Each vial was only thawed once on the day of testing to make the appropriate dose concentrations (0.25 µg/µl, 0.5 µg/µl, 1.5 µg/µl) using 0.05 M acetic acid as vehicle, and were kept on ice until administration. Working concentrations were chosen based on Thompson *et al.*, (2008) and scaled for the typical weight of chicks at 48 hr post-hatch. In the dose response experiment, we tested the three doses stated above to determine a minimum dose that would not impair locomotor activity and used information from a study on rats (Hong *et al.*, 2015) as a guideline for estimating the minimal lethal dose (MLD); our highest dose was less than half the MLD. On any given test day, vehicle and all three dose groups (for the dose response experiment) or vehicle and 25 µg SP groups, were tested to control for any possible incubator batch and/or day effects.

Chicks were injected five minutes prior to the beginning of the preference test with 100 µl of either vehicle or one of three substance P doses (25 µg, 50 µg, 150 µg) into the intraperitoneal cavity and returned to the closed box. Five minutes later, the chick was transferred to the running wheel setup and the preference test began immediately. The time to test post-injection was chosen based on (Boix *et al.*, 1992; Shaikh *et al.*, 1993; Hasenöhrl *et al.*, 1994; Thompson *et al.*, 2008).

In the substance P dose response experiment, in addition to preference scores, we were interested in examining whether higher SP doses reduced the chicks' locomotor activity and we assessed this by comparing group means for total number of revolutions. After analyzing these results, we decided upon a minimum

acceptable number of revolutions of 25, to be considered for subsequent data analysis - because fewer rotations were unlikely to reflect an actual preference for either stimuli. After the dose response experiment, we chose 25 μg of SP as the dose to continue testing so we added more animals (N = 12 chicks) to increase the final sample sizes of vehicle and 25 μg dose groups. We report number of revolutions for all chicks tested and for group comparisons of hen preference scores we included chicks that ran 25 revolutions or more, based on these criteria, 15 chicks (out of 63) were excluded.

*Intact group*

To establish a non-injected baseline group ('intact condition'), 38 chicks were tested in the preference test for the predisposed stimulus without receiving any type of injection prior to preference testing. All other conditions from egg incubation to testing were otherwise identical to other treatment groups.

*Preference test for the predisposed stimulus*

All testing took place between the hours of 11:00 and 20:00. After being removed from the incubator, the chick was placed in a closed box and transported to the testing room, always ensuring no exposure to light or visual stimuli occurred prior to the preference test. In a dimly lit room, the chick was removed from the box and placed directly into the running wheel and the preference test began immediately (intact group) or injections were performed (drug treatment groups described below) and returned to the closed box for 5 min before beginning the preference test. The testing area consisted of a rectangular enclosure with a base that was 153 cm long, 63 cm wide, and 52 cm tall walls [an illustration of a comparable setup is depicted in

(Sgadò *et al.*, 2018)]. The interior floor and walls were black and there was no top-facing side. Inside the enclosure, the running wheel (diameter, 25 cm) was placed in the middle and on either side at a fixed distance, stimuli were placed on a rotating platform (30 rpm) and illuminated by light that originated from directly above the stimulus (40W lightbulb in an aluminum covered lampshade, diameter 21 cm) and from the front-right with a 40W lightbulb directed at the stimulus). The light from above was diffused with a semi opaque white plastic board, upon which the lampshade was directly placed. The predisposed stimulus was a stuffed hen and the non-predisposed stimulus was a control stimulus. Stuffed hens were obtained from a local taxidermist and were chosen on the basis of resemblance to the original stuffed jungle fowl stimulus used in the preference test that led to the discovery of social predispositions in chicks (McCabe *et al.*, 1981; Johnson & Horn, 1988) and recent studies (Mayer *et al.*, 2016; Versace *et al.*, 2017; Sgadò *et al.*, 2018) have validated the use of these stimuli in replicating the results from the original findings (Fig. 1). The placements of the stimuli (left or right) were counterbalanced in each condition. A digital camera was suspended above the running wheel to record the chick running. The running wheel was equipped with an automatic counter that recorded the number of revolutions taken in the left and right directions, respectively. The circular sides of the running wheel were covered in black paper on the inside so the chick could only see in the direction of stimuli and directly above and below itself. Once placed inside the running wheel, the chick ran freely in the running wheel for 30 min, this was the total duration of the preference test. The experimenter documented the number of left and right revolutions at 10 minute intervals.

For each chick, the behavioral preference for the stuffed hen was calculated as *number of revolutions in the direction of the stuffed hen / (number of revolutions in the direction of the stuffed hen + number of revolutions in the direction of the control stimulus)*. This preference was then converted to a percentage (hereinafter referred to as "hen preference score"), such that a score greater than 50% indicated a preference for the stuffed hen. For each treatment group a mean preference score was calculated from individual values and a significant departure from chance level (50%) was tested with a one-sample two-tailed t-test.

*Statistical analyses*

Statistical analyses were performed in GraphPad version 7.0. In all tests, a *p*-value at $<0.05$ was considered significant. Tests for unequal variances between groups were performed with an F-test and distributions were assessed with the D'Agostino-Pearson normality test (omnibus K2). Comparisons of means across groups were performed using one-way ANOVA tests if variance and distribution assumptions for parametric tests were satisfied, otherwise we used non-parametric Kruskal-Wallis (KW) tests. Significant results from KW tests were followed by multiple comparisons with Dunn test corrections. In cases where two groups were compared, we used Student t-tests if parametric assumptions were satisfied, otherwise we applied Mann-Whitney tests. For substance P data, a two-way ANOVA test on the preference scores data was performed, with sex and drug treatment as factors, to determine differences between males and females, followed by Bonferroni corrected multiple comparisons. Adjusted *p-v*alues were reported from these post hoc tests. Unless otherwise noted, group means are expressed as mean ± SEM.

**Results**

*Effects of mesotocin and vasotocin on locomotor activity*

Drug treatment had an effect on locomotor activity (KW, p < 0.0001, H = 24.46) (Fig. 3). Vasotocin-injected animals ran little to not at all at both the 1 nmol and 0.1 nmol doses compared to the saline group (Dunn's tests: SAL vs. AVT 1 nmol, p<0.0001; SAL vs. AVT 0.1 nmol, p = 0.013) whereas the mesotocin-injected group did not differ from saline-injected controls in number of revolutions (Dunn's test: SAL vs. MT 1 nmol, p>0.99). Because vasotocin-injected chicks did not pass minimum running inclusion criteria we did not calculate hen preference scores for this group.

*Effects of mesotocin on preference for the predisposed stimulus*

Mesotocin-injected chicks had higher hen preference scores than saline-injected controls (Student t-test, $t_{19}$ = 2.13, p = 0.046) (Fig. 4A). Mean hen preference scores were as follows: SAL group (N = 9), 60.35 ± 2.037; MT 1 nmol group (N = 12), 69.3 ± 3.288. Mean hen preference scores for both groups were significantly different from chance (one sample t-test: SAL group $t_8$ = 5.08, p = 0.001; MT group $t_{11}$ = 5.86, p = 0.0001). We calculated hen preference scores for every 10 min interval of the 30 min test and did not find any effect of time [$F_{(2,38)}$ = 0.58, p = 0.56]; drug [$F_{(1,19)}$ = 2.77, p = 0.11] or an interaction effect [$F_{(2,38)}$ = 0.04, p = 0.95] (Fig. 4B). There was no correlation between number of revolutions and hen preference scores in either group (SAL: Pearson r = 0.44, p = 0.23; MT: Pearson r = -0.13, p = 0.67) (Fig. 4C).

*Substance P dose response experiment*

Thirty-seven chicks were tested in the dose response experiment. The mean hen preference scores for the predisposed stimulus in vehicle (N = 13), 25 μg (N = 8), 50 μg (N = 10) and 150 μg (N = 6) groups were 59.69 ± 4.53, 57.55 ± 5.25, 57.93 ± 4.48 and 45.05 ± 9.99, respectively. None of the group means were significantly different from 50% (one-sample t-tests: 25 μg group: $t_7$ = 1.438, p = 0.19; 50 μg group: $t_9$ = 1.768, p = 0.11; 150 μg group: $t_5$ = 0.495, p = 0.64), but the vehicle group was on the margin of significance (one-sample t-test $t_{12}$ = 2.137, p = 0.054). The mean hen preference scores for the drug injected groups did not differ from the vehicle group (KW, H = 0.61, p = 0.89). For number of revolutions across groups there was a main group effect (KW, H = 9.01, p = 0.029) and multiple comparison tests showed that chicks in the 150 μg group ran significantly less than those in the vehicle group (Dunn's test, p = 0.021). When we examined how many chicks in each group ran more than 25 revolutions, in the highest dose group only 33% (2 out of 6 chicks tested) had passed this criterion compared to more than 90% in all other groups. Based on these results, we decided to proceed with the lowest dose (25 μg) because this dose did not produce a significant difference in the number of revolutions compared to the vehicle group.

*Substance P effects on preference for the predisposed stimulus*

Overall, there was no significant difference in hen preference scores between chicks injected with 25 μg of SP compared to controls (Mann-Whitney, p = 0.64). The mean hen preference score for vehicle-injected animals (53.89 ± 3.06, N = 16) did not differ from chance (one-sample t-test $t_{15}$ = 1.26; p = 0.22) but SP-injected animals

did show a mean hen preference score (56.62 ± 2.52, N = 18) in favor of the stuffed hen that was significantly different from chance ($t_{17}$ = 2.62; p = 0.017). In post-hoc analyses, we were interested in examining whether preference scores for the predisposed stimulus varied by sex. A two-way ANOVA analysis of the hen preference scores data, with sex and drug treatment as factors, revealed an interaction effect on the margin of significance (two-way ANOVA, $F_{(1,30)}$ = 3.278, p = 0.08) indicating that perhaps males and females could differ in their response to drug treatment, but there were no main effects for sex ($F_{(1,30)}$ = 2.55, p = 0.12) or drug treatment ($F_{(1,30)}$ = 0.067, p = 0.79).

When the data was segregated by sex, the results of the one-sample t-test of mean preference scores against chance were as follows: vehicle-injected males ($t_9$ = 0.285, p = 0.78); SP-injected males ($t_9$ = 3.169, p = 0.011); vehicle-injected females ($t_5$ = 3.184, p = 0.024), SP-injected females ($t_6$ = 3.180, p = 0.019). Thus, it appears that SP-injections were able to restore the male preference for the hen to resemble the typical response seen in intact animals. In contrast, females in both the vehicle and SP-injected groups, showed a preference for the stuffed hen that was comparable to that of intact females.

*Intact predisposition*

Out of the 38 chicks tested one chick was excluded because it had splayed legs which impeded proper walking and another because of technical problems with the running wheel during the test. The mean hen preference score for intact chicks, (N = 36) was 55.35 ± 2.12 and was significantly different from chance (one sample t-test, $t_{35}$ = 2.519, p = 0.016). Post hoc descriptive statistics of intact chicks grouped

based on preference were as follows: twenty-three chicks preferred the hen (mean hen preference score 62.37 ± 2.02), twelve chicks preferred the control stimulus (mean hen preference score 42.36 ± 1.79) and one chick showed no preference for either stimulus.

## Discussion

In this study, we investigated whether manipulating endogenous nonapeptide levels in the brain, and separately, substance P peripherally, affects social predispositions in chicks, that is, the tendency of visually naïve chicks to prefer to approach a stuffed hen over a control stimulus. Our main finding is that chicks that received bilateral intracranial mesotocin injections had greater hen preference scores than saline-injected chicks. Despite conserved distribution of substance P neurons and its association with the dopaminergic reward system, we did not find any effect of peripheral injections on approach response in chicks, contrary to what we predicted according to the so-called primitive social approach circuit model proposed by Thompson et al. (2008). In agreement with previous studies (Johnson & Horn, 1988; Mayer *et al.*, 2016; Versace *et al.*, 2017) the majority of intact chicks demonstrated the social predisposition preference for the stuffed hen and this result was also observed in saline and mesotocin injection-treated animals. We did not find any correlation between hen preference scores and number of revolutions in the running wheel in the mesotocin-injected group, indicating that greater hen preference scores were not driven by an increase in locomotor activity. In fact, there was no difference in locomotor activity between mesotocin-injected chicks and controls in our study. Vasotocin-injected chicks on the other hand, became hypoactive and therefore hen preference in this treatment condition could not be examined. Other

studies have also reported that equimolar doses of mesotocin and vasotocin produce different effects on certain movements and behaviors. For example, in 5-day-old chicks unilateral (left) intracranial mesotocin, but not vasotocin, increased time spent preening relative to saline injected controls (Masunari *et al.*, 2016); and in 4-day-old chicks intracranial vasotocin treatment at the same dose as in our experiment, reduced number of vocalizations, steps and defecations and increased the number of wing-flaps relative to saline injected controls (Tachibana *et al.*, 2004).

Many examples across phylogenetically distant taxa show that soon after birth, features associated with animate beings are attention-drawing which begs the question of whether homologous neuroanatomical areas, neuron types and/or neuromodulators are responsible for mediating this response. For example, human newborns (Morton & Johnson, 1991), infant monkeys (Sugita, 2008) and newly hatched *Gallus gallus* chicks (Rosa-Salva *et al.*, 2010) demonstrate a predisposition to attend to faces and face-like stimuli, which suggests there may be some functional similarities in the underlying neural circuitry that enables face-detection (Rosa-Salva *et al.*, 2015). However, brain areas that are known to be, for example, essential for face detection in humans and face-like preference in human newborns (Buiatti *et al.*, 2019) lack one-to-one homologies with avian brains, which makes the cross species comparison at the neuroanatomical level challenging. Even most recent efforts aimed at identifying neurons with firing responses selective to faces within brain nuclei of the tectofugal visual pathway in pigeons using *in vivo* recordings during stimuli presentation reported such neurons were not detected (Clark *et al.*, 2019). However, studies that have mapped expression of c-Fos following predisposition tests show differential activation associated with increased preference for

predisposed stimuli in nodes of the social behavior network, namely the lateral septum, preoptic area and nucleus taeniae (a partial homolog of the medial amygdala) (Mayer *et al.*, 2016; Lorenzi *et al.*, 2017; Mayer, Rosa-Salva, Morbioli, *et al.*, 2017; Mayer, Rosa-Salva, & Vallortigara, 2017).

In several bird species, these brain nuclei have been shown to bind nonapeptide ligand analogs, be enriched for expression of mRNAs of specific nonapeptide receptors and to contain cells and processes that are immunoreactive for the avian VT4 receptor (see Table 1 for nomenclature) (Goodson *et al.*, 2009; Leung *et al.*, 2009, 2011; Selvam *et al.*, 2015; Wilson *et al.*, 2016; Ondrasek *et al.*, 2018). For example, in both the white-throated sparrow and zebra finch the lateral septum showed high intensity of staining for VT3 receptor mRNA, a receptor which has highest homology to the mammalian oxytocin receptor. In contrast, expression of the VT4 receptor, an ortholog of the mammalian vasopressin V1a receptor, was not detected in the lateral septum in either species (Leung *et al.*, 2011) despite evidence that BSTM AVT neurons in the zebra finch do project there and further, that dense AVT-immunoreactive fibers are present in the lateral septum in another bird species (Goodson, Evans, *et al.*, 2004). The nucleus taeniae on the other hand, showed low expression of VT3 in the white-throated sparrow and no detectable expression in the zebra finch (Leung *et al.*, 2011) demonstrating here (and also elsewhere throughout the brain) that there are species differences in 'where' nonapeptide receptors are expressed. Similarly, in the winter flocking field sparrows and dark-eyed juncos, the lateral septum and arcopallium show high optical density of radiolabeled ornithine vasotocin analog binding (which is supposed to show where VT3 receptors are expressed) (Wilson *et al.*, 2016). In the chicken however, the lateral septum contains

cells and processes that span across the entire lateral septum that are VT4-immunoreactive (Selvam *et al.*, 2015). It is important to note that in birds, out of the four known nonapeptide receptors, the distribution of VT4 and VT3 receptors in the brain has been studied most extensively. These two receptors have been documented to be expressed throughout the entire brain, in many other areas besides the aforementioned SBN nodes, such as within the hypothalamus, thalamus and brainstem. Therefore, we cannot ascertain that the effects of mesotocin injections on the stuffed hen preference is mediated solely by the lateral septum, for instance, because one or more of the other brain nuclei that express these receptors could also be important for the effects reported here.

We found that mesotocin injections produced a greater hen preference in the predisposition test, which suggests that under natural conditions during the sensitive period for imprinting, mesotocin signaling, potentially mediated by the VT3 receptor, may be critical for guiding attention and directing the appropriate approach response towards conspecifics. Several studies have demonstrated that in rodents, both oxytocin and vasotocin are important for social recognition. For example, oxytocin knockout male mice are unable to recognize females they have been repeatedly exposed to before, even though their ability to recall associations with other odors remains intact (Ferguson *et al.*, 2000). This effect was further narrowed to the medial amygdala, where oxytocin treatment was able to rescue the ability to recognize previously encountered females (Ferguson *et al.*, 2001). In addition, other studies have shown that vasopressin signaling is also important for recognizing conspecifics. For example, a null mutation in the AVPR1A gene, which encodes the vasopressin V1a receptor, results in impaired social recognition (Bielsky & Young, 2004) and in

wild type mice, delivery of an antagonist for the vasopressin receptor V1a in the lateral septum, but not in the medial amygdala, produces a social recognition deficit. Importantly, in the case of the AVPR1A null mutant mice, social recognition can be rescued if the AVPR1A gene is re-expressed in the lateral septum (Bielsky *et al.*, 2005). While similar examples such as these do not exist in birds (i.e. transgenic knockout lines) one study in zebra finches has shown that vasotocin knockdown, specifically in BSTM AVT neurons, which project to the lateral septum, reduces their preference to spend time in close proximity to groups of conspecifics (Kelly *et al.*, 2011). However, in the same species, partner preference is not affected by bilateral injections to the lateral ventricles of any of the following: vasotocin, mesotocin, a vasopressin receptor antagonist and an oxytocin receptor antagonist (Goodson, Lindberg, *et al.*, 2004). Although vasotocin had an overall hypoactive effect and we could not examine its effect in the social predisposition test, we cannot rule out a role for vasotocin in the natural approach response towards the mother hen. Further knowledge on the distribution of nonapeptide receptors in the brain at this critical life stage would help shed light on this issue.

In fact, it has been proposed that in birds, vasotocin may exert oxytocin-like effects (e.g. on ovipositioning) by acting via the VT3 receptor, which has high homology to the mammalian oxytocin receptor (Gubrij *et al.*, 2005)  In this scenario, it would be possible then that delivery of vasotocin could have activated both VT3 and VT4 receptors and that this led to an overall reduction in locomotor activity. In contrast, delivery of mesotocin may have only activated the VT3 receptor and to a much lesser extent, the VT4 receptor (given documented promiscuity of nonapeptides binding to their non-cognate receptors), and therefore no reduction in

locomotor activity was observed. Our findings make clear the effect of mesotocin on the preference for a stuffed hen over a control stimulus, but whether this effect is mediated by mesotocin binding to the VT3 receptor alone, or additionally to any of the other vasotocin receptors (VT1, VT2, VT4) remains to be studied. Future studies could examine, for example, how hen preference scores differ from the results reported here, when antagonists for VT3 or VT4 are delivered intracranially prior to mesotocin injections. Therefore, one caveat of this study is that we did not test whether the effects of mesotocin on hen preference can be blocked by the use of an oxytocin receptor antagonist. We were challenged in that the chicks in our study were not fitted with a cannula, which allows repeated injections to the same site (i.e. receptor antagonist injection followed shortly after by nonapeptide injection) therefore with the data presented here we cannot determine specifically through which of the nonapeptide receptors mesotocin exerted its effects on hen preference. Future studies can address this as well as test whether an oxytocin receptor antagonist delivered alone results in a decrease in hen preference compared to saline injected controls.

Here we show that before imprinting occurs, the first approach towards hen-like stimuli can be intensified by increasing levels of mesotocin in the brain. Social predispositions in filial imprinting of precocial bird species, since they were first described [e.g. (Horn & McCabe, 1984; Johnson & Horn, 1988)] have been thought of as a key element that entails directed attention towards conspecifics to ensure proper filial imprinting will subsequently take place. Because in the wild successful imprinting confers a survival advantage to chicks, the notion that natural selection must have also played an important role in shaping the behavioral responses that

social predispositions are comprised of, is implied. If the behavior is under selection, then brain structures and function underlying the behavior are also under selection. Our findings suggest that the activity of mesotocin neurons and sensitivity to mesotocin throughout the brain are candidates for further investigation into how they may be critical for enabling social predispositions. Given mesotocin can bind not only to VT3, but also with lower affinity to the behaviorally relevant VT4 (and perhaps even to VT1 and VT2 as well) further work aimed at discerning where in the brain and which of these receptors is associated with mediating the effect on hen preference on social predispositions remains to be studied. Furthermore, the effects of mesotocin on social predispositions may be a particular case of precocial species, and perhaps not extendable to altricial species, such as the zebra finch. We hypothesized that peripheral injections of substance P in visually naïve chicks would result in reduced approach towards predisposed stimuli. We did not find any effect of substance P on hen preference scores, which suggests that the mediating effect of substance P on social approach is not as widespread (across taxa) as previously proposed (Thompson *et al.*, 2008). Alternatively, there is still the possibility that this so-called primitive circuit is still present and functional in regulating affiliative behaviors in adult birds, but not in newly hatched chicks. Of note is that we tested chicks on the second day post-hatch and spinal cord SP neurons have been documented to be immunohistochemically detectable at day 4 post-hatch, but not on day 1 post-hatch in *Gallus gallus* chicks (Du *et al.*, 1987). Moving forward, continued research on the effects of nonapeptides, especially early in life, along with mapping of the neuroanatomical distribution of their receptors across several bird species (Leung *et al.*, 2009, 2011; Ondrasek *et al.*, 2018) will allow a more in depth

understanding of their roles in shaping behaviorally diverse repertoires towards conspecifics.

**Conflicts of interest**

The authors have no conflicts of interest to declare.

**Author contributions**

JL conceptualized the project, designed and carried out experiments, analyzed data and wrote the manuscript, MGS contributed a substantial resource to the design and revised the manuscript, GV supervised the project and revised the manuscript.

**Data accessibility**

The article's data can be accessed by request to the corresponding author.

**Abbreviations**
| | |
|---|---|
| ANOVA | analysis of variance |
| AVP | arginine vasopressin |
| AVPR1A | mammalian vasopressin V1a receptor |
| AVT | arginine vasotocin |
| BSTM | medial part of the bed nucleus of the stria terminalis |
| BW | body weight |
| Hp | hippocampus |
| IP | intraperitoneal |
| IC | intracranial |
| KW | Kruskal-Wallis |
| LS | lateral septum |
| LV | lateral ventricle |
| MLD | minimal lethal dose |
| MT | mesotocin |
| MW | Mann-Whitney |
| PVN | paraventricular nucleus |
| SAL | saline |
| SEM | standard error of the mean |
| SP | substance P |
| V1a | vasopressin receptor subtype V1a |
| W | watts |

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**Table 1. Avian and mammalian nomenclature of nonapeptide receptors**

| Avian | Mammalian |
| --- | --- |
| VT1 | V2 |
| VT2 | V1b |
| VT3 | OTR |
| VT4 | V1a |

**Figures**

**Figure 1. Visual stimuli**

Photographs of control stimulus and stuffed hen used in this study. Both stimuli possess the same body parts, colors and texture. In the control stimulus, body parts are arranged in a disorganized fashion and the typical outline of the head and neck is absent. In preference tests, visually naïve chicks tend to show a predisposed preference for the stuffed hen (predisposed stimulus) over the control stimulus.

**Figure 2. Experimental timeline and representative bilateral intracranial injection validation**

From hatching to the time of preference testing, chicks were always maintained in darkness inside incubators. On the first day of life, chicks were kept in individual compartments inside the incubator and were acoustically stimulated for 4 hr and preference tests were performed the next day. Chicks that received either intraperitoneal (IP) or bilateral intracranial injections were injected 5 min prior to the beginning of the test. B) Photographs of a chick brain that showed successful bilateral intracranial injection, areas with blue coloration indicate where the injected solution traveled. Brain is shown as viewed from above, the dorsal posterior part of the telencephalon shows blue staining. The middle image is a coronal cross-section of the same brain shown in the left image, the approximate section plane is illustrated, and blue staining in tissue lining the lateral ventricles is evident. The right image shows an illustration of major structures in middle image. Abbreviations: Hp = hippocampus, LV = lateral ventricle, LS = lateral septum.

**Figure 3. Effects of mesotocin and vasotocin on number of revolutions in the running wheel**

Chicks given bilateral intracranial injections of vasotocin (AVT) ran little to not at all at both 1 nmol and 0.1 nmol doses compared to the saline (SAL) group, whereas mesotocin (MT) at the 1 nmol dose had no effect on running compared to saline (SAL) controls. Dunn's p-values: *** $p<0.0001$; *$p<0.05$.

**Figure 4. Mesotocin-injected chicks had greater hen preference scores compared to controls**

A) Among chicks that showed the predisposition for the stuffed hen, mesotocin (MT) injected chicks had greater hen preference scores compared to saline (SAL) injected controls. B) Hen preference scores were calculated for each 10 min interval of the 30 min of testing. C) No correlation was found between hen preference scores and number of revolutions in either the MT group (Pearson r = -0.13, p = 0.67) or the SAL group (Pearson r = 0.44, p = 0.23). Error bars indicate ± standard error of the mean.

**Stuffed hen**                    **Control stimulus**

**A**



ip or ic injection
5 min before
test

0          24          ↓          48     Hours
                                          post-hatch

4 hr          30 min          Verify ic
acoustic          preference          injection
stimulation          test          site

**B**



Hp
X X
LV          LV
LS