AVIAN MODELS FOR SOCIAL COHESION

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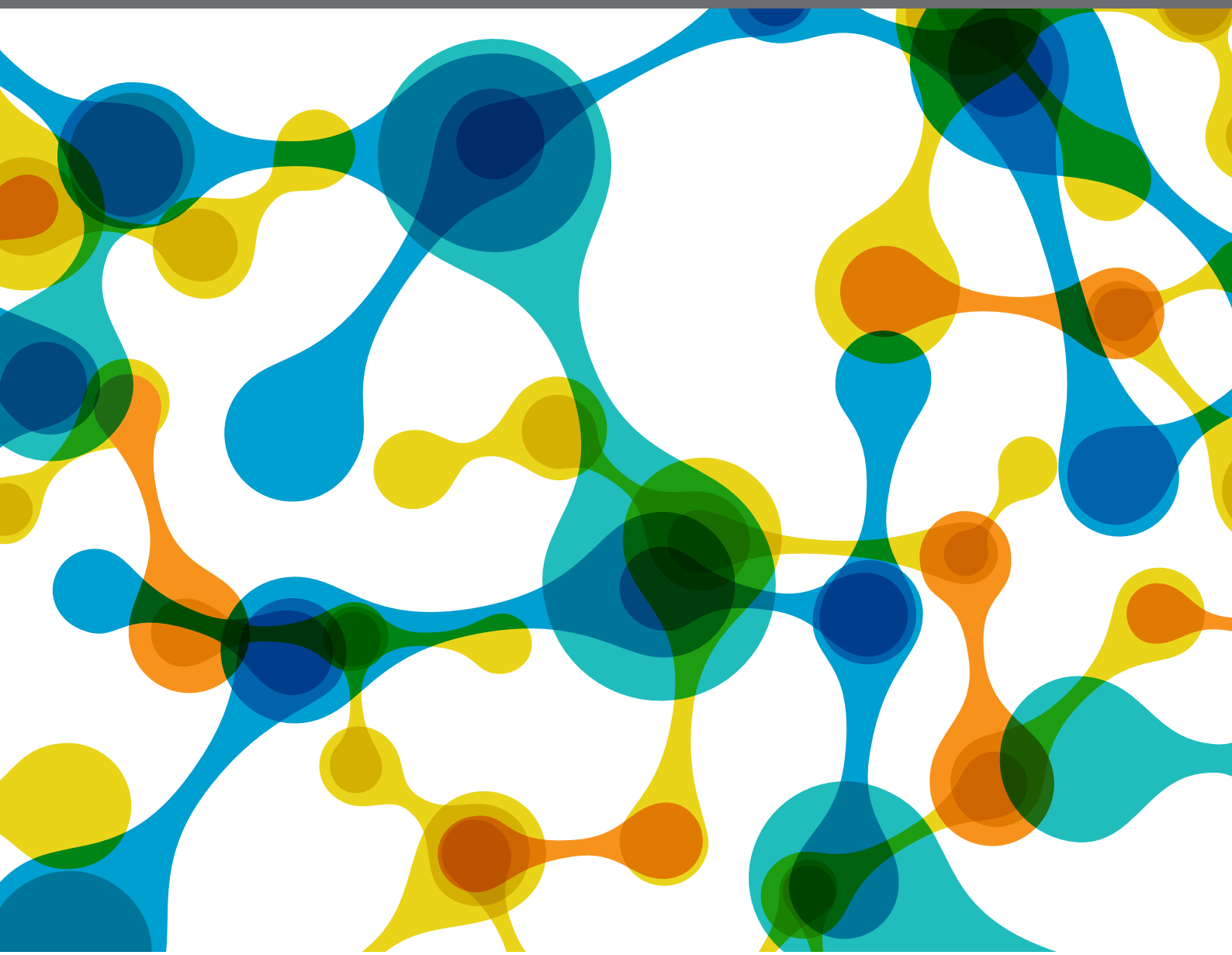

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AVIAN MODELS FOR SOCIAL COHESION

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Animals living in groups are often linked to group or family members stronger than to other conspecifics, and form stronger coalitions (often based on genetic relatedness) within such groups. Effective cooperation within a group requires the preference for proximity of group members, suppression of aggression toward conspecifics, an ability to perceive and respond to social signals and to change (often synchronize) behavior accordingly.

Birds have long been used for a number of investigations involving sensory perception, learning, feeding strategies and vocal communication. Recently, they have been proposed as ideal model species even for psychiatric disorders affecting social cohesion, such as autism spectrum disorder.

The physiological mechanisms and neural systems underlying different forms of sociability (sexual and parental bonding, group preference, nesting, care for offspring, migration) can often be studied easier in birds, since their social behavioral repertoire, as a taxon (but sometimes also as individuals), is more diverse than that of mammals. By contrast with laboratory rodents, birds rely less on olfactory cues. Rather, they tend to use visual and acoustic signals for social interactions, much like humans.

Comparative approach and evolutionary relevance of studies using avian species have already yielded valuable results in several fields of neuroscience: learning and memory (imprinting), acoustic communication (birdsong), neurogenesis (seasonal changes in the song network). With the advent of robust novel methods in molecular biology, genomics and proteomics, information technology and electronic engineering; and also based upon an ever improving battery of behavioral tests, avian research in social cohesion has likely gained a new impetus.

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EDITORIAL
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Editorial: Avian Models for Social Cohesion

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Keywords: bird, affiliation, imprinting, social perception, flocking, ASD, forebrain evolution

Editorial on the Research Topic

Avian Models for Social Cohesion

As a cover term, social cohesion comprises different levels of explanation for a fundamental feature of numerous vertebrate species: the ability to form groups. Birds have long been considered good examples for sociability, with iconic capabilites such as birdsong, migration, or diverse mating systems and parental behavior. Apart from the fascination evoked by the apparent complexity of avian affiliatory behaviors, an impressive number of studies have been focusing on the underlying mechanisms. It is becoming increasingly evident that the validity of molecular, neurophysiological, and neuroanatomical correlates of avian social behaviors extends far beyond the realm of birds. More recent research has further corroborated the value of avian models for normal or impaired social cohesion, even for certain social deficits occurring in humans.

The present selection of articles represents molecular, behavioral, evolutionary, and theoretical approaches of investigation. According to their focus, the articles can be assigned to the following main categories: (i) Imprinting as one fundamental tool in early bond formation; (ii) Predispositions and socially relevant perception mechanisms; (iii) Adaptive processes underlying flocking; (iv) Evolutionary and developmental trends determining sociability in vertebrates. In this editorial summary we, as Guest Editors, attempt to provide a progress report in those fields, illuminating The Research Topic for the Reader by bringing them, if momentarily, into the limelight.

Ad (i) By revisiting visual imprinting, the review by McCabe discusses this process in the framework of filial bond forming and, in more general terms, social recognition. It also gives a promising account of new perspectives in the investigation of physiological mechanisms underlying imprinting. One such recently discovered factor determining the sensitive period for imprinting is triiodothyronine (T3). The study by Aoki et al. further elucidates the molecular basis of this T3 dependent regulatory mechanism by demonstrating an increase in GABA-A receptor expression and a contrasting decrease in GABA-B receptor expression in the early post-hatch period. The authors concluded that GABA-B receptors facilitate imprinting downstream to T3, whereas GABA-A receptors contribute to termination of the imprintable period. The paper by Miura et al. further elaborates on the role of T3 by showing that, as one possible mechanism to prolong the sensitive period for imprinting, T3 also promotes the manifestation of predisposed preference for biological motion (BM) in 1-day-old (but not in 4-day-old) chicks. Partial re-sensitization of BM preference and filial imprinting with T3 corroborate its role in the flexibility of the imprintable period. Another aspect of imprinting concerns the association with memory formation for the imprinting stimulus (with relevance to social recognition). The study by Tiunova et al. demonstrated an elevation of c-fos induction in the hippocampus on first presentation of the imprinting stimulus, but not after memory retrieval, while in the IMM, mediorostral nidopallium/mesopallium and hyperpallium densocellulare, c-fos activation was induced by retrieval of only the remote

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but not of recent memory. The results cast new light on a perplexing problem of an apparent "migration" of the socially relevant memory trace from acquisition to consolidation.

Ad (ii). A key element of social recognition involves categorical representation of biologically relevant percepts such as facial features. The study by Clark et al. was based on electrophysiological recordings from four relevant forebrain structures associated with the tectofugal visual system (entopallium, mesopallium ventrolaterale, nidopallium frontolaterale, area temporo-parieto-occipitalis) in a discrimination task of pigeons. No "face-selective" neurons were detected in any of the regions, suggesting a predisposition of birds for a more global combination of features, also subserving perception of faces. Early predispositions are implicated in the recognition of conspecifics, social partners, or predators. Similar processes likely occur in human newborn infants, and are potentially impaired in newborns at risk of Autism Spectrum Disorder (ASD). Using a domestic chick model, Lorenzi et al. found that, similarly to impairments of preference for static social stimuli, valproic acid (VPA) exposure *in ovo* specifically affects also the preference for animate motion stimuli. The study underlines the importance of predispositions in the development and early diagnosis of ASD in human neonates. The study by Zachar et al. focuses on another aspect of VPA-related effects. While the preference for large vs. small groups of conspecifics, and for partners with intact facial features over those with blurred faces, as well as early adaptive learning, were unaffected by VPA, social exploration and the recognition of familiar conspecifics were attenuated after VPA treatment. The findings suggest an importance of early social exploration in the development of ASD.

Ad (iii). How and why birds prefer their conspecifics is reviewed by Riters et al. with a comprehensive overview of group forming behaviors. Flocking offers distinct adaptive benefits for gregarious species of birds without an immediate reward (survival, food reward, mating) for the individual. Thus, in the long run, such behavior has to be promoted by reward (positive social interactions that "feel good") and by reinforcement (reducing the negative affective state due to social isolation). Both modalities can be controlled by the mu opioid receptors in the medial preoptic area, connected with the periaqueductal gray and ventral tegmental area. Based on experimental evidence from starlings (*Sturnus vulgaris*), the paper reviews and extends current knowledge on the motivation to form non-sexual social groups.

Ad (iv). Sociability necessitates an interplay between different neural systems. The large-scale review by Medina et al. summarizes the phylogenetic processes leading to divergent yet comparable neuroanatomical systems in birds and mammals. The account concentrates on two interrelated brain networks instrumental in sociability: one including the pallial (basolateral) amygdala, temporal and temporoparietal neocortices, and orbitofrontal cortex, involved in social perception and decision-making, and another comprising the medial extended amygdala, ventromedial striatum (nucl. accumbens), and ventromedial hypothalamus, related to affiliation. The study gives a detailed comparison of available neuroanatomical, evolutionary and developmental data between the relevant neural systems of mammals and their sauropsid equivalents.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Thyroid Hormone Sensitizes the Imprinting-Associated Induction of Biological Motion Preference in Domestic Chicks

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Filial imprinting is associated with induction of predisposed preference to animations that bear visual features of Johansson's biological motion (BM), and the induction is limited to a few days after hatching. As thyroid hormone (3,5,3′-triiodothyronine, T_3) plays a critical role in determining the sensitive period of imprinting, we examined if exogenously applied T_3 (or iopanoic acid, IOP; a selective inhibitor for converting enzymes) could also sensitize (or desensitize) the BM induction. Chicks were trained by using a non-BM stimulus (rotating red toy) according to a conventional imprinting procedure. Trained chicks were tested for preference to a point-light BM animation (walking chick) over a non-BM animation (linear motion), and for the preference for the familiarized stimulus (red toy) over an unfamiliar one (yellow toy). In 1-day chicks, those injected with IOP showed significantly lower scores than controls on both BM and imprinting tests. In 4-days chicks, those injected with T_3 showed higher scores than control, but the difference in BM score was not significant. Imprinting and the accompanying T_3 surge may be necessary for the predisposed BM preference to appear in 1-day chicks. Even after the conventional sensitive period is over, exogenous T_3 can partly re-sensitize the BM preference as it does imprinting.

Keywords: biological motion, imprinting, thyroid hormone, chicks, sensitive period

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INTRODUCTION

Filial imprinting constitutes the earliest step of the social attachment formation in precocial birds such as chickens and ducks (Lorenz, 1935; Hess, 1958; Matsushima et al., 2003; Horn, 2004). We have recently reported critical roles played by thyroid hormone (3,5,3′-triiodothyronine, T_3) (Yamaguchi et al., 2012). Imprinting upregulates gene expression of the converting enzyme (type 2 iodothyronine deiodinase, Dio2) in newly-hatched (1-day old) chicks. Infusion of exogenous T_3 (intra-venous injection) augments the imprinting score in 1-day chicks, and it reopens the sensitive period even in 4-days old chicks. We have argued that the imprinting primes the memory formation mechanism associated with imprinting. Once successfully trained, chicks can be re-imprinted to novel object for a substantially longer period than the sensitive period assumed so far. Furthermore, thyroid hormone would reorganize the neural mechanisms for visual perception. The chicks could thus develop durable social cohesion selectively to live animals such as their mother hen, even after they were initially imprinted to non-animate artifact.

Accordingly, newly-hatched chicks have an innately predisposed preference to Johansson's biological motion (BM) even without any visual experiences (Vallortigara et al., 2005). When exposed to motion pictures composed of light points, 1-day old chicks developed a distinct BM preference even when the picture was not of a BM nature (Miura and Matsushima, 2012); a video clip composed of randomly moving light points was similarly effective, but the induction of the BM preference did not occur in aged chicks (5-days old) suggesting a sensitive period as for conventional imprinting. When induced by non-specific motion pictures, the BM preference was tightly associated with imprinting (Miura and Matsushima, 2016) such that those chicks with a higher BM preference showed a higher imprinting score. Furthermore, we found a significant positive correlation between the induced BM score and the level of Dio2 expression in telencephalon of 1-day old chicks (Takemura et al., 2018). The BM preference is associated with imprinting probably via thyroid hormone actions, however, direct evidence is not yet available as to the causal relationships among these events.

In this study, we experimentally manipulated the thyroid hormone level and examined if the BM preference is accordingly controlled. The 1-day old chicks, which have an endogenously high level of T_3 (Yamaguchi et al., 2012), were treated with systemic injection of Dio2 inhibitor (iopanoic acid, IOP). The 4-days old chicks with a low endogenous T_3 level were supplemented with exogenous applied T_3. These chicks and the respective control groups were trained by non-BM object (red toy) and tested for BM preference and imprinting scores.

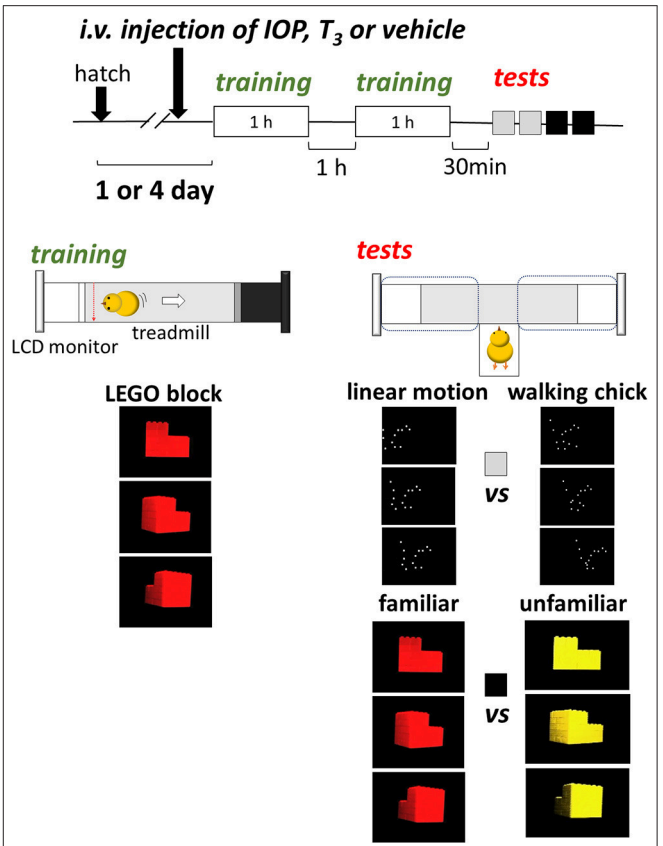

FIGURE 1 | Experimental procedures. On the day of training (1- or 4-days post-hatch), chicks received intravenous injection of IOP (iopanoic acid), T_3 (triiodo-thyronine), or respective vehicle at *ca.* 30 min before the training started. The chick was placed on a treadmill with one LCD monitor that displayed a rotating plastic toy for 1 h twice with a 1-h interval. At 30 min after the training, the chick was tested for BM preference (walking chick vs. linear chick) twice for 5 min each, and for imprinting (familiar vs. unfamiliar). The period in which the chick stayed in the left and right arm was recorded.

MATERIALS AND METHODS

Fertilized eggs of white leghorn strain were purchased from a local hatchery and incubated in darkness until hatching. A total of 46 chicks (21 males and 25 females) were used. Of these, we discarded 4 chicks (3 males and 1 females) that did not walk in training and tests. We merged both sexes, as no sex difference appeared at population level. Experiments were conducted under the guidelines and approval of the Committee on Animal Experiments of Hokkaido University (approval number: #16-0080). The guidelines are based on the national regulations for animal welfare in Japan (Law for Humane Treatment and Management of Animals, after a partial amendment No. 68, 2005).

For training, we used an I-shaped maze with a 50-cm-long treadmill at the center and a pair of LCD monitors (size 10.4", type LCM-T102AS, Logitec Co., Japan) at both ends (**Figure 1**). For display, free viewer software (APlayer, version 6.0) was used on PC. The width of the presentation was set at 9 cm on the monitor. During training, only one of the monitors was used. An infrared sensor and a transparent Plexiglass partition were placed at a point 10 cm from the active monitor. When chicks ran and hit the sensor, the treadmill moved and drew the chick backward by *ca.* 30 cm at a time. During the 1-h pause period between the two training sessions, chicks were stored in a dark incubator.

For tests, both monitors were used for binary choice. The partitions were removed and the treadmill was turned off. The subject chick was placed in a start box and allowed to freely choose between the two arms each equipped with an LCD monitor. Side of presentation alternated in two tests, and the side of the first test was counter-balanced among individuals. We recorded the total stay time in each arm (dashed lines) for a period of 5 min. The behavior of the subject chick was monitored through a CCD camera (250 kilo pixels) placed on the ceiling.

Chicks were exposed to a video clip of a toy made of red LEGO® blocks rotating along its vertical axis (see **Supplementary Videos** (LEGO block (red), LEGO block (yellow), Walking chick (white) and Linear motion (white)). For BM scores, we used a point-light animation that mimicked a "walking chick" used in our previous study (Miura and Matsushima, 2016; Takemura et al., 2018). One frame of the "walking chick" animation was used to generate "linear motion" of a constant speed from the left to the right of the screen. For imprinting scores, we tested chicks using the red toy video ("familiar") and a yellow toy ("unfamiliar"). For statistical analysis, R (version 2.12.0) was used for student *t*-test for comparisons between two groups. We also constructed

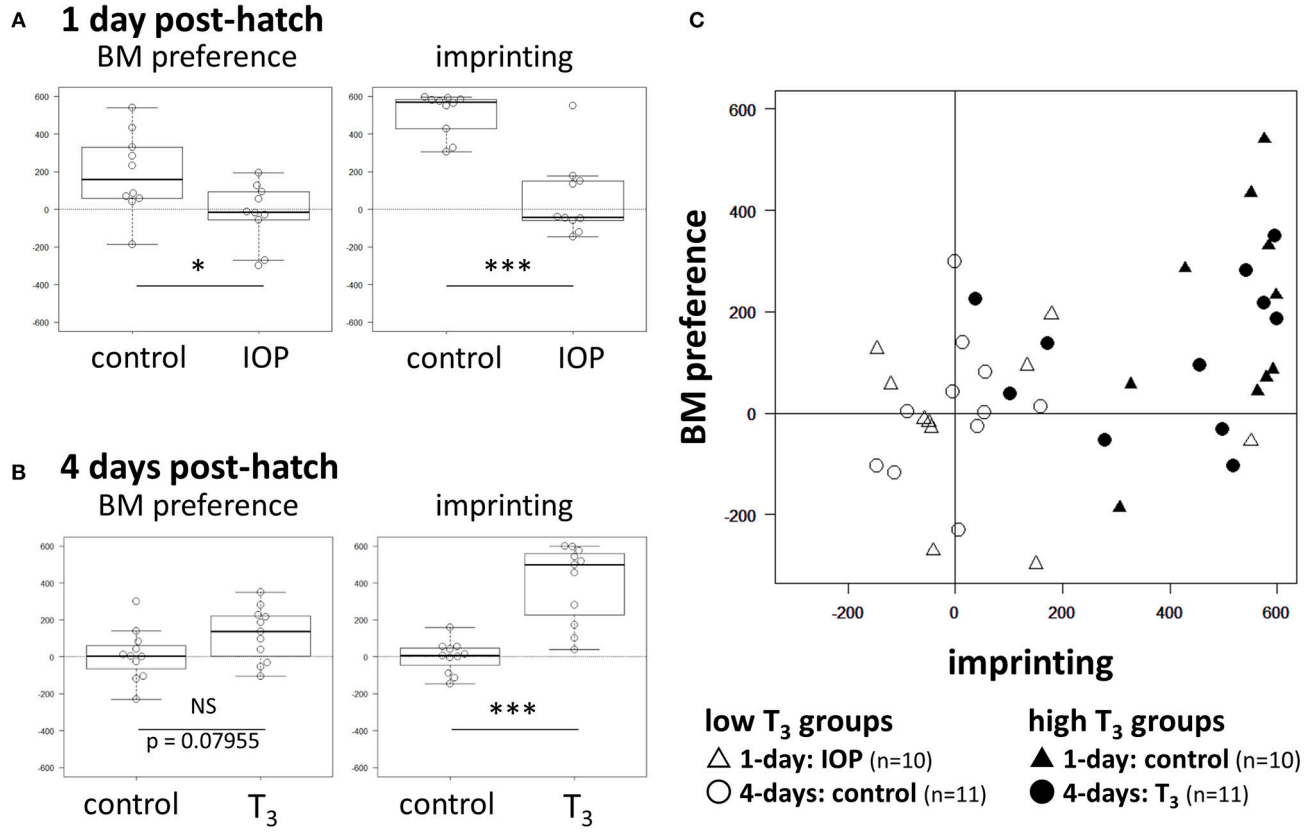

FIGURE 2 | Effects of IOP and T_3 injection on BM preference and imprinting score. **(A)** Results of 1-day chicks. **(B)** Results of 4-days chicks. NS (no significant difference), *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$). **(C)** BM score of individual chick was plotted against the imprinting score. Open symbols denote the low T_3 groups; day-1 chicks with IOP injection (△), day-4 control chicks (○). Filled symbols denote the high T_3 groups; day-1 control (▲), day-4 chicks with T_3 injection (•).

generalized linear models (GLMs) and evaluated these models based on AICs.

Single intravenous injection of T_3, IOP or respective vehicle (200 μl per chick) was accomplished by using 26G hypodermic injection needle to the leg tibia vein at *ca.* 30 min before the beginning of training. IOP (Tokyo Chemical Industry) was dissolved in 0.05M NaOH solution at 1 mM, and then rebuffered to pH = 8.5 by 0.2M HCl. T_3 (Sigma-Aldrich) was dissolved in 0.0002M NaOH and 0.9% NaCl at 10 μM.

RESULTS

In 1-day old chicks, IOP groups showed significantly lower scores both in BM preference ($t = -2.4784$, $df = 18$, $p = 0.023$) and imprinting ($t = -6.0682$, $df = 18$, $p < 10^{-5}$) than control that received vehicle injection (**Figure 2A**; see **Supplementary Table 1** for individual data). In 4-days old chicks, the T_3 group showed higher BM scores than control with a suggestive but not significant difference ($t = 1.8473$, $df = 20$, $p = 0.080$). On the other hand, imprinting scores were significantly higher in the T_3 group ($t = 5.8393$, $df = 20$, $p < 10^{-4}$) (**Figure 2B**). We conclude that T_3 is necessary and sufficient (at least partially) for the induction of BM preference in successfully imprinted chicks.

To examine if BM preference is associated with imprinting depending on the T_3 level, we plotted the individual BM preference score against the imprinting score after merging data obtained from these four groups (**Figure 2C**). In low T_3 groups (open symbols), both imprinting scores (X-axis) and BM preference scores (Y-axis) were distributed around 0. In high T_3 groups (filled symbols), on the other hand, those chicks with a higher imprinting score tended to show a higher BM score. Further statistical analysis using GLM (generalized linear model) revealed that interaction term of the T_3 level and the imprinting score gave the appropriate account of the BM preference; see **Supplementary Statistics (Table 2)** for details. We conclude that thyroid hormone sensitizes the induction of BM preference in a manner associated with the degree of imprinting.

DISCUSSION

We examined the effects of experimentally manipulated T_3 on BM preference and imprinting scores. In high T_3 groups, those chicks with a high imprinting score tended to show a high

BM preference in close accordance with our previous reports (Miura and Matsushima, 2016; Takemura et al., 2018). We may reasonably suppose that the thyroid hormone reorganized mechanisms responsible for visual perception. We must however notice that chicks with a high BM score always had a high imprinting score, but those with a high imprinting score sometimes failed to show a high BM preference (**Figure 2C**). In addition, the BM preference scores were generally lower in magnitude than the imprinting scores (Miura and Matsushima, 2016; Takemura et al., 2018). T_3 may primarily act on the brain mechanisms responsible for imprinting (such as those in intermediate medial mesopallium, IMM) (Horn, 2004), which subsequently sensitizes the BM preference through some unspecified downstream mechanisms. Alternatively, our test procedure for the BM preference could be simply not optimal and we underestimated it. Further efforts must be paid to develop better procedure to measure the BM preference.

The neural substrate for BM preference has not yet been specified, but several candidate areas have been suggested. Using an immediate early gene (c-Fos) as marker, Mayer et al. (2016a,b) found septum, hypothalamus, and amygdaloid areas (arcopallium and nucleus taeniae of the amygdala; Arco and TnA) are selectively activated by visual exposure to live conspecifics. Though IMM does not have direct projections to most of these limbic areas, Arco may poly-synaptically mediate the actions of IMM down to nucleus accumbens, septum, hypothalamic nuclei, and midbrain tegmentum including optic tectum (Csillag, 1999; Montagnese et al., 2004; Hanics et al., 2017; Xin et al., 2017). This scenario fits well with a more general idea that detection of biologically important visual inputs (such as facial emotions) occurs through fast-but-robust processes in the sub-cortical (sub-pallial) pathway (Inagaki and Fujita, 2017). Alternatively, dorsal projections from IMM actions to the hyperpallium (intermediate hyperpallium apicale, IMHA; Aoki et al., 2015) may also receive visual inputs from the pallial thalamo-fugal area such as visual Wulst (Nakamori et al., 2010). Reciprocal interactions between IMHA and IMM (both show a high level of Dio2 expression; Yamaguchi et al., 2012) may be responsible for the associated BM preference and imprinting sensitized by T_3. It is critically important to specify the brain areas responsible for the predisposed BM preference.

AUTHOR CONTRIBUTIONS

TM conceived the study. TM, NA, SY, KH, and TM designed the experiment. MM accomplished the experiment. MM and TM analyzed the data. TM wrote the draft of manuscript. MM, NA, SY, and KH revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01740/full#supplementary-material

Supplementary Table 1 | Behavioral data of individuals.

Supplementary Statistics | Statistical analysis (GLM analysis) of the data shown in **Figure 2C**.

Supplementary Videos | "**Video 1:** LEGO block (red)", "**Video 2:** LEGO block (yellow), "**Video 3:** Walking chick (white)", "**Video 4**: Linear motion (white)": WMV type video clip data used for training and tests. Chicks were trained using "LEGO block (red)", and the BM preference was tested in binary choice between "Walking chick (white)" and "Linear motion (white)". Thereafter, the imprinting was tested in binary choice between "LEGO block (red)" as familiar and "LEGO block (yellow) as unfamiliar."

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GABA-A and GABA-B Receptors in Filial Imprinting Linked With Opening and Closing of the Sensitive Period in Domestic Chicks (*Gallus gallus domesticus*)

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Filial imprinting of domestic chicks has a well-defined sensitive (critical) period lasting in the laboratory from hatching to day 3. It is a typical model to investigate the molecular mechanisms underlying memory formation in early learning. We recently found that thyroid hormone 3,5,3′-triiodothyronine (T_3) is a determinant of the sensitive period. Rapid increases in cerebral T_3 levels are induced by imprinting training, rendering chicks imprintable. Furthermore, the administration of exogenous T_3 makes chicks imprintable on days 4 or 6 even after the sensitive period has ended. However, how T_3 affects neural transmission to enable imprinting remains mostly unknown. In this study, we demonstrate opposing roles for gamma-aminobutyric acid (GABA)-A and GABA-B receptors in imprinting downstream of T_3. Quantitative reverse transcription polymerase chain reaction and immunoblotting showed that the GABA-A receptor expression increases gradually from days 1 to 5, whereas the GABA-B receptor expression gradually decreases. We examined whether neurons in the intermediate medial mesopallium (IMM), the brain region responsible for imprinting, express both types of GABA receptors. Immunostaining showed that morphologically identified putative projection neurons express both GABA-A and GABA-B receptors, suggesting that those GABA receptors interact with each other in these cells to modulate the IMM outputs. The roles of GABA-A and GABA-B receptors were investigated using various agonists and antagonists. Our results show that GABA-B receptor antagonists suppressed imprinting on day 1, while its agonists made day 4 chicks imprintable without administration of exogenous T_3. By contrast, GABA-A receptor agonists suppressed imprinting on day 1, while its antagonists induced imprintability on day 4 without exogenous T_3. Furthermore, both GABA-A receptor agonists and GABA-B receptor antagonists suppressed T_3-induced imprintability on day 4 after the sensitive period has ended. Our data from these pharmacological experiments indicate that

GABA-B receptors facilitate imprinting downstream of T_3 by initiating the sensitive period, while the GABA-A receptor contributes to the termination of the sensitive period. In conclusion, we propose that opposing roles of GABA-A and GABA-B receptors in the brain during development determine the induction and termination of the sensitive period.

Keywords: filial imprinting, sensitive period, GABA-A receptor, GABA-B receptor, thyroid hormone

INTRODUCTION

Newly hatched chicks undergo filial imprinting, a process in which they memorize and follow their mother in order to receive care (Lorenz, 1937; Vallortigara and Versace, 2018). The domestic chick (*Gallus gallus domesticus*) serves as a useful model for early learning and memory (Rose, 2000; Matsushima et al., 2003; Horn, 2004; Vallortigara, 2012a,b; Versace et al., 2018). Imprinting has clearly a sensitive or critical period after which chicks cannot be imprinted (Hess, 1959). The molecular mechanisms of memory formation in imprinting have been investigated intensively (Horn, 2004; Yamaguchi et al., 2008a,b, 2010, 2011; Solomonia and McCabe, 2015). We previously revealed that the thyroid hormone 3,5,3′ -triiodothyronine (T_3) functions as a starter and recoverer of the sensitive period (Yamaguchi et al., 2012). After hatching, imprinting training induces rapid inflow of T_3 into the brain, which makes the chicks imprintable. Intravenous T_3 injection into the intermediate medial mesopallium (IMM), a critical brain area for imprinting acquisition (McCabe et al., 1981), makes chicks imprintable even after the sensitive period has closed. For instance, chicks injected with T_3 on day 1 can also be imprinted on days 4–8. We call these phenomena induced by T_3 injection "memory priming" (MP). Downstream of T_3, the protein Wnt-2b is involved in the memory formation of imprinting (Yamaguchi et al., 2018). Pena and colleagues recently showed that T_3 activates the mechanistic target of rapamycin (mTOR), which has been implicated in long-term potentiation (LTP) and long-term memory, in the IMM neurons and that mTOR activation by an Akt activator made day 4 chicks imprintable similar to the described T_3 effects (Batista et al., 2018). However, the roles of neurotransmitters underlying the neuronal mechanisms downstream of T_3 remain unknown. In a previous study, the exogenous injection of transmitters or hormones, e.g., norepinephrine, serotonin, dopamine, and testosterone, did not influence the imprintability of chicks, suggesting that they cannot be substituted for T_3 (Yamaguchi et al., 2012).

Because mTOR signaling impairs GABAergic transmission (Weston et al., 2012), gamma-aminobutyric acid (GABA) is a candidate for neurotransmitters involved in T_3 signaling during imprinting. Two types of GABA receptors, the ionotropic GABA-A receptor and the metabotropic GABA-B receptor, have different properties (Matsumoto, 1989) and are thought to be involved in memory processes (Venault et al., 1986; Chapouthier and Venault, 2002; Heaney and Kinney, 2016). In humans, an anterograde amnesia is caused by administration of the GABA-A receptor modulator diazepam (Lister, 1985; Mejo, 1992). In mice, learning is impaired after enhanced GABA-A signaling by diazepam. By contrast, the reduction of GABA-A signaling by an inverse agonist (methyl beta-carboline-3-carboxylate) enhances the memory processes in learning tasks. In the juvenile brains of mammals, the neural network development relies on the appropriate modulation of GABAergic neurons (Wu and Sun, 2015). In chicks, inhibitory GABAergic neurons are likely to be involved in filial imprinting. For example, the intraperitoneal injection of the GABA-A receptor modulator diazepam reduces the preference to the imprinting object (Venault et al., 1986). After 2 h of training, expression of the immediate-early gene *Fos* is increased in *Fos*-positive GABA-containing neurons of the IMM (Ambalavanar et al., 1999). In brain slices containing the left IMM, the GABA release in the presence of potassium is positively correlated with the preference score after 2 h of training (McCabe et al., 2001).

Therefore, we hypothesized that GABA-A and GABA-B receptors play a role as key determinants of the sensitive period for imprinting downstream of T_3. We predicted that the expression levels of these two types of GABA receptors change around the sensitive period and that the balance between the two receptor types contributes to the beginning and the termination of the sensitive period. In this study, we determined the levels of GABA-A and GABA-B receptors and examined whether the GABA receptors are involved in imprinting using various GABA receptor agonists and antagonists. We found through these pharmacological experiments that GABA-B receptor signaling is necessary for imprinting, while GABA-A receptor signaling suppresses imprinting acquisition. We propose that the GABA-A and GABA-B receptor balance during development influences the start and end time points of the sensitive period.

MATERIALS AND METHODS

Animals

The experiments were conducted under the guidelines of the national regulations for animal welfare in Japan and with the approval of the committee on animal experiments of Teikyo University (approval number: 12-019). In this study, 415 newly hatched domestic chicks of the Cobb strain (*G. gallus domesticus*) were used. Fertilized eggs were obtained from a local supplier (3-M, Aichi, Japan) and incubated at 37°C for 21 days. After hatching, the chicks were placed in dark plastic enclosures in a breeder at 30°C to prevent light exposure (Izawa et al., 2001).

Gene Expression Analysis Using Quantitative Reverse Transcription Polymerase Chain Reaction

Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as reported previously (Yamaguchi et al., 2008a; Takemura et al., 2018). Briefly, the telencephalons of 1-, 3-, and 5-day-old chicks reared in the dark were dissected under anesthetizing them using a ketamine (Daiichi Sankyo, Tokyo, Japan)-xylazine (Sigma-Aldrich Co., St. Louis, MO, United States) cocktail. Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, United States). Total RNA (1 μg) was treated with RNase-free DNaseI (Invitrogen) and used for quantitative RT-PCR. The relative expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers used were as follows: *GABA-A receptor subunit alpha 1* (XM_025154781), 5′-TGGGCTGGCAACCATTG -3′ (sense) and 5′- GCTTTGTTTCTGGCTTAACTTCTTTG -3′ (antisense); *GABA-B receptor subunit 2* (XM_015282399), 5′-TGACAATTTGGCTTGGGATTG -3′ (sense) and 5′-GGCTAAGAAACAACCAAATAACATCA -3′ (antisense); and *GAPDH* (XM_204305), 5′-TGGAGCCCCTGCTCTTCA-3′ (sense) and 5′-GGAACAGAACTGGCCTCTCACT-3′ (antisense).

Immunoblot Analysis

An immunoblot analysis was performed as described previously (Yamaguchi et al., 2007). In brief, the telencephalons from 0- and 5-day-old chicks reared in the dark were dissected after anesthesia. For the detection of GABA-A receptors, an anti-GABA-A receptor subunit alpha 1 rabbit polyclonal antibody was used as the primary antibody (ab33299, 1:1,500; Abcam plc, Cambridge, United Kingdom), while an anti-rabbit horseradish peroxidase-conjugated antibody (1:1,000; GE Healthcare, Chicago, IL, United States) was used as the secondary antibody. To detect GABA-B receptors, an anti-GABA-B receptor subunit 2 rabbit monoclonal antibody (ab75838, 1:1,500; Abcam plc) was used, while an anti-rabbit horseradish peroxidase-conjugated antibody (1:1,000, GE Healthcare) was used as the secondary antibody. Data of each sample were normalized to the expression of beta-actin as detected by an anti-beta-actin mouse monoclonal antibody (A5316, 1:1,000, Sigma-Aldrich Co.). The band intensities were quantified using ImageJ (National Institutes of Health, Bethesda, MD, United States), and the ratios of the band intensities were calculated.

Immunohistochemistry

Chicks on day 0 were transcardially perfused with 4% paraformaldehyde in phosphate buffered saline (PBS) under deep anesthesia using a ketamine-xylazine cocktail. The brains were post-fixed with the same fixative for 24 h and immersed in 30% sucrose in PBS. The brain tissues were then cut into 18-μm-thick sections using a cryostat. For fluorescent staining, the sections including the IMM (Kuenzel and Masson, 1988) were blocked with 3% normal pig serum for 1 h and incubated with anti-GABA-A receptor subunit alpha 1 goat polyclonal antibody (sc-31403, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, United States) and anti-GABA-B receptor subunit 2 rabbit monoclonal antibody (ab75838, 1:250; Abcam plc) for 24 h at 4°C. The sections were then incubated with Alexa Fluor 546-conjugated anti-goat antibody (1:250; Thermo Fisher Scientific K.K., Waltham, MA, United States), Alexa Fluor 488-conjugated anti-rabbit antibody (1:250; Thermo Fisher Scientific K.K.), and Hoechst 33342 (Thermo Fisher Scientific K.K.). Fluorescent images were obtained using a confocal microscope (FV-10i; Olympus, Tokyo, Japan).

In vivo Injection

The injection was performed as described previously (Yamaguchi et al., 2011) with modifications. Chicks were anesthetized with a 1% isoflurane/air mixture and mounted on a stereotaxic apparatus. The skin was cut, and a small piece of the skull's surface was incised. The dura mater was cut to expose the telencephalon. Stereotaxic coordinates for the IMM were as follows: 2.9 mm anterior to the bregma, 1.3 mm lateral to the midline, and 2.3 mm deep (Kuenzel and Masson, 1988). We slowly (13.4 nL/min) injected for 35 min GABA receptor drugs using an auto-nanoliter injector (Nanoject I; Drummond Scientific Co., Broomall, PA, United States). The GABA receptor drugs were GABA-A agonist: muscimol 5 mM (Wako Chemicals, Tokyo, Japan); GABA-A antagonist: bicuculline 5 mM (Wako), picrotoxin 5 mM (Wako); GABA-B agonist: baclofen 20 μM (Wako); GABA-B antagonist: CGP52432 1 mM (Tocris Bioscience, Bristol, United Kingdom); GABA 5 mM (Wako). The doses of the chemicals were determined with reference to Knudsen et al. (1993); Fedele et al. (1997); Campbell et al. (1999). Control chicks were subjected to a sham operation in which only the syringe was inserted into the IMM under anesthesia. The chicks were returned to the dark chamber at 30°C for 30 min to allow them to recover from the anesthesia. For the intravenous injection of baclofen, 200 μM baclofen was dissolved in PBS. For the intravenous injection of T_3, 10 μM T_3 (Sigma-Aldrich Co.) was dissolved in 0.002 M NaOH and 0.9% NaCl. In the experiment shown in **Figure 4B**, a low dose of bicuculline (0.33 mM) was injected into the IMM, and a low dose of baclofen (13.3 μM) was injected intravenously. The GABA receptor drugs used in each experiment are listed in **Supplementary Table S1**.

Behavioral Training and Testing

Training for imprinting was performed according to the method of Izawa et al. (2001) with modifications. A hand-made training chamber (8 cm wide, 43 cm long, 15 cm high) was equipped with a rubber belt controlled by a microcomputer (RCX2.0; LEGO Co., Tokyo, Japan). Thirty minutes after the injection or the sham operation, two 1-h training sessions were conducted. An imprinting object (a blue LEGO block, 4.7 cm × 6.2 cm × 5.0 cm) was in one side of the training chamber. During training, the imprinting object rotated clockwise and anticlockwise repeatedly for 30 s with pauses of 10 s in between and was illuminated by a 100 W fiber optic light during the rotation. An infrared sensor was placed 20 cm in front of the imprinting object. If the chicks crossed the sensor, the belt moved toward the opposite side of the imprinting object, they did so again and again. We

counted how many times the chicks crossed the infrared sensor during the training. The chick was not tested if the number was <500 for the sum of two training sessions. In our experiments, the injection of various chemicals did not impair the locomotor activities of the injected chicks. The locomotor activity was measured as previously described (Yamaguchi et al., 2012). In the simultaneous choice test, we used a T-maze with a 20-cm-long main arm and a 69-cm-long sidearm. The imprinting object (a blue LEGO block) and a novel control object (a brown LEGO block) were positioned at the end of each sidearm of the T-maze. After a chick started from the main arm, we counted the stay time of the approach area of each object during testing for 120 s. Except for the time the chicks stayed in the approach areas, they spent time in the intermediate area between two approach areas. We ran the tests four times and averaged the approach time. We then calculated a preference score by subtracting the approach time of the control object from the approach time of the imprinting object. After the behavioral experiments, the animals were sacrificed with an overdose of isoflurane.

Statistical Analyses

For statistical analyses, we used R software for Windows (version 3.3.2; The R Foundation for Statistical Computing, Vienna, Austria) as previously described (Yamaguchi et al., 2018) or MATLAB for Windows (The Mathworks, Inc., Natick, MA, United States). Gene expression data are reported as mean ± standard error of the mean (SEM). All other data

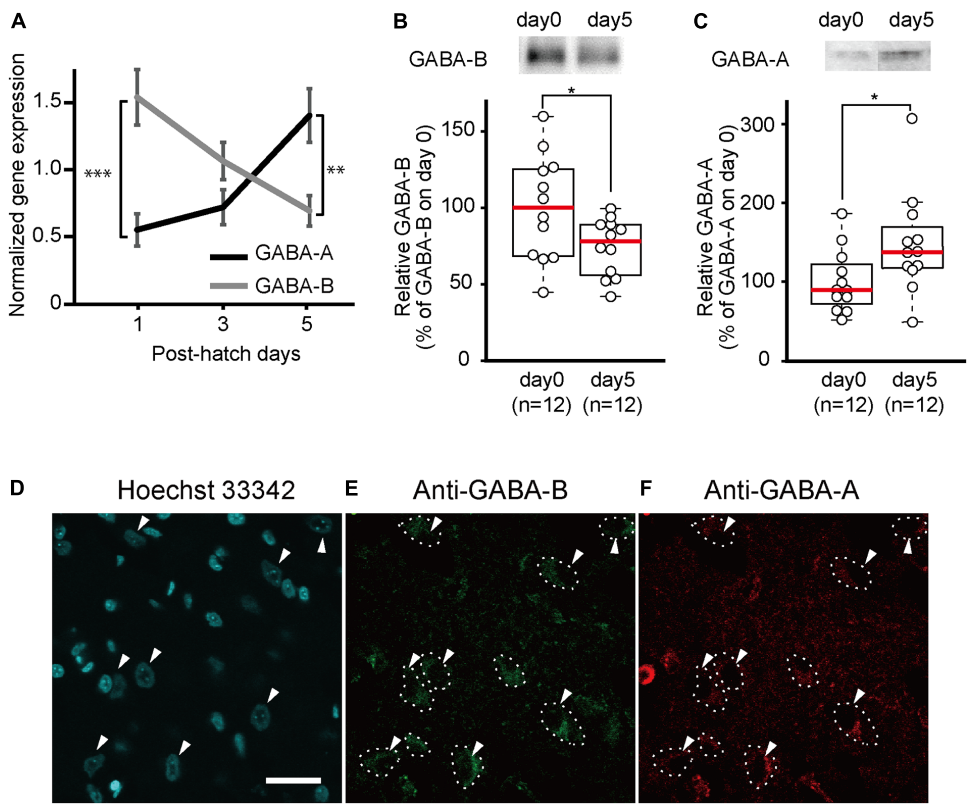

FIGURE 1 | GABA-A receptor expression increases and GABA-B receptor expression decreases between day 1 and day 5. **(A)** Gene expression of GABA-A and GABA-B receptors in the telencephalons of 1-day-old (GABA-A: $n = 8$; GABA-B: $n = 9$), 3-day-old (GABA-A: $n = 8$; GABA-B: $n = 9$), and 5-day-old (GABA-A: $n = 7$; GABA-B: $n = 7$) chicks measured by quantitative RT-PCR. Black and gray indicates gene expression of GABA-A and GABA-B receptors, respectively. On day 1, the expression level of the GABA-B receptor is higher than that of the GABA-A receptor. The gene expression of the GABA-A receptor increases from days 1 to 5, whereas the gene expression of the GABA-B receptor decreases. On day 5, the GABA-A receptor expression level is higher than that of the GABA-B receptor. The gene expression was normalized to that of GAPDH [two-way analysis of variance; factor A: type of receptor; factor B: day, $F_{A\ (1,42)} = 2.52$, n.s.; $F_{B\ (2,42)} = 0.64$, n.s.; $F_{interaction\ (2,42)} = 14.42$, $p < 0.001$; $F_{A\_day1(1,42)} = 19.15$, $^{***}p < 0.001$; $F_{A\_day3(1,42)} = 2.31$, n.s.; $F_{A\_day5(1,42)} = 9.90$, $^{**}p < 0.01$; $F_{B\_GABA-A(2,42)} = 7.99$, $p < 0.01$; $F_{B\_GABA-B(2,42)} = 7.07$, $p < 0.01$]. **(B)** The protein expression levels of GABA-B receptors measured by immunoblotting. The expression of GABA-B receptors is presented as the percentage of the average GABA-B receptor expression on day 0. GABA-B receptors are significantly more expressed on day 0 than on day 5. (t-test, $t = 2.18$; $^{*}p < 0.05$). **(C)** The protein expression of GABA-A receptors measured by immunoblotting. The expression levels of GABA-A receptors are shown as the percentage of the average GABA-A receptor expression on day 0. GABA-A receptor levels are significantly higher on day 5 compared to day 0. (t-test, $t = 2.26$, $^{*}p < 0.05$). Images in **(B,C)** have been spliced together for illustrative purposes. The original data are shown in **Supplementary Figure S1**. **(D–F)** A sample was stained with Hoechst 33342 and immunostained with anti-GABA-A and anti-GABA-B antibodies. **(D)** The cell nuclei in the IMM are stained by Hoechst 33342. The arrowheads indicate the cell body of putative projection neurons. **(E)** The neurons in which GABA-B receptors are expressed are enclosed by dashed lines. **(F)** Dashed lines indicate neurons in which GABA-A receptors are expressed. GABA-A receptors are expressed in the same neurons as in **(E)**. Scale bar, 20 μm. n.s., not significant.

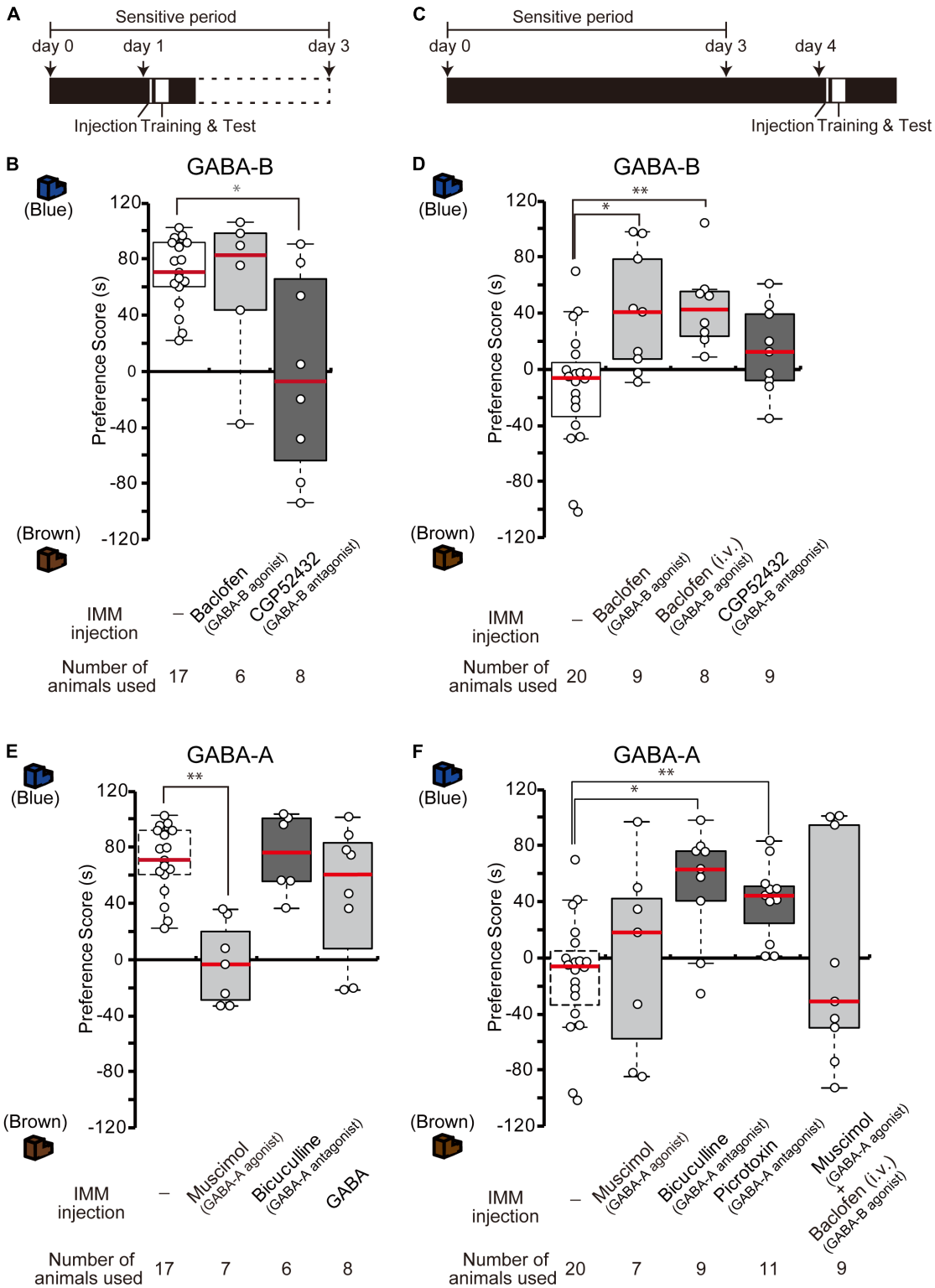

FIGURE 2 | GABA-B receptors are necessary for imprinting, while GABA-A receptors suppress imprinting. **(A)** Schematic representation of the experimental schedule used in **(B,E)**. The chicks were bilaterally injected with drugs into the IMM right before training on day 1 during the sensitive period. **(B)** The chicks were injected with GABA-B receptor agonist or antagonist before training on day 1. The preference scores of the chicks injected with the GABA-B receptor antagonist CGP52432 are significantly lower than those of sham control chicks (Steel's test, $t = 2.38$, $^*p < 0.05$). The preference scores of chicks injected with the GABA-B

(Continued)

FIGURE 2 | Continued
receptor agonist baclofen are not significantly different from those of control chicks (Steel's test, $t = 0.56$, n.s.). **(C)** Representation of the experimental design used in **(D,F)**. The chicks were injected with drugs into the IMM or intravenously right before the first training on day 4. **(D)** The chicks were injected with a GABA-B receptor agonist or antagonist before training on day 4. The preference scores of the chicks injected with the GABA-B receptor agonist baclofen into the IMM or intravenously were significantly higher than those of the control chicks (Steel's test, IMM, $t = 2.81$, $^{*}p < 0.05$; intravenously, $t = 3.20$, $^{**}p < 0.01$). The preference scores of chicks injected with the GABA-B receptor antagonist CGP52432 do not differ from those of control chicks (Steel's test, $t = 1.58$, n.s.). **(E)** The chicks were injected with a GABA-A receptor agonist or antagonist before training on day 1. The preference scores of the chicks that were injected with the GABA-A receptor agonist muscimol are significantly lower than those of the control chicks (Steel's test, $t = 3.53$, $^{**}p < 0.01$). The preference scores of the chicks injected with the GABA-A receptor antagonist bicuculline or GABA are not significantly different from those of the control chicks (Steel's test, bicuculline, $t = 0.56$, n.s.; GABA, $t = 0.81$, n.s.). The sham control chicks' data shown in **(B)** are duplicated for comparison. **(F)** The chicks were injected with a GABA-A receptor agonist or antagonist before training on day 4. The preference scores of the chicks injected with either the GABA-A receptor antagonist bicuculline or picrotoxin are significantly higher than those of the sham control chicks (Steel's test, bicuculline, $t = 2.96$, $^{*}p < 0.05$; picrotoxin, $t = 3.61$, $^{**}p < 0.01$). The preference scores of chicks that were injected with the GABA-A receptor agonist muscimol are not different from those of the control chicks (Steel's test, $t = 0.66$, n.s.). The preference scores of chicks that were injected with both the GABA-A agonist muscimol and the GABA-B agonist baclofen are not different from those of the control chicks (Steel's test, $t = 0.18$, n.s.). The data of the control chicks shown in **(D)** are duplicated here for comparison. n.s., not significant.

are presented as box plots. The number of animals used is indicated in each figure or legend. The equality of variance of each data point was checked by the *F*-test or Bartlett's test. Since variances were not different in the quantitative RT-PCR data, we used the parametric two-way analysis of variance. Since variances were not different in the immunoblotting data, we used Student's *t*-test. Since variances were significantly different in some data of the behavioral experiments, we used as a non-parametric test Steel's multiple comparisons. *p*-values < 0.05 were considered significantly different. The *p*-values are shown in the **Supplementary Table S2**. We also determined Cohen's *d* or η^2 as effect size in the parametric analysis (Cohen, 1988). To determine the *r* value as the effect size for the non-parametric analysis, we calculated it from the *Z*-value of the Mann–Whitney *U* test according to the following formula: $r = Z/\sqrt{n}$. The effect sizes are shown in the **Supplementary Table S3**.

RESULTS

Developmental Changes in the Gene Expression of GABA Receptors

To measure the developmental changes in GABA-A and GABA-B receptor gene expressions after hatching, we conducted quantitative RT-PCRs. RNA was extracted from the brains of newborn chicks at days 1, 3, and 5. On day 1, the gene expression of the GABA-B receptor was significantly higher than that of the GABA-A receptor (**Figure 1A**). From days 1 to 5, the gene expression of the GABA-A receptor gradually increased, whereas that of the GABA-B receptor decreased. On day 5, the gene expression of the GABA-A receptor was significantly higher than that of the GABA-B receptor.

Developmental Changes in the Protein Expression of GABA Receptors

To measure the developmental changes in GABA-A and GABA-B receptors in the telencephalon on days 0 and 5, we conducted immunoblotting using antibodies directed against GABA-A or GABA-B receptors. The amount of GABA-B receptors on day 0 in the telencephalon was significantly higher than that on day 5 (**Figures 1B** and **Supplementary Figure S1A**). In contrast, the amount of GABA-A receptors was significantly higher on day 5 than that on day 0 (**Figures 1C** and **Supplementary Figure S1B**). These results were consistent with the gene expression according to the quantitative RT-PCR experiments. This led us to the assumption that abundant GABA-B receptors on day 0 may facilitate imprinting at the start of the sensitive period while GABA-A receptors on day 5 suppress imprinting at the end of the sensitive period.

Expression of GABA-A and GABA-B Receptors in IMM Neurons

Neurons in the IMM, a brain region responsible for imprinting acquisition, may express both GABA-A and GABA-B receptors and have opposing roles in imprinting. We conducted immunostaining using anti-GABA-A or anti-GABA-B antibody in brain slices containing the IMM region. Cell nuclei were stained by Hoechst 33342. Two types of cells in the IMM were distinguished based on their size (**Figure 1D**). Neurons with a cell body diameter > 15 μm were putatively projection neurons (Patel and Stewart, 1988; Tombol et al., 1988). These neurons in the IMM project to the arcopallium (Bradley et al., 1985) and intermediate hyperpallium apicale (IMHA) (Aoki et al., 2015). Among them, the pathway from the IMM to the IMHA plays critical roles in imprinting acquisition and recall (Aoki et al., 2015). As shown in **Figures 1E,F**, the majority of the larger neuronal cells expressed both GABA-A and GABA-B receptors, suggesting that the two receptor types may interact in the projection neurons to modulate the input from T_3 in the IMM.

Effects of GABA-B Receptor Agonists and Antagonists on Imprinting

The results from the GABA-B receptor expression experiment suggest that the abundant GABA-B receptors on day 1 may mainly mediate and facilitate imprinting. To examine whether the blockade of GABA-B receptors prevents day 1 chicks from imprinting, a GABA-B receptor antagonist (CGP52432) was injected into the IMM right before the training on day 1 (**Figure 2A**). The preference scores of chicks injected with the antagonist CGP52432 were significantly lower than those of the sham control chicks (**Figure 2B**). The preference

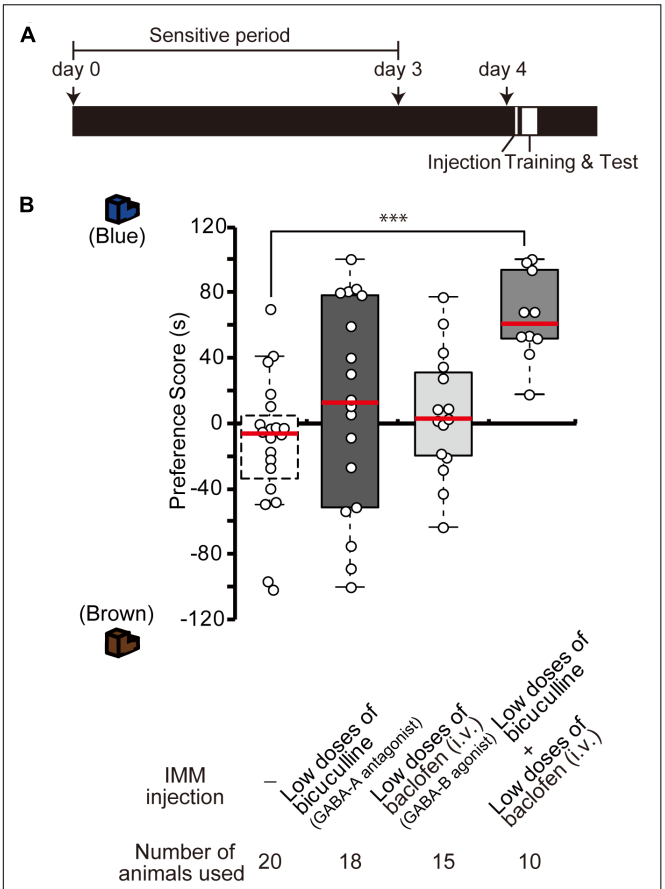

FIGURE 3 | Interference of GABA-A and GABA-B signaling during imprinting. **(A)** Schematic representation of the experimental schedule used in **(B)**. The chicks were injected with drugs into the IMM or intravenously right before the first training on day 4. **(B)** The chicks were injected before training on day 4. The preference scores of the chicks injected with low doses of the GABA-A receptor antagonist bicuculline or low doses of the GABA-B receptor agonist baclofen are not different from those of control chicks (Steel's test, bicuculline, $t = 1.29$, n.s.; baclofen, $t = 1.37$, n.s.). The preference scores of chicks that were injected with low doses of both chemicals are significantly higher than those of the control chicks (Steel's test, $t = 3.96$, $^{***}p < 0.001$). The data of sham control chicks shown in **Figure 2D** are duplicated for comparison. n.s., not significant.

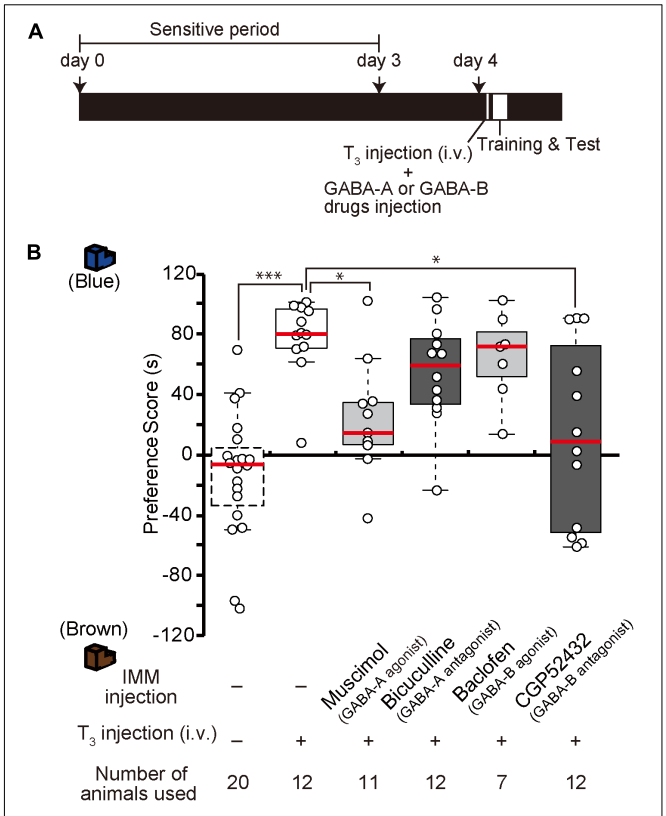

FIGURE 4 | Enhancement of GABA-A signaling or reduction of GABA-B signaling on day 4 impairs the imprintability on day 4 induced by T_3 injection on day 4. **(A)** The experimental schedule is shown schematically. GABA-A or GABA-B drugs were injected bilaterally into the IMM, and T_3 was injected intravenously before the first training on day 4 after the sensitive period. **(B)** The preference scores of the chicks that were intravenously injected with T_3 on day 1 are significantly higher than those of the control chicks (Steel's test, $t = 4.43$, $^{***}p < 0.001$). The preference scores of the chicks injected with the GABA-A receptor agonist muscimol and T_3 are significantly lower than those of T_3-injected chicks (Steel's test, $t = 2.89$, $^{*}p < 0.05$). The preference scores of the chicks injected with the GABA-B receptor antagonist CGP52432 and T_3 are also significantly lower than those of T_3-injected chicks (Steel's test, $t = 2.59$, $^{*}p < 0.05$). The preference scores of the chicks injected with both the GABA-A receptor antagonist bicuculline and T_3 or both the GABA-B receptor agonist baclofen and T_3 are not different from those of the T_3-injected chicks (Steel's test, bicuculline, $t = 1.84$, n.s.; baclofen, $t = 0.92$, n.s.). The data of the sham control chicks shown in **Figure 2D** are duplicated here for comparison. n.s., not significant.

scores of chicks injected with the agonist baclofen were not significantly different from those of the control chicks (**Figure 2B**). These results suggest that the molecular signaling of GABA-B receptor is necessary for imprinting acquisition on day 1.

We previously showed that the T_3 levels in the brain decrease until day 4 after hatching, but that exogenous T_3 injection extends the imprintable period even beyond the end of the sensitive period on day 4 (Yamaguchi et al., 2012). As the expression of GABA-B receptors also decreases until day 4, this decrease might be related to the end of the imprintable period. To examine whether the functional enhancement of GABA-B receptor makes day 4 chicks imprintable without an exogenous T_3 administration, we injected the GABA-B receptor agonist baclofen into the IMM right before the training on day 4 (**Figure 2C**). The preference scores of the chicks injected with the agonist baclofen were significantly higher than those of the sham control chicks (**Figure 2D**). The intravenous injection of baclofen showed a similar effect on day 4 chicks (**Figure 2D**). These findings suggest that the GABA-B receptor agonist baclofen made day 4 chicks imprintable without exogenous T_3 application. On the other hand, the preference scores of chicks injected with the antagonist CGP52432 were not significantly different from those of control chicks (**Figure 2D**). Taken together, these results suggest that the GABA-B receptor agonist baclofen substituted for the role of T_3 and that GABA-B receptor signaling was downstream of T_3.

Effects of GABA-A Receptor Agonists and Antagonists on Imprinting

Due to the low expression of GABA-A receptors on day 1, these receptors may perform a different role than GABA-B receptors in the course of imprinting. To identify the role of GABA-A receptors, we injected the GABA-A receptor agonist muscimol into the IMM right before imprinting training on day 1 (**Figure 2A**). The preference scores of the chicks injected with the agonist muscimol were significantly lower than those of the sham control chicks (**Figure 2E**). By contrast, the preference scores of chicks injected with the GABA-A receptor antagonist bicuculline were not significantly different from those of control chicks (**Figure 2E**). These results suggest that an enhancement of GABA-A receptors suppresses imprinting processes. Furthermore, we injected GABA into the IMM right before imprinting training on day 1 (**Figure 2A**); however, the preference scores of the chicks injected with GABA were not significantly different from those of sham control chicks (**Figure 2E**). This result indicates that imprintability on day 1 does not depend on the GABA concentration but rather on the GABA-B receptor expression. GABA-A receptors might not have shown their suppressive role in imprinting due to their insufficient expression levels on day 1.

By contrast, the increased expression of GABA-A receptors on day 4 may prevent imprinting in chicks. Thus, we examined whether GABA-A receptor blockade would influence imprinting in chicks on day 4. The GABA-A receptor antagonists bicuculline or picrotoxin were injected into the IMM right before training on day 4 (**Figure 2C**). The preference scores of the chicks injected with these GABA-A receptor antagonists were higher than those of sham control chicks (**Figure 2F**). The preference scores of chicks injected with the GABA-A receptor agonist muscimol were not significantly different from those of control chicks (**Figure 2F**). These results demonstrate that a reduction in GABA-A receptor signaling made day 4 chicks imprintable without administration of T_3 and that GABA-A receptor signaling was downstream of T_3. When we injected both the GABA-A agonist muscimol and the GABA-B agonist baclofen at the same time, the chicks could not be imprinted (**Figure 2F**), probably because the ability of GABA-B receptors to accelerate imprinting was erased by the suppressive role of GABA-A receptors.

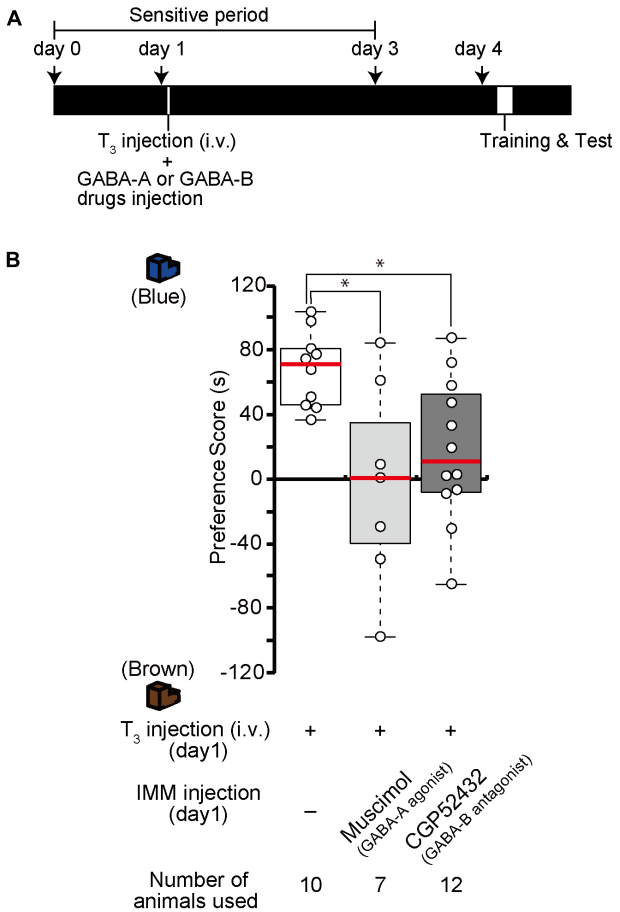

FIGURE 5 | Enhanced GABA-A signaling or reduced GABA-B signaling on day 1 impairs memory priming on day 4 induced by T_3 administration on day 1. **(A)** The experimental design is shown schematically. The chicks were injected with the GABA-A agonist muscimol or the GABA-B antagonist CGP52432 into the IMM and intravenously injected with T_3 on day 1, then trained and tested on day 4. **(B)** The preference scores of the chicks that were injected with both T_3 and the GABA-A receptor agonist muscimol are significantly lower than those of T_3-injected chicks (Steel's test, $t = 2.24$, $^*p < 0.05$). The preference scores of the chicks that were injected with T_3 and the GABA-B receptor antagonist CGP52432 are also significantly lower than those of T_3-injected chicks (Steel's test, $t = 2.63$, $^*p < 0.05$).

Interaction Between the Roles of GABA-A and GABA-B Receptors in Imprinting

To examine whether GABA-A receptor antagonist and GABA-B receptor agonist influence synergistically the imprinting in chicks, day 4 chicks were injected with low doses of the GABA-B agonist baclofen and the GABA-A antagonist bicuculline (**Figure 3A**). Either drug alone did not influence the imprinting (**Figure 3B**), but the combination of the two low-dose drugs clearly modulated the imprinting in chicks (**Figure 3B**). This indicates that GABA-A and GABA-B signaling interact synergistically with each other to enable imprinting.

Effects of GABA Receptor Drugs on the Imprintability Induced by T_3 Administration Before Training on Day 4

In our previous study, we found that after T_3 administration chicks are imprintable even beyond the sensitive period (Yamaguchi et al., 2012). To examine whether GABA-A receptor signaling is downstream of T_3, the GABA-A receptor agonist muscimol was injected into the IMM, and T_3 was injected intravenously right before training on day 4 (**Figure 4A**). The preference scores of chicks injected with both the GABA-A receptor agonist and T_3 were significantly lower than those of control chicks injected with T_3 alone (**Figure 4B**). This result means that GABA-A receptor signaling impaired the

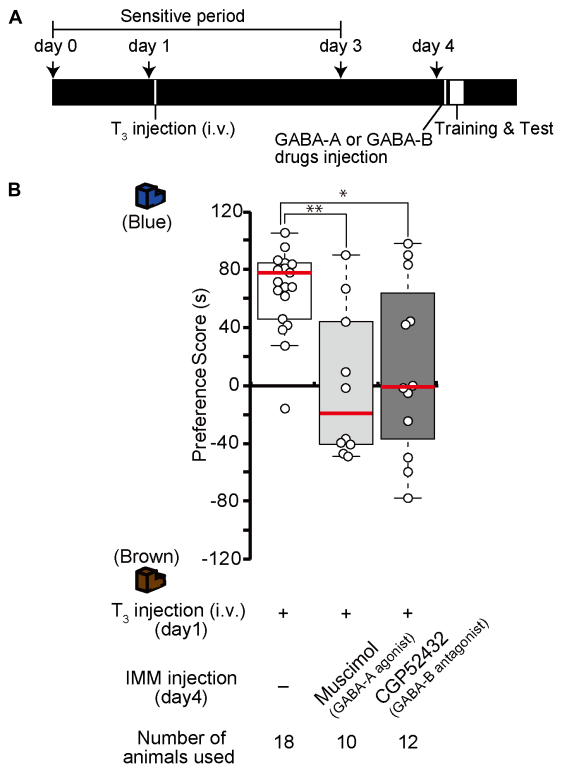

FIGURE 6 | Enhancement of GABA-A signaling or reduction of GABA-B signaling on day 4 impairs memory priming on day 4 induced by T_3 injection on day 1. **(A)** Schematic representation of the experimental design. The chicks were intravenously injected with T_3 on day 1 and bilaterally injected with the GABA-A agonist muscimol or the GABA-B antagonist CGP52432 into the IMM shortly before a first training on day 4. **(B)** The preference scores of the chicks that were injected with T_3 and the GABA-A receptor agonist muscimol are significantly lower than those of the T_3-injected chicks (Steel's test, $t = 2.97$, $^{**}p < 0.01$). The preference scores of the chicks that were injected with T_3 and the GABA-B receptor antagonist CGP52432 are also significantly lower than those of T_3-injected chicks (Steel's test, $t = 2.24$, $^{*}p < 0.05$).

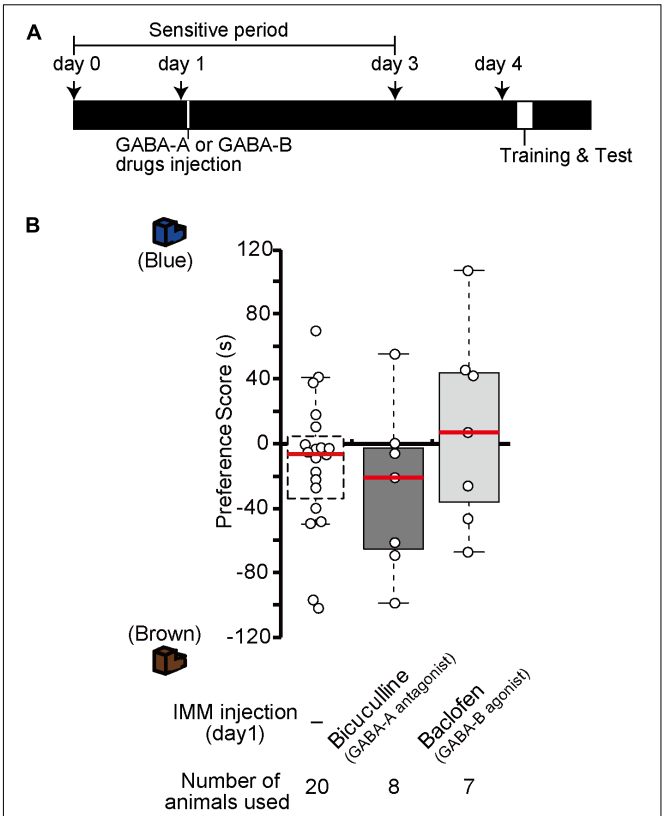

FIGURE 7 | In the absence of exogenous T_3, GABA-A receptor antagonists or GABA-B receptor agonists do not induce memory priming. **(A)** The schedule of the experiment. The chicks were injected with the GABA-A antagonist bicuculline or the GABA-B agonist baclofen into the IMM on day 1 and trained and tested on day 4. **(B)** The preference scores of the chicks injected with the GABA-A antagonist bicuculline or the GABA-B agonist baclofen are not different from those of the control chicks (Steel's test, bicuculline, $t = 0.86$, n.s.; baclofen, $t = 0.83$, n.s.). The data of sham control chicks shown in **Figure 2D** are duplicated for comparison. n.s., not significant.

imprintability induced by T_3 and was downstream of T_3. To examine whether the GABA-B receptor signaling is also downstream of T_3, the GABA-B receptor antagonist CGP52432 was injected into the IMM, while T_3 was intravenously injected right before the training on day 4. The preference scores of chicks injected with both the GABA-B receptor antagonist and T_3 were significantly lower than those of control chicks injected with T_3 alone (**Figure 4B**). This result means that GABA-B receptor signaling downstream of T_3 is necessary for acquiring imprinting. The preference scores of chicks injected with both the GABA-A receptor antagonist bicuculline and T_3 or both the GABA-B receptor agonist baclofen and T_3 were not different from those of T_3-injected chicks.

Effects of GABA Receptor Drugs Before T_3 Injection on Day 1 to Induce MP

The effects of one injection of exogenous T_3 on imprinting were shown to last for more than 1 week (Yamaguchi et al., 2012). We call this phenomenon MP. To examine whether GABA-A receptor agonists or GABA-B receptor antagonists impair MP, we injected the GABA-A receptor agonist muscimol or the GABA-B receptor antagonist CGP52432 prior to the intravenous administration of T_3 on day 1 (**Figure 5A**). The preference scores of these chicks were lower than those of the T_3-injected control chicks (**Figure 5B**). These results indicate that GABA-A or GABA-B receptor signaling is involved at an earlier phase of MP.

Effects of GABA Receptor Drugs on Day 4 Chicks Injected With T_3 on Day 1

Because the effects of T_3 injection on imprinting last for more than 1 week, structural and/or neural changes may occur in the IMM after T_3 injection. To examine whether GABA-A receptor agonists or GABA-B receptor antagonists impair imprinting after such structural changes have already occurred in the brain, chicks were intravenously injected with T_3 on day 1 and injected with the GABA-A receptor agonist

muscimol or the GABA-B receptor antagonist CGP52432 into the IMM right before the training on day 4 (**Figure 6A**). The preference scores of these chicks were lower than those of T_3-injected control chicks (**Figure 6B**). These results suggest that GABA-A or GABA-B receptor signaling is involved at a later stage of MP execution just before the imprinting training in addition to its role at the earlier step of MP described above.

GABA Receptors as MP Executor in Imprinting

Injection of GABA-A receptor antagonists or GABA-B receptor agonists before the training made chicks imprintable on day 4 without T_3 administration (**Figure 2**). To examine whether the effects of GABA-A receptor antagonists or GABA-B receptor agonists lasts for 4 days similar to the T_3 effects in MP, we injected the GABA-A receptor antagonist bicuculline or the GABA-B receptor agonist baclofen on day 1, then trained and tested the chicks on day 4 (**Figure 7A**). The preference scores of these chicks were not different from those of the sham control chicks (**Figure 7B**). This result shows that the effects of either GABA-A receptor antagonists or GABA-B receptor agonists do not last for 4 days such as in MP. This indicates that GABA-B receptor signaling is necessary for acquiring the imprinting ability downstream of T_3 but insufficient to induce MP. Most likely, GABA-B receptor signaling is only partly involved in T_3 signaling, e.g., in neural transmission or intracellular molecular signaling, but not in T_3-induced structural changes of neurons.

DISCUSSION

Chicks become imprintable to recognize their mothers and siblings at the appropriate time of the sensitive period. During that period, intracerebral T_3 levels are critical to induce imprinting. Here, we showed using pharmacological approaches that the inhibitory neurotransmitter GABA contributes to the imprinting process via two types of receptors downstream of T_3. Both ionotropic GABA-A and metabotropic GABA-B receptors play important roles at the start and the end of the imprinting-sensitive period. GABA binds to GABA-A and GABA-B receptors with similar affinities (Enna and McCarson, 2013). This suggests that the balance in GABA-A and GABA-B receptor expression can be a critical factor for mediating the intracellular signaling in the course of imprinting. This idea is consistent with the

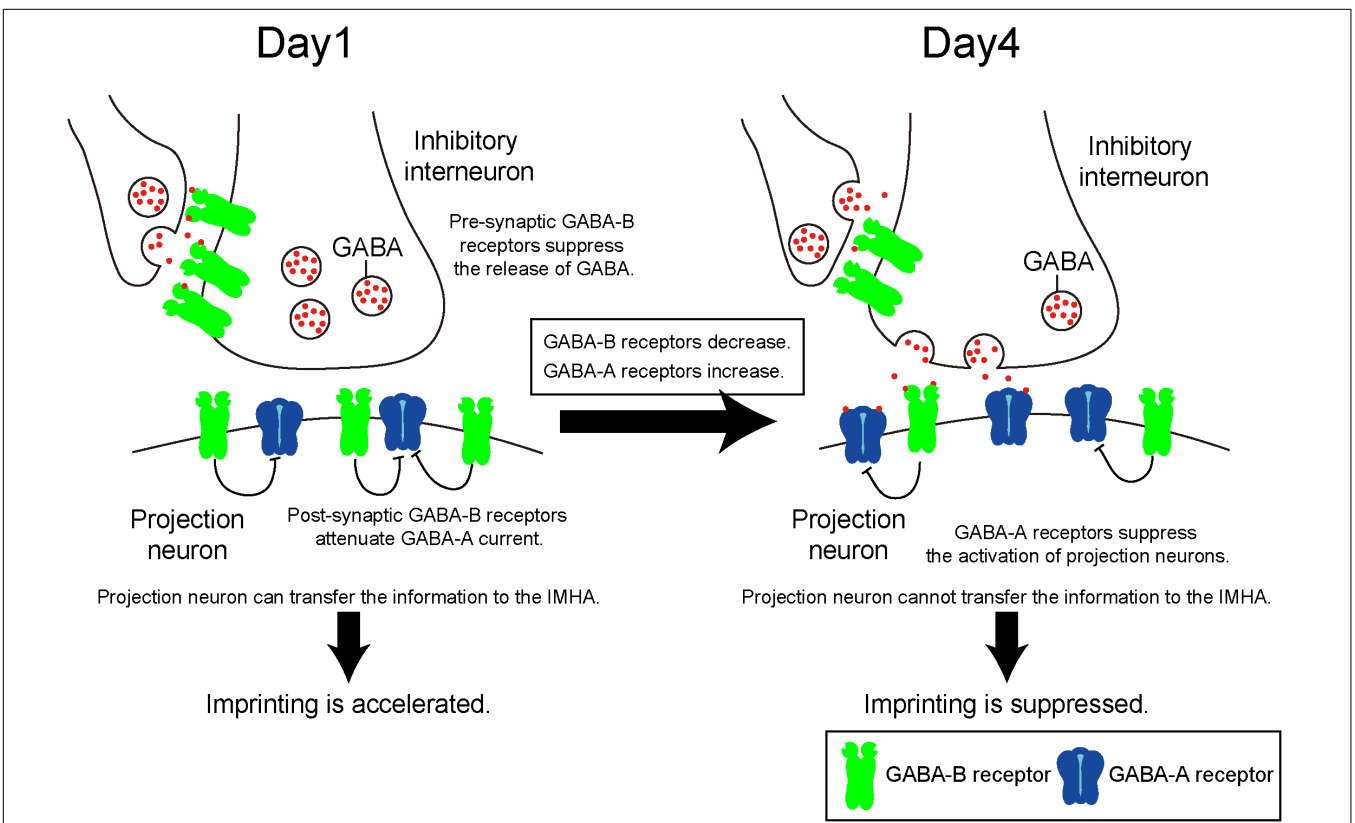

FIGURE 8 | Quantitative balance of GABA-A and GABA-B receptors in the IMM determines the induction and termination of the sensitive period of imprinting. On day 1, GABA-B receptors are abundant compared to GABA-A receptors. Both pre- and post-synaptic GABA-B receptors may impair currents evoked by GABA-A receptor. Pre-synaptic GABA-B receptors suppress GABA release to projection neurons. Post-synaptic GABA-B receptors attenuate GABA-A receptor currents. From day 1 to day 4, the expression levels of GABA-B receptors decrease over time, while those of GABA-A receptors increase. Thus, GABA-A receptors become dominant and suppress neural activities of the projection neurons.

experiments using chicks injected with various receptor agonists and antagonists in the present paper. During the development, the role of GABA in imprinting is likely to shift as the quantitative balance of the two GABA receptors changes over time. As a consequence, GABA-B receptor signaling in the IMM facilitates imprinting behavior on day 1, while GABA-A receptor signaling suppresses imprinting on day 4. Considering that the GABA-B receptor is abundant on day 1, it may be involved in the start of the sensitive period. By contrast, the GABA-A receptor is abundant on day 5, suggesting that it may be involved in the termination of the sensitive period.

GABA-A receptors in mice function as post-synaptic Cl^- permeable heteropentameric ion channels that cause hyperpolarization (Jacob et al., 2008). The action of GABA-A receptors leads to a decreased depolarization induced by glutamatergic receptors, which is involved in the LTP that accompanies learning and memory. A previous paper has shown that imprinting in chicks is impaired after intraperitoneal injection of the GABA-A receptor modulator diazepam (Venault et al., 1986). In the present study, the GABA-A agonist muscimol suppressed imprinting behavior, and the expression levels of GABA-A receptors increased until day 5 when the sensitive period had already ended. These findings suggest that increased GABA-A receptor signaling suppresses IMM neuron activation, which impairs imprinting and terminates the sensitive period (**Figure 8**).

By contrast, the GABA-B receptor is a G protein–coupled receptor that is pre- and post-synaptically expressed (Gassmann and Bettler, 2012). Pre-synaptic GABA-B receptors reduce the Ca^{2+} influx through voltage-gated calcium channels, which inhibits neurotransmitter release. Accordingly, pre-synaptic GABA-B receptors reduce GABA release to post-synaptic GABA receptors (Deisz and Prince, 1989), suppressing the inhibitory action of post-synaptic GABA-A receptors. In addition, an electrophysiological experiment revealed that post-synaptic GABA-B receptors suppress the inhibitory action of GABA-A receptors of neurons in the mammalian amygdala and retina (Shen et al., 2017). Taken together, we hypothesize that during imprinting pre- and post-synaptic GABA-B receptors suppress the post-synaptic function of GABA-A receptors in different ways (**Figure 8**).

Thyroid hormone receptors are expressed in neurons of the IMM (Yamaguchi et al., 2012). T_3 may mediate both GABA-A and GABA-B receptor signaling to facilitate imprinting in chicks. T_3 reportedly reduces GABA-A receptor-evoked currents in the mammalian brain (Puia and Losi, 2011), suggesting that it may directly reduce the electrophysiological activity of GABA-A receptors. On the other hand, the phosphorylation level of nucleoside-diphosphate kinase 2 (NDPK2) is upregulated by T_3 (Yamaguchi et al., 2016). NDPK2 is known to function downstream of the phosphoinositide 3-kinase (PI_3K), which sends signals to open K^+ channels (Srivastava et al., 2009), resulting in an enhanced post-synaptic GABA-B action. T_3 may activate GABA-B receptors in projection neurons that were suppressed by GABA-A receptors.

Using immunostaining, we revealed that a significant number of larger neurons in the IMM are putative projection cells (Patel and Stewart, 1988; Tombol et al., 1988) that express both GABA-A and GABA-B receptors. This finding suggests that they are the projection neurons that receive GABA secreted from the pre-synapses of inhibitory interneurons. Projection neurons in the IMM project to the arcopallium and the IMHA (Bradley et al., 1985; Aoki et al., 2015). Our recent study shows that the neural connections from the IMM to the IMHA are critical for memory formation and recall in imprinting (Aoki et al., 2015). Information is likely to be transferred from the IMM to the IMHA neurons through the action of Wnt protein mediated by GABA receptors signaling, which is involved in the memory formation of imprinting (Yamaguchi et al., 2018). Additionally, mTOR whose activity is mediated by Wnt signaling (Ma et al., 2011) was recently demonstrated as an intracellular mediator downstream of T_3 signaling in the course of imprinting (Batista et al., 2018). This suggests that GABA receptor signaling in the IMM mediates mTOR in IMHA neurons through Wnt protein signaling to induce imprinting.

Exogenous T_3 administration induces imprintability even after the sensitive period has ended and extends the sensitive period for more than 1 week (Yamaguchi et al., 2012). In this study, GABA-B signaling was necessary for MP, while GABA-A signaling suppressed MP. However, chicks injected with either a GABA-B agonist or a GABA-A antagonist on day 1 could not be imprinted on day 4, indicating that both drugs fail to induce MP without the support of T_3. Thus, GABA receptors are necessary but not sufficient for MP completion. GABA-A receptor antagonist and GABA-B receptor agonist may not induce in IMM neurons the structural changes that are necessary to accomplish MP.

CONCLUSION

In the current study, we demonstrated that metabotropic GABA-B receptor signaling in the IMM is necessary for the acquisition of imprinting behavior, while ionotropic GABA-A receptor signaling suppresses imprinting. The quantitative balance between GABA-A and GABA-B receptors determines the duration of the imprinting-sensitive period. On day 1, when GABA-B receptors are abundant, chicks can be imprinted. By contrast, on day 4, when the GABA-A receptor expression level increases, chicks cannot be imprinted. Thereby, developmental changes in the GABA receptor balance determine the opening and the closing of the sensitive period.

AUTHOR CONTRIBUTIONS

NA designed the study, conducted experiments, and wrote the manuscript. KH contributed to interpretation of data and wrote the manuscript. SY, TF, CM, EF, and TM contributed to data collection and interpretation and critically reviewed the manuscript. All authors approved the final version of the manuscript, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01837/full#supplementary-material

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Searching for Face-Category Representation in the Avian Visual Forebrain

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Visual information is processed hierarchically along a ventral ('what') pathway that terminates with categorical representation of biologically relevant visual percepts (such as faces) in the mammalian extrastriate visual cortex. How birds solve face and object representation without a neocortex is a long-standing problem in evolutionary neuroscience, though multiple lines of evidence suggest that these abilities arise from circuitry fundamentally similar to the extrastriate visual cortex. The aim of the present experiment was to determine whether birds also exhibit a categorical representation of the avian face-region in four visual forebrain structures of the tectofugal visual pathway: entopallium (ENTO), mesopallium ventrolaterale (MVL), nidopallium frontolaterale (NFL), and area temporo-parieto-occipitalis (TPO). We performed electrophysiological recordings from the right and left hemispheres of 13 pigeons while they performed a Go/No-Go task that required them to discriminate between two sets of stimuli that included images of pigeon faces. No neurons fired selectively to only faces in either ENTO, NFL, MVL, or TPO. Birds' predisposition to attend to the local-features of stimuli may influence the perception of faces as a global combination of features, and explain our observed absence of face-selective neurons. The implementation of naturalistic viewing paradigms in conjunction with electrophysiological and fMRI techniques has the potential to promote and uncover the global processing of visual objects to determine whether birds exhibit category-selective patches in the tectofugal visual forebrain.

Keywords: conspecific recognition, extrastriate cortex, tectofugal pathway, face-selective neurons, face cell, face-category, pigeon visual forebrain, object representation

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INTRODUCTION

Birds derived their visual forebrain structures from an archosaur reptile (diapsid amniotes including living crocodilians and extinct dinosaurs) roughly 320 million years ago (Jarvis et al., 2005). Accordingly, the avian visual system exhibits a nuclear organisation that bears almost no resemblance to the six-layered mammalian neocortex, and is instead composed of densely clustered neuronal cell bodies (Reiner et al., 2004; Briscoe et al., 2018). The organisation of the mammalian neocortex is heavily implicated with the emergence of mammals' advanced capability to integrate complex sensory information for the robust perception and recognition of visual objects, particularly for faces (Quiroga, 2016; Hawkins et al., 2017). Despite the absence of a laminar neocortex, birds also exhibit a remarkable ability to perceive and recognise the faces of conspecifics

(Watanabe and Ito, 1990; Nakamura et al., 2003; Patton et al., 2010) and humans (Soto and Wasserman, 2011; Marzluff et al., 2012) across transformations in lighting, distance and viewpoint. The neuronal mechanisms by which visual categories are represented in the avian visual system is currently undetermined, but likely arises from circuitry that is homologous with associative cell-types found in the mammalian extrastriate visual cortex (Atoji and Karim, 2014; Briscoe et al., 2018).

In mammals, incoming visual information from primary visual areas is processed hierarchically along a ventral ('what') pathway that terminates in an extrastriate region known as the anterior inferior-temporal (IT) cortex (Ungerleider and Haxby, 1994; Freiwald and Tsao, 2010). Increasingly complex and view invariant representations of biologically relevant object categories, such as scenes (Kornblith et al., 2013), body parts (Pinsk et al., 2009), and faces (Tsao et al., 2003; Freiwald and Tsao, 2010) emerge in extrastriate cortex. For example, electrophysiological recordings from six functionally connected regions (known as 'face-patches') of macaque extrastriate cortex contain populations of neurons that respond with extreme selectively to faces (Tsao et al., 2003; Tsao et al., 2006; Moeller et al., 2017). Face-selective neurons in posterior 'face-patches' represent low-level dimensions of facial features in specific viewpoints (Freiwald et al., 2009; Issa and DiCarlo, 2012), whereas face-selective neurons in the most anterior 'face-patch' represent more complex dimensions of facial features across any viewpoint (Freiwald and Tsao, 2010; Chang and Tsao, 2017). Thus, the macaque 'face-patch' system generates a highly view-invariant 3-D representation of facial identity.

A wealth of neurobiological evidence suggests that pigeons also process visual ('what') information hierarchically (Nguyen et al., 2004; Stacho et al., 2016), and that despite a nuclear architecture, the functional connectivity of the pigeon visual system is astoundingly similar to the macaque visual system (De Groof et al., 2013; Shanahan et al., 2013). Like mammals, birds have two visual pathways. The thalamofugal pathway, homologous to the mammalian geniculo-striate pathway, projects from the retina to the principal optic nuclei and then to the visual wulst (Hodos and Karten, 1970; Shimizu and Bowers, 1999). The tectofugal visual pathway, in contrast, is homologous with the mammalian colliculo-pulvinar-cortical pathway, and travels from the retina to the optic tectum and then to the nucleus rotundus (nRT) of the thalamus, then to a primary visual structure known as the entopallium (ENTO), and finally to three visual association areas: the mesopallium ventrolaterale (MVL), nidopallium frontolaterale (NFL), and area temporo-parieto-occipitalis (TPO) (Husband and Shimizu, 1999; Krützfeldt and Wild, 2005).

Surprisingly, only a single study (Scarf et al., 2016) has investigated whether neurons in ENTO exhibit a category level representation of faces, but failed to demonstrate the presence of any face-selective neurons. In fact, very little is known about the response properties of neurons downstream of ENTO in NFL, MVL, and TPO, but preliminary recordings from NFL (Johnston et al., 2017) and MVL (Azizi et al., 2019) suggest that these association structures are functionally homologous with the mammalian extrastriate visual cortex. To determine if a 'face-patch' system also exists in the avian brain, it is necessary to understand how neurons in the association regions of the avian visual forebrain respond to complex visual categories. We performed bilateral electrophysiological recordings from ENTO, NFL, MVL, and TPO of freely moving pigeons during a Go/No-Go task that required them to discriminate between two stimulus sets consisting mainly of images depicting the face-region of two different pigeons. We hypothesised that neurons would respond selectively to images of faces, indicating biologically relevant percepts are encoded by neurons in the visual forebrain of birds.

MATERIALS AND METHODS

Subjects

Thirteen pigeons (*Columba livia*) with previous experience on visual delayed-matching-to-sample tasks served as experimental subjects. The pigeons were housed in a colony room maintained at 20°C. Each pigeon had *ad libitum* access to grit and water and were fed a combination of wheat, peas, and corn. All 13 pigeons were maintained at approximately 85% of their free-feeding weight and individually housed for the duration of the experiment. All experimental procedures were approved by the University of Otago Animal Ethics Committee and conducted in accordance with the University of Otago's Code of Ethical Conduct for the Manipulation of Animals.

Apparatus

Pigeons were trained and tested in standard operant boxes measuring 36 cm wide, 32.5 cm high, and 34.5 cm deep. Stimuli were presented on a 17-inch monitor set at a resolution of 1284 × 1024. Situated in front of the monitor was a Carroll Touch infrared touch frame (EloTouch, baud rate 9600, transmission time 20 ms) that registered the XY coordinates of all the pecks. To prevent accidental activation of the touch frame by the pigeon's body, a transparent Perspex panel with a single square opening (2.5 cm × 2.5 cm) was placed in front of the touch frame. Stimuli were presented on the monitor and the pigeons were required to peck at the image. A food hopper was positioned underneath the floor directly in front of the centre of the screen and 110 mm below the lower centre hole.

Stimuli

Twenty different images were used as stimuli and were taken using a Cannon DS126291 (12.2 Megapixel) digital camera and edited using Paint Shop Pro (Version 7) computer software. The colour pallets of each stimulus were approximately matched to ensure consistent brightness. Each set contained images of two different pigeon's faces, hereafter referred to as Bob and Larry. The set of 10 Bob stimuli was composed of: a portrait face, portrait face (eyes occluded), portrait face (minimalistic line drawing), portrait face (scrambled), profile face, profile face (eyes occluded), profile face (minimalistic line drawing), profile face (scrambled), checkerboard geometric pattern and spots geometric pattern (see **Figure 1A**). The set of 10 Larry stimuli was composed of: a portrait face, portrait face (eyes occluded), portrait face (minimalistic line drawing), portrait

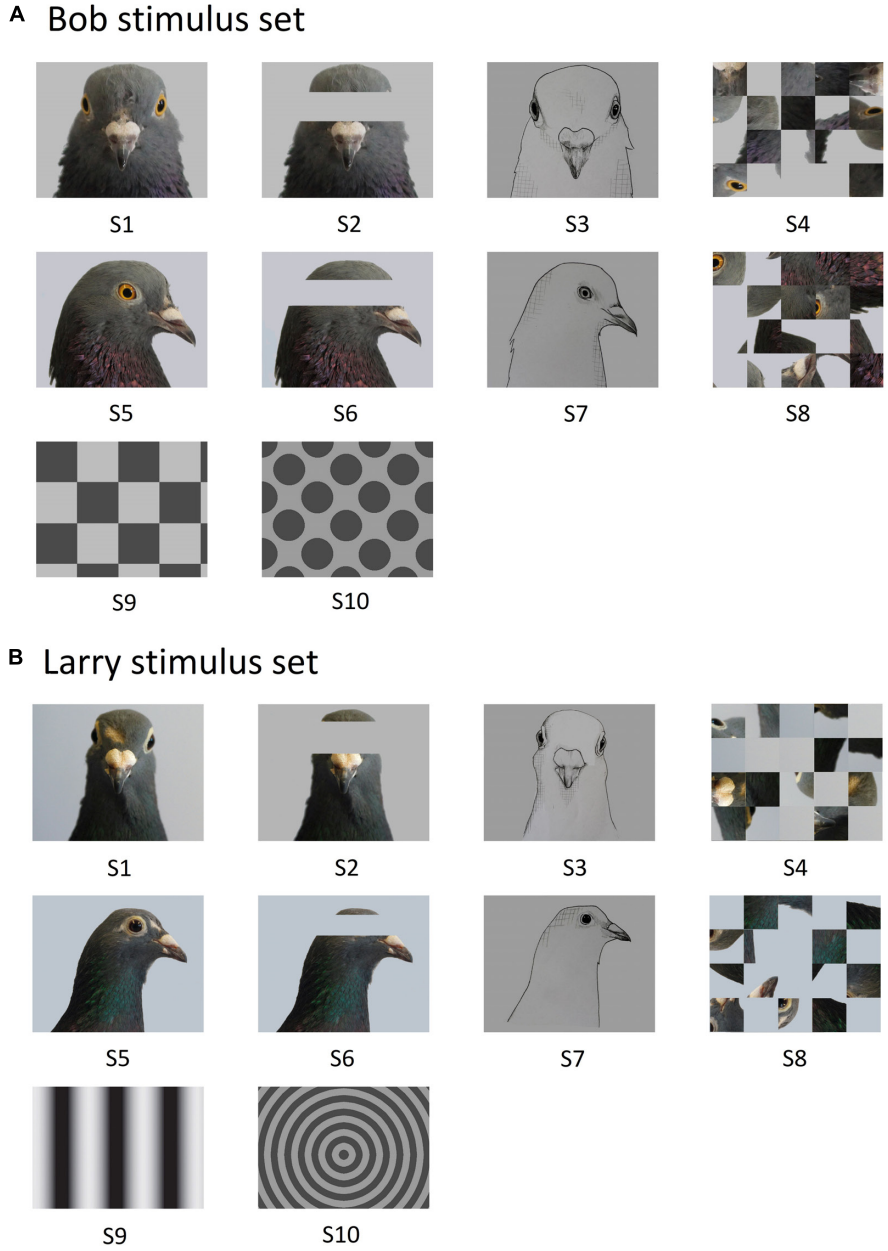


FIGURE 1 | The 10 stimuli comprising image set Bob **(A)** and Larry **(B)**. Portrait face (S1), portrait face, eyes occluded (S2), portrait face, minimalistic line drawing (S3), portrait face, scrambled (S4), profile face (S5), profile face, eyes occluded (S6), profile face, minimalistic line drawing (S7), profile face, scrambled (S8), checkerboard geometric pattern or sine grating geometric pattern (S9), and spots geometric pattern or concentric circles geometric pattern (S10).

face (scrambled), profile face, profile face (eyes occluded), profile face (minimalistic line drawing), profile face (scrambled), sine grating geometric pattern and concentric circle geometric pattern (see **Figure 1B**). All stimuli were presented against a black background.

Behavioural Task

The pigeons were initially trained to eat grain from the food hopper. Next they were autoshaped to respond to a white dot three times to receive a reward. The pigeons were then trained on a Go/No-Go task to discriminate between the Bob and Larry stimuli. The procedure on a typical trial was as follows (see **Figure 2**). At the end of a 5 s inter trial interval (ITI), an orienting stimulus (white dot) was displayed. Three pecks to the orienting stimulus turned it off and initiated a 2 s pause period. Pecks during the pause period extended the pause period duration by 2 s. Following the pause period, either a Bob or Larry stimulus was displayed for at least 5 s. During trials in which an S+ stimulus was displayed, the first peck after 5 s resulted in 2 s access to grain, accompanied by a 1000-Hz tone and the illumination of

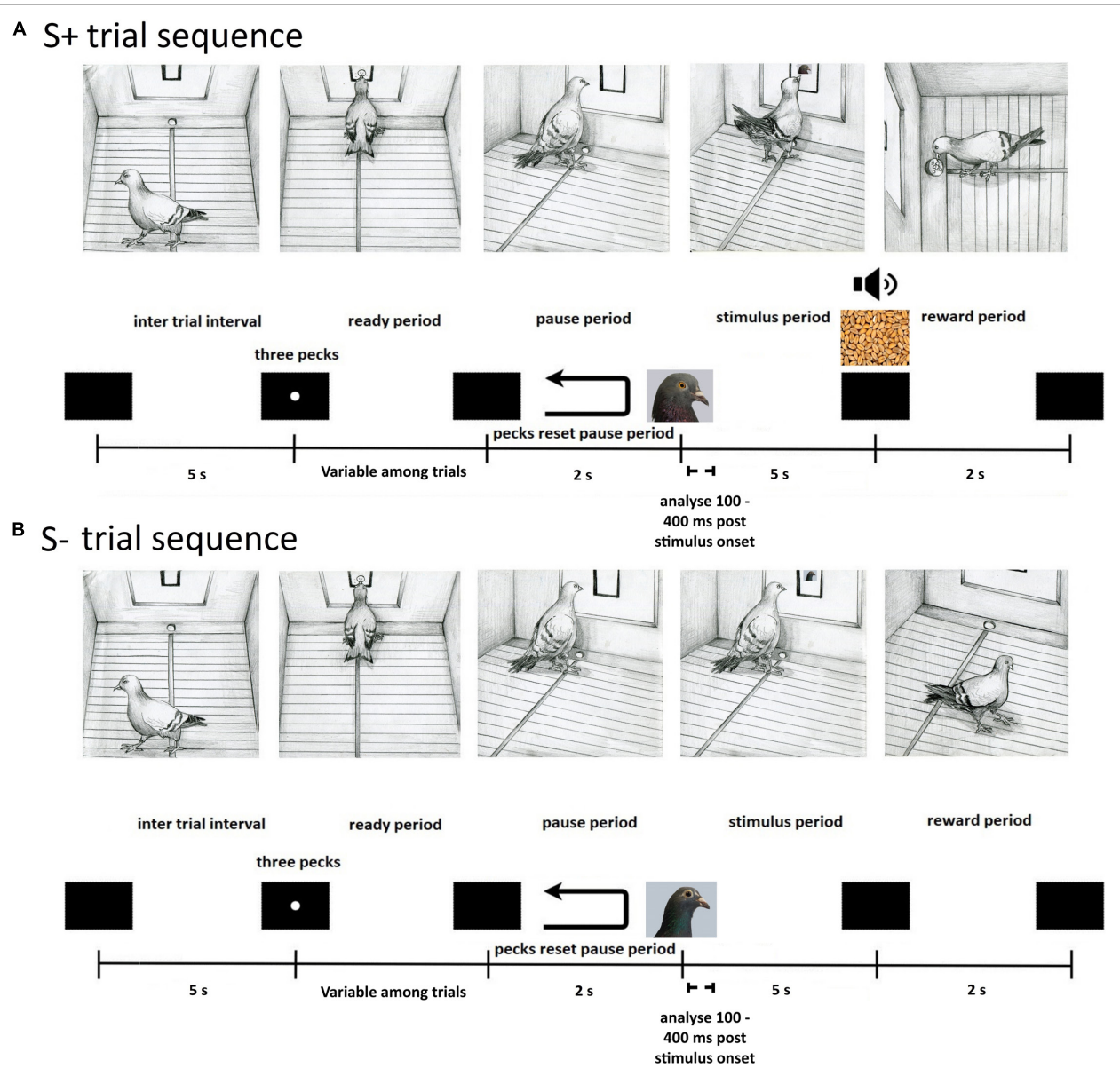


FIGURE 2 | Depiction of the sequence of events within a single experimental trial for S+ **(A)** and S– **(B)** stimuli. **(A)** S+ trials began with a black screen during the inter trial interval, lasting 5 s, after which an orienting stimulus appeared. The pigeon was then required to peck the orienting stimulus three times during the ready period to initiate a 2 s pause period with a black screen. An S+ stimulus was then displayed within the central response key on the screen for a minimum of 5 s during the stimulus period. A peck was required to deliver a grain reward from the hopper (2 s) that was paired with a tone cue (1000-Hz) and illumination of the hopper, followed by the inter trial interval. On unrewarded S+ trials, an equivalent 2 s period was paired with a tone cue (1000-Hz) and illumination of the hopper, letting pigeons know they responded correctly. **(B)** S– trials began with a black screen during the inter trial interval, lasting 5 s, after which an orienting stimulus appeared. The pigeon was then required to peck the orienting stimulus three times during the ready period to initiate a 2 s pause period with a black screen. An S– stimulus was then displayed within the central response key on the screen for 5 s during the stimulus period, followed by the inter trial interval.

the hopper. On S− trials, the stimulus turned off after the 5 s presentation period.

A session consisted of 240 trials, taking approximately 1 h and 20 min to complete, with each of the 20 images presented 12 times in a random order. Due to the large number of trials, correct S+ trials were rewarded approximately 70% of the time. Unrewarded S+ trials also consisted of a 2 s period during which the 1000-Hz tone was played and the hopper light was illuminated, but no reward was delivered. Discrimination training ended when the subjects attained a discrimination ratio (DR) of 0.75 in a session. The DR was calculated as the number of responses to the S+ stimuli divided by the number of responses to both the S+ and S− stimuli. Responses were accumulated only during the 5 s presentation period. Upon reaching criterion, pigeons were implanted with a movable microdrive. Seven birds were trained with image set Bob as the S+ stimuli (M5, M20,

K1, K18, K22, Z11, and Bev), while for the other seven the image set Larry served as the S+ stimuli (M14, Z28, K11, K21, Z19, Z28, and Q42).

Surgery

Once the pigeons were reliably completing the task with at least a DR of 0.75, stereotaxic surgery was performed to install a movable microdrive into the target brain area (Bilkey et al., 2003). A mixture of Ketamine (30 mg/kg) and Xylazine (6 mg/kg) was injected into the pigeon's legs as an anaesthetic. The feathers on the head were then removed. The pigeons were placed in a Revzin stereotaxic adapter (Karten and Hodos, 1967) to immobilise the head and a topical anaesthetic (10% Xylocaine) was applied to the scalp. The skin overlying the skull was retracted exposing the skull, and six stainless steel screws were inserted into the skull. One of these screws served as the ground screw. A hole was drilled above the targeted area, as defined by Karten and Hodos (1967), and the dura was removed. A microdrive housing the electrodes was lowered into the hole until the tips of the electrodes were positioned above ENTO, NFL, MVL, and TPO. The microdrive was then secured to the skull using dental acrylic, and the wound was sutured closed. Xylocaine was applied again before the pigeons were placed into a padded and heated recovery cage. The pigeon remained in the recovery cage until it had returned to an active state, and then returned to their home cage where they were given another 7 days to recover before experimental sessions began.

Neuronal Recording

The microdrives housed eight 25 μm Formvar-coated nichrome wires (California Fine Wire, Grover Beach, CA, United States) used to measure single neuron activity. For each experimental session we searched for activity on any one of the eight wires and used one of the remaining wires as the indifferent. The signals were amplified (x10000) using a GrassP511K amplifier (Grass Instruments, Quincy, MA, United States) and 50 Hz noise was eliminated. A CED (Cambridge Electronic Design, Cambridge, United Kingdom) electrophysiology system with Spike2 software stored and analysed the data. Cells were isolated using CED's template matching capacity (thereby eliminating artefacts) sampling at a rate of 20000 Hz. The only selection criterion was that the isolated neuron had a signal-to-noise ratio of no less than 2:1. A separate computer controlled the behavioural task and sent codes to the CED system to align key task events. Following each recording session, the electrodes were advanced approximately 40 μm before the pigeon was returned to their home cage. If we did not record from any neural activity the electrodes were moved approximately 20 μm, and the animal was returned to its cage. Recording sessions took approximately 1 h to complete. Pigeons completed one session daily for 5 days a week.

Neural Analysis

All of the data for each neuron was loaded into MATLAB (version R2016B) for data analysis using custom written MATLAB code. Neurons were required to exhibit mean firing rates > 0.2 Hz during the ITI period across an entire experimental session to be included in the analysis. To be included for further analysis, a neuron had to be visually responsive, firing at a significant level (Paired *t*-test: modified Bonferroni, $p < 0.02$; Keppel and Zedeck, 1982) to at least one of the 10 S+ stimuli relative to the baseline ITI activity. We next compared each recorded neuron's baseline ITI mean firing rates with the mean firing rate elicited by the 10 S+ stimuli during the stimulus period (100–400 ms post stimulus onset) over the 12 stimulus presentations. We performed a comprehensive series of analysis steps to investigate the possibility that activity reflective of stimuli may be present in the first 100 ms of the Stimulus period. We calculated all neurons firing rates for every trial during the first two bins (50 ms bin size) of the stimulus period, and an equivalent two bin window in the middle of the ITI. *T*-tests between the first 100 ms of the stimulus period and ITI window showed that 19/405 (4.96%: uncorrected) and only 1/405 (0.24%: Holm–Bonferroni, $p < 0.0001$) total neurons with a strict Bonferroni correction showed significantly different firing rates between these periods. Therefore, while these analyses suggest that activity reflective of stimuli emerges approximately 100 ms post stimulus, it is important for future studies to consider that such representations may emerge at a slightly earlier time course in some structures of the tectofugal visual pathway.

We used custom written MATLAB code to find trials where the difference between the time of stimulus onset and the first peck made to the stimulus was <400 ms and excluded these trials from further analysis. Finally, each visually responsive neuron was classified as either excitatory (firing more to the onset of the stimulus than the baseline ITI level) or inhibitory (firing less to the onset of the stimulus than the baseline ITI level). Lastly, we assigned neurons a classification (excitatory or inhibitory) based on a binomial probability distribution of the total number of excitatory and inhibitory responses relative to the total number of stimuli that the neuron fired to at a significant level. We compared the total numbers of visually responsive versus non-visually responsive, and excitatory versus inhibitory cells between each region, hemisphere, and anteroposterior position. Each of these comparisons was performed by either using a Chi-Squared test (Holm–Bonferroni, $p < 0.008$), or a Fishers Exact test (Holm–Bonferroni, $p < 0.005$) if the frequency of sampled neurons in a group was <5. We used a total of 6 Chi-squared tests and 9 Fishers Exact tests for these comparisons.

Stimulus Selectivity

Once we determined that a neuron was visually responsive as per the previous criteria, we next assessed whether any neurons exhibited selective responses to faces such as those found in the category-selective 'face-patches' in macaque extrastriate cortex (Freiwald et al., 2009; Freiwald and Tsao, 2010). We also determined if neurons responded selectively only to geometric stimuli, indicating that these neurons may be encoding low-level features of our visual stimuli (Koenen et al., 2016). The selectivity patterns of isolated neurons' responses to the 10 S+ stimuli (either Bob or Larry) during the ITI and stimulus period were determined using a series of 10 paired *t*-tests with a modified Bonferroni correction (conservative alpha of $p < 0.02$; Keppel and Zedeck, 1982).

We classified neurons as face-selective according to six possible classifications (see **Table 1**) based on how the neuron responded to the S+ stimuli. The first three classifications involved significant responses only to the intact face images. We classified neurons as face-selective (F) based on the following criteria. Neurons that responded significantly to only S1 (portrait face) were considered viewpoint selective, and were categorised as F-1 selective, those that fired at a significant level only to S5 (profile face) were also considered viewpoint selective and were categorised as F-2 selective, and those that fired at a significant level to S1 (portrait face) and S5 (profile face) were considered viewpoint invariant and were classified as F-3 selective.

The second three classifications involved significant responses to the geometric stimuli (see **Table 1**). We classified neurons as geometric-selective based on the following criteria. Neurons that fired at a significant level to S9 (checkerboard or sine grating) and no other stimulus were classified as G-1 selective, those that fired at a significant level to S10 (spots or concentric circle) and no other stimuli were classified as G-2 selective, and those that fired at a significant level only to S9 (checkerboard or sine grating) and Stim 10 (spots or concentric circle) were categorised as G-3 selective.

Stimulus Selectivity: Population Analysis

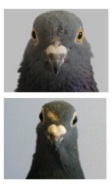
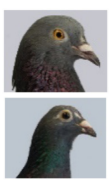
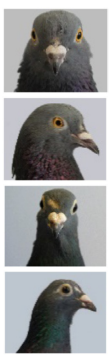
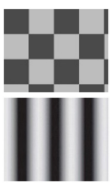
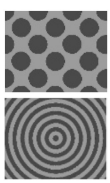
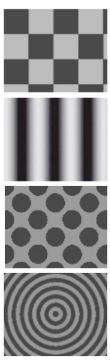
The mammalian ventral visual stream is organised in a series of hierarchical processing stages that encode increasingly explicit information on object identity and category (Kriegeskorte et al., 2008b; Yamins et al., 2014). Therefore, viewpoint invariant representations of object categories (e.g., faces and animals) can be extracted at the level of a neuronal population in IT cortex, but not from a population at lower stages of the visual hierarchy (e.g., V1/V2; Yamins et al., 2014). As described in Kriegeskorte et al. (2008a), Representational Similarity Matrices (RSM) are a tool used to extract categorical representations by comparing a correlate of neural activity (in our case the population firing rates) associated with each pair of visual stimulus conditions between multiple brain regions. An RSM is composed of a symmetric matrix of cells that each contain a number reflecting the similarity/dissimilarity of firing rates between each pair of stimulus conditions averaged across a neuronal population. The similarity of stimulus conditions is measured as correlation distance (R) over space (1 for total correlation, 0 for no correlation, and −1 for total anticorrelation). When stimuli are organised by category along the rows of the matrix, the resulting RSM reflects the position of each stimulus in the high-dimensional space of the neuronal population.

Computational modelling suggests that the pigeon visual system is organised in a hierarchical feed-forward progression (Soto and Wasserman, 2012), and electrophysiological recordings indicate that category level information may be represented at a population level in association visual forebrain regions (Azizi et al., 2019). We therefore generated an RSM for the ENTO, MVL, NFL, and TPO to investigate whether any of these regions exhibited a population coding of conspecific's faces as a perceptual category. Neuronal data during the stimulus period (100–400 ms post stimulus onset) on S+ and S− trials was used for the RSM analysis. The RSM stimulus period included S− trial data, and therefore differed from that used for stimulus selectivity (only S+ trial data) so that the RSM reflected each isolated neuron's responses to the entire stimulus set. We then computed Pearson correlation coefficients (Pearson's *R*) of the average firing rate during the stimulus period, for each stimulus, with the average firing rates elicited by all other stimuli. These correlation coefficients were used to generate an RSM for each neuron that was stored in a 3-D array. Individual neuron's Pearson correlations were then averaged together to create an RSM for ENTO ($n = 65$), MVL ($n = 37$), NFL ($n = 28$), and TPO ($n = 12$).

Histology and Electrode Track Reconstruction

When the electrodes reached the end of ENTO, NFL, MVL, or TPO, the final recording position was marked by sending a

TABLE 1 | Classification of face-selective and geometric-selective neurons.

Face-selective			Geometric-selective		
F-1	**F-2**	**F-3**	**G-1**	**G-2**	**G-3**
Fired at a significant level to S1	Fired at a significant level to S5	Fired at a significant level to S1 and S5	Fired at a significant level to S9	Fired at a significant level to S10	Fired at a significant level to S9 and S10
					

15 mA (9V) current through each electrode for 10 s to create an electrolytic lesion at the tip of each electrode. The pigeons were then anaesthetised deeply with isoflurane and perfused with physiological saline and 10% formalin. The brains were removed and kept for five days in 10% formalin in 30% sucrose. They were then frozen and cut into 40 μm sections. Cresyl violet was used to stain every 5th section of the brain. The position of the recorded neurons was located by using the position of the electrolytic lesion, track reconstructions, and depth records.

RESULTS

Electrode Positions

All electrode tracks were within the borders of the targeted ENTO, NFL, MVL, and TPO regions (Karten and Hodos, 1967; Stacho et al., 2016). The histological track placements are shown in **Figure 3**. For ENTO, two pigeons (M14 and M20) had microdrives installed at positions AP ± 9.5, and ML ± 6.0, and two pigeons (M5 and Z26) at positions AP ± 10.5 and ML ± 6.0. For MVL, two pigeons (Z11 and Z19) had microdrives installed at positions AP ± 9.75 and ML ± 6.7, and two pigeons (K22 and K25) at positions AP ± 11.25 and ML ± 6.7. For NFL, two pigeons (K1 and K11) had microdrives installed at positions AP ± 12.25 and ML ± 6.0, and two pigeons (K18 and K21) at positions AP ± 12.75 and ML ± 6.3. For TPO, one pigeon (Q42) had a microdrive installed at position AP ± 9.5 and ML ± 8.8, and two pigeons (Z28 and BEV) AP ± 8.25 and ML ± 9.5. Although for several birds we were unable to recover the electrode track placements, all recovered track locations were within ± 0.6 mm of their intended AP and ML implant co-ordinates (see **Figure 3**).

Behavioural Performance

The discrimination ratio (DR) was calculated from the number of correct pecks to the S+ stimuli divided by the total number of S+ and S− pecks. We then averaged the DR across the total number of sessions each bird completed to find its mean DR. All

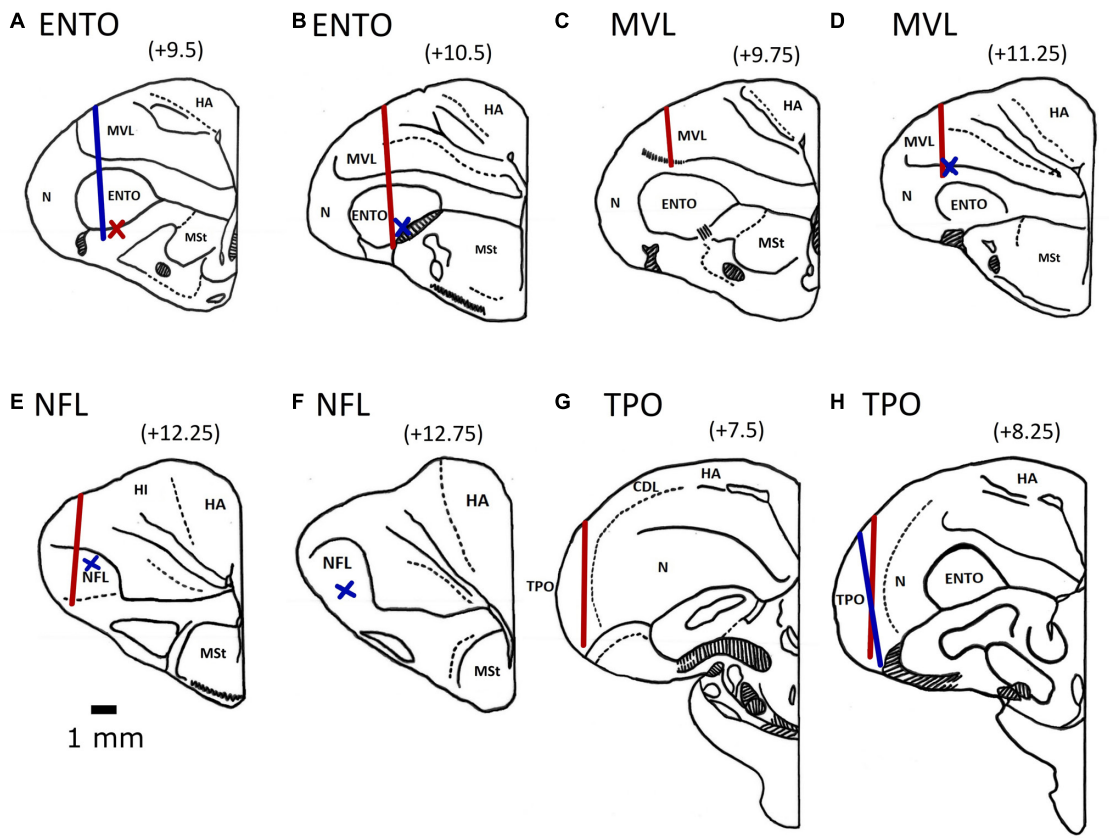


FIGURE 3 | Electrode track position reconstructions for the two ENTO birds at AP + 9.5 **(A)** and AP + 10.5 **(B)**, the MVL bird at AP 9.75 **(C)** and two birds at AP + 11.25 **(D)**, two NFL birds at AP + 12.25 **(E)** and one bird implanted at AP + 12.75 **(F)**, the TPO bird implanted at AP + 7.5 **(G)** and two birds implanted at AP + 8.25 **(H)**. Both left and right electrode tracks are displayed on the same section. Tracks for birds with implants in the left hemisphere are displayed by the blue line and for birds with implants in the right hemisphere by a red line. We determined the termination point of electrode tracks that were unable to be recovered based on depth records. Termination points for birds with implants in the left hemisphere are displayed by a blue cross and for birds with implants in the right hemisphere by a red cross. All electrodes were advanced to the top of the intended region before recording began. The following are the brain regions as defined by Reiner et al. (2004): MSt, medial striatum; HA, hyperpallium apicale; HD, hyperpallium denso-cellulare; HI, hyperpallium intercalatum; Hp, hippocampus; MVL, mesopallium ventrolateral; N, nidopallium; NFL, nidopallium frontolaterale.

13 birds achieved a high level of performance, exhibiting DRs of 96% (M14), 94% (M5), 92% (Z26), 85% (M20), 88% (K1), 88% (K11), 94% (K18), 97% (K22), 91% (Z11), 92% (Z19), 83% (Z28), 96% (Q42), and 84% (Bev) correct responses. There was no difference in the DRs for birds whose S+ stimulus was the Bob set (92%) compared to birds whose S+ stimulus was the Larry set (88%), [Paired *t*-test, $t = 1.16$ (6), $p = 0.30$]. We next compared all birds' DR performance for the face images (95%) with the four line-drawn face images (79%), occluded-eyes face images (95%), and scrambled face images (96%) across all sessions. All birds DR performance was significantly lower for the four line-drawn stimuli compared with the four intact face stimuli [Paired *t*-test, $t = 4.54$ (12), $p = <0.001$]. There were no significant differences between birds' DR performance for the four intact face images compared with the four face images with occluded-eyes [Paired *t*-test, $t = 0.025$ (12), $p = 0.74$], and the four scrambled face images [Paired *t*-test, $t = 0.02$ (12), $p = 0.40$].

Basic Response Properties of ENTO, MVL, NFL, and TPO

All four regions exhibited a large proportion of neurons that were visually responsive to at least one of the S+ stimuli, verifying that each region is heavily involved in processing tectofugal visual information (see **Table 2**). Although visual responsivity was greatest in MVL (40%), followed by ENTO (37%), NFL (32%), and TPO (23%), a Chi-squared test revealed no significant differences in the relative numbers of visually responsive neurons between the four areas [$\chi^2(3) = 3.72$, $p = 0.29$].

The percentages of cells in each of the four areas that displayed excitatory or inhibitory activity is also shown in **Table 2**. Both ENTO and MVL exhibited a greater proportion of excitatory neurons than inhibitory neurons, whereas both NFL and TPO displayed the opposite trend. There were no significant differences in the relative numbers of excitatory and inhibitory neurons isolated in ENTO (Fishers Exact test: $p = 0.08$), MVL (Fishers Exact test: $p = 0.42$), or NFL (Fishers Exact test: $p = 0.82$). All visually responsive neurons isolated in TPO were inhibitory.

TABLE 2 | Proportion of visually responsive, excitatory and inhibitory neurons in ENTO, MVL, NFL, and TPO.

Region	Visually responsive	Excitatory	Inhibitory
ENTO	65/176 (37%)	38/65 (58%)	27/65 (42%)
MVL	37/93 (40%)	22/37(60%)	15/37(40%)
NFL	28/88 (32%)	13/28 (46%)	15/28 (54%)
TPO	12/48 (25%)	0/12 (0%)	12/12 (100%)

Hemispheric Comparisons Between ENTO, NFL, MVL, and TPO

Previous ENTO studies have demonstrated that pigeons exhibit significantly greater visually responsive neurons in the left hemisphere compared to the right hemisphere (Verhaal et al., 2012). In contrast, although far fewer studies have been conducted, there is little evidence of lateralisation in MVL, NFL, and TPO (Stacho et al., 2016). We compared the characteristics of visually responsive neurons between the left hemisphere and right hemisphere of ENTO, MVL, NFL, and TPO to evaluate any possible differences in visual function between hemispheres. The results are shown in **Table 3**.

ENTO and TPO exhibited a greater proportion of visually responsive neurons in the left hemisphere relative to the right hemisphere. In contrast, MVL and NFL showed a greater number of visually responsive neurons in the right hemisphere compared with the left hemisphere. No significant differences in the number of visually responsive neurons were found between the right and left hemisphere of ENTO (Fishers Exact test: $p = 0.27$), MVL (Fishers Exact test: $p = 0.12$), NFL (Fishers Exact test: $p = 0.04$), or TPO (Fishers Exact test: $p = 0.08$).

We also compared the total number of visually responsive neurons classified as excitatory and inhibitory across the left and right hemisphere of ENTO, NFL, MVL, and TPO. ENTO and NFL exhibited a greater proportion of excitatory neurons in the left hemisphere than in the right hemisphere, and a greater proportion of inhibitory neurons in the right hemisphere compared with the left hemisphere. MVL exhibited a greater proportion of excitatory neurons in the right hemisphere relative to the left hemisphere, and a greater proportion of inhibitory neurons in the left hemisphere compared with the right hemisphere. No excitatory neurons were isolated in TPO, and we found that most of the inhibitory neurons were in the left hemisphere compared with the right hemisphere. We found a significant difference in the proportion of excitatory and inhibitory neurons between the left and right hemisphere of MVL [Chi Squared test: $\chi^2(3) = 12.163$, $p = <0.007$], and NFL [Chi Squared test: $\chi^2(3) = 41.88$, $p = <0.0000001$] but not ENTO [Chi Squared test: $\chi^2(3) = 4.462$, $p = 0.21$].

Comparisons Between Anterior–Posterior ENTO and MVL

We also compared the characteristics of visually responsive neurons between the anterior and posterior regions of ENTO and MVL to examine any potential differences in visual processing.

TABLE 3 | Proportion of visually responsive, excitatory, and inhibitory neurons in the left and right hemisphere of ENTO, MVL, NFL, and TPO.

Region	Left overall	Right overall	Left excitatory	Right excitatory	Left inhibitory	Right inhibitory
ENTO	39/95 (41%)	26/81 (32%)	24/39 (62%)	14/26 (53%)	15/39 (38%)	12/26 (47%)
MVL	10/35 (29%)	27/58 (47%)	3/10 (30%)	19/27 (70%)	7/10 (70%)	8/27 (30%)
NFL	11/49 (22%)	17/39 (44%)	11/11 (100%)	2/17 (12%)	0/11 (0%)	15/17 (88%)
TPO	11/34 (32%)	1/14 (7%)	0/11 (0%)	0/1 (0%)	11/11 (100%)	1/1 (100%)

Insufficient numbers of visually responsive neurons were isolated at the anterior aspect of NFL (AP 12.75; $n = 3$) and posterior aspect of TPO (AP 7.5; $n = 3$) for functional comparisons to be made. The results are shown in **Table 4**.

The overall proportion of visually responsive neurons was greater in posterior relative to anterior ENTO, and this difference was significant (Fishers Exact test: $p = <0.0001$). In contrast, there was little evidence for a difference in the number of visually responsive neurons isolated from anterior relative to posterior MVL (Fishers Exact test: $p = 0.46$).

We also compared the total numbers of visually responsive cells classified as excitatory and inhibitory across the anterior and posterior aspect of ENTO and MVL. ENTO and MVL exhibited a greater proportion of excitatory neurons in the anterior compared with posterior regions. In contrast, the posterior ENTO and MVL showed a greater proportion of inhibitory neurons compared with anterior regions. We found no significant differences in the proportion of excitatory and inhibitory neurons between anterior and posterior ENTO [Chi Squared test: $\chi^2(3) = 3.84$, $p = 0.27$], and MVL [Chi Squared test: $\chi^2(3) = 8.909$, $p = 0.03$].

Stimulus Selectivity

We assessed the stimulus selectivity exhibited by visually responsive neurons in ENTO, MVL, NFL, and TPO and the results are show in **Table 5**.

A total of 15/141 (11%) visually responsive neurons isolated across ENTO, MVL, NFL, and TPO were classified as face-selective. Most of the face-selective neurons were found in ENTO and the majority (10/15; 67%) of these face-selective cells responded to the portrait face image. Fewer numbers of face-selective neurons were found in MVL, NFL, and TPO and fired either to portrait (F-1) or profile (F-2) face stimuli, but not to both face images (F-3, see **Table 5**). While the 15 face-selective neurons fired at significant levels only to faces, they did not exhibit the selectivity of that shown by face-selective neurons in macaque extrastriate cortex (Tsao et al., 2006) and also responded to other visual stimuli (see **Figure 4A** for an example cell). Therefore, the neurons we classified as "face-selective" are not homologous with face-selective neurons in macaques, and their exact contribution to vision remains undetermined.

A total of 13/141 (9%) visually responsive neurons isolated across ENTO, MVL, NFL, and TPO responded selectively to geometric stimuli and were classified as geometric-selective (see **Figure 4B** for an example cell). Two of the geometric-selective neurons in ENTO and a single neuron in MVL and NFL only fired to the concentric circle or spots. Two of the geometric-selective neurons in all regions fired to either the checkerboard or gratings patterns. The low number of total neurons that we classified as "geometric-selective," and small number of geometric stimulus manipulations means that we are unable to determine exactly what stimulus dimensions these cells responded to.

Stimulus Selectivity: Population Analyses

On the basis of single-unit analysis, we found no evidence for face-selective neurons in ENTO, MVL, NFL, and TPO. One possibility is that, as in primates, face-category information is represented at a population level in the pigeon visual forebrain. We therefore generated Representational Similarity Matrices (RSMs) for each of our targeted regions (ENTO, NFL, MVL, and TPO) in order to assess weather face-category information was represented at a population level. We observed no evidence of a clustering of similar correlations for face images in ENTO (**Figure 5A**), MVL (**Figure 5B**), NFL (**Figure 5C**), or TPO (**Figure 5D**) that was indicative of a categorical population representation like that found in primate extrastriate cortex (Kiani et al., 2007; Kriegeskorte et al., 2008b).

We verified that there was no population level representation of face-information in any region by comparing the relative differences in the normalised firing rates elicited by faces, eye-manipulations, scrambled faces and geometric stimuli between ENTO, NFL, MVL, and TPO. Relative differences in firing rates for each region were calculated by taking the mean of the normalised firing rates for each visually responsive neuron to faces, and subtracting the resulting values from the means of eye-manipulation, scrambled, and geometric stimuli. There

TABLE 4 | Proportion of visually responsive, excitatory, and inhibitory neurons in anterior and posterior regions in ENTO and MVL.

Region	Anterior	Posterior	Anterior excitatory	Posterior excitatory	Anterior inhibitory	Posterior inhibitory
ENTO (AP 9.5 vs. AP 10.5)	13/66 (20%)	52/110 (47%)	8/13 (61%)	30/52 (58%)	5/13 (39%)	22/52 (42%)
MVL (AP 9.75 vs. AP 11.25)	11/23 (47%)	26/70 (37%)	9/11 (82%)	13/26 (50%)	2/11 (18%)	13/26 (50%)

TABLE 5 | Number of face-selective and geometric-selective neurons in ENTO, MVL, NFL, and TPO.

			Face-selective			Geometric-selective		
	Total face-selective	Total geometric-selective	F-1	F-2	F-3	G-1	G-2	G-3
ENTO	10/65 (15%)	5/65 (8%)	7	2	1	2	2	1
MVL	2/37 (5%)	3/37 (8%)	0	2	0	1	2	0
NFL	2/28 (7%)	3/28 (11%)	1	1	0	1	2	0
TPO	1/11 (9%)	2/11 (18%)	0	1	0	0	2	0

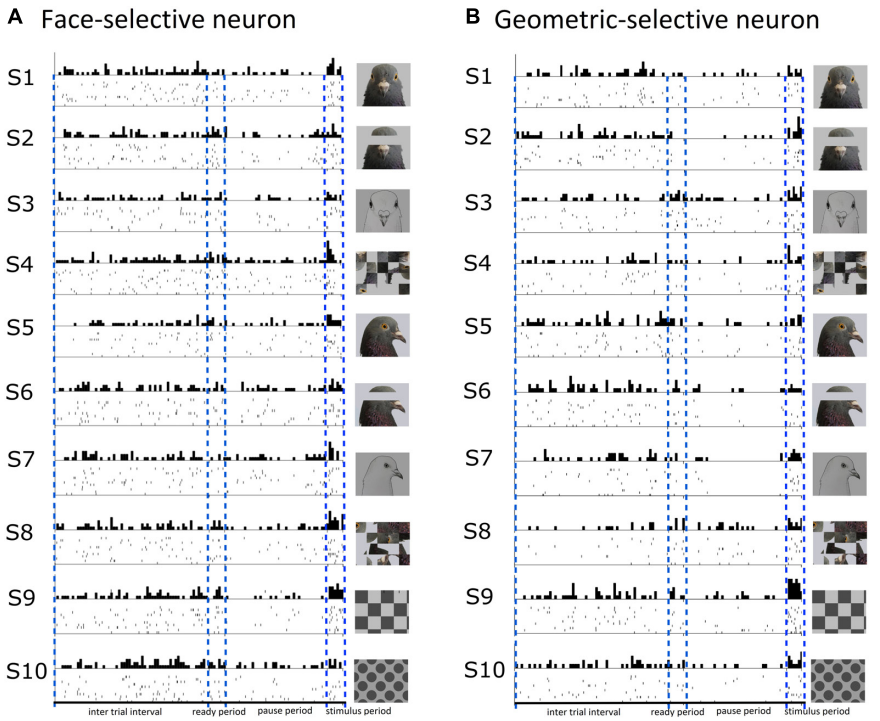


FIGURE 4 | Raster and histogram plots of a neuron classified as face-selective **(A)** and geometric-selective **(B)**. **(A)** Example of a neuron in the posterior entopallium that exhibited excitatory firing at a significant level to the stimulus S1 (portrait face) relative to the ITI, but also responded to the other stimuli during the stimulus period. **(B)** A neuron in the posterior entopallium that fired in an excitatory manner at a significant level to a geometric stimulus S9 (checkerboard) relative to the ITI.

were no significant differences in the mean firing rates for faces, compared to eye-manipulation [Kruskal–Wallis test (3): $\chi^2(2) = 4.11$, $p = 0.249$], scrambled [Kruskal–Wallis test (3): $\chi^2(2) = 4.79$, $p = 0.187$], and geometric stimuli [Kruskal–Wallis test (3): $\chi^2(2) = 1.15$, $p = 0.765$] between ENTO ($n = 65$), MVL ($n = 37$), NFL ($n = 28$), and TPO ($n = 12$).

DISCUSSION

We performed bilateral electrophysiological recordings from ENTO, NFL, MVL, and TPO of freely moving pigeons during a Go/No-Go task that required discriminating between two stimulus sets that included images of the faces of two different pigeons. ENTO, NFL, MVL, and TPO all exhibited a relatively large proportion of neurons (between 21 and 37%) responsive to at least one visual stimulus, verifying that each region is heavily involved in the appraisal of tectofugal visual information. In stark contrast to the human (Nasr and Tootell, 2012; Schalk et al., 2017), macaque (Tsao et al., 2006; Chang and Tsao, 2017), marmoset (Hung et al., 2015), and sheep (Kendrick and Baldwin, 1987) extrastriate visual cortex, very few neurons in the pigeon brain fired selectively to faces (15 of 407 total neurons: 4%). These 15 neurons did not respond to faces at levels comparable to mammalian face-selective neurons (Tsao et al., 2006) and therefore we do not consider them to be functionally homologous. The absence of face-selective neurons suggests that birds' solution to the challenges of object representation may be mechanistically different to mammalian species, but can also be explained by divergences in birds' visual physiology and behaviour in visual discrimination tasks.

How do Birds Construct Visual Representations of Objects?

Birds are perhaps the most visually complex vertebrate species (Shimizu and Bowers, 1999; Jarvis et al., 2005) and like primates, possess exceptional foveal visual acuity for analysing object features (Tyrrell et al., 2014). Recent breakthroughs in our understanding of high-level vision in macaque IT cortex have shown that patches of stimulus-selective neurons emerge in a large swath of foveal extrastriate cortex from V4 through to anterior IT cortex. Stimulus-selective neurons can be selective to object class (e.g., faces: Tsao et al., 2003), or for other stimulus dimensions (e.g., curved or rectangular stimuli: Yue et al., 2014). Ethologically relevant visual percepts exhibit the greatest representation of stimulus-selective neurons in extrastriate cortex (Kriegeskorte et al., 2008b; Moeller et al., 2017). Electrophysiological recordings from the macaque 'face-patch' system have revealed that increasingly complex and view-invariant representations of visual objects are encoded by neurons in stimulus-selective patches (Freiwald and Tsao, 2010) and that the computational goal of such neurons is to measure the feature dimensions of visual objects (Chang and Tsao,

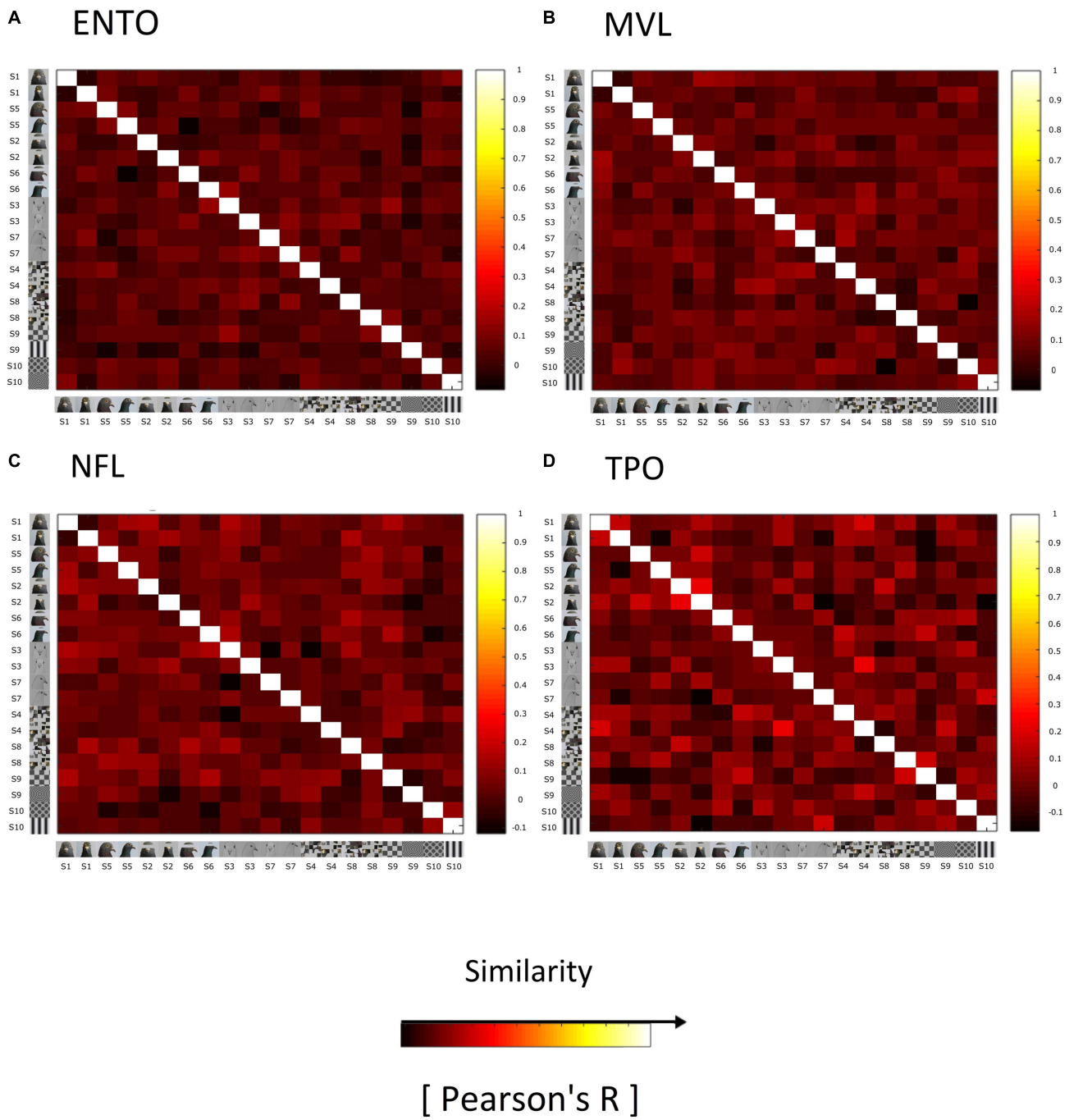


FIGURE 5 | Representational similarity matrices (RSM) showing the similarity of responses elicited by the stimuli for all visually responsive neurons in **(A)** ENTO, $n = 65$, **(B)** MVL, $n = 37$, **(C)** NFL, $n = 28$, and **(D)** TPO, $n = 11$. The similarity of responses for each stimulus is measured as Pearson correlation across space (Pearson's R), averaged across all sessions for each region. The colour code reflects the similarity of spatial correlations (see colour bar; +1 being total similarity, 0 for no correlation, and –1 as total anticorrelation) computed separately for each RSM (for R-values and their histograms). The Bob and Larry examples of individual stimuli are organised into Face (S1, S5, S2, S6, S3, and S7) and Non-Face (S4, S8, S9, and S10) categories along the rows of the matrix. RSM analysis failed to demonstrate a clustering of strong correlations for faces relative to non-face stimuli in ENTO, MVL, NFL, or TPO, in contrast to the face-representation exhibited in monkey and human IT Cortex (Kriegeskorte et al., 2008b).

2017). If the hierarchical organisation of object representation demonstrated in macaque extrastriate cortex is conserved across vertebrate species, then birds may also exhibit clusters of category-selective neurons in association forebrain structures of the tectofugal visual pathway. While we found no evidence to suggest that face-selective neurons exist in ENTO, MVL, NFL,

and TPO, our sampled neurons' high responsivity to visual stimuli indicates that these regions may perform homologous functions to extrastriate visual cortex.

Is the Avian Face a Socially Significant Stimulus for Birds?

A possible explanation for our inability to locate category-selective neurons is that the freely moving behavioural paradigm and close-proximity of the visual stimuli in an operant chamber inhibit the processing of faces as a global configuration of feature dimensions. While neurons in the pigeon visual forebrain exhibit large receptive fields like in extrastriate cortex (Kimberly et al., 1971; Xiao et al., 2006), pigeons preferentially attend to and analyse the local features of visual stimuli over their global configuration in visual discrimination tasks (Cavoto and Cook, 2001; Aust and Braunöder, 2015). Pigeons' predisposition to discriminate between object categories based on local features is associated with a left-hemispheric dominance of the tectofugal visual pathway in visual discrimination and learning (Yamazaki et al., 2007; Xiao and Güntürkün, 2009; De Groof et al., 2013). Moreover, electrophysiological recordings from ENTO suggest that the association of visual stimuli with reward progressively increases the number of visually responsive neurons in the left hemisphere and modulates their capacity to differentiate between rewarded and unrewarded visual stimuli (Verhaal et al., 2012). As a result, top-down dopaminergic visuo-motor feedback via projections with the nidopallium caudolaterale (NCL) and motor structures may cause visually guided behaviour in birds to be increasingly driven by local perceptual cues to solve the visual discrimination over time (Schultz, 2016; Güntürkün et al., 2018). An important implication of birds' local-precedence effect is that our pigeons may have learned to discriminate between stimulus sets by attending to a component feature, colour, or background patterns predictive of the S+ images. For instance, Cook et al. (2013) trained pigeons to discriminate between line drawings of birds and mammals and subsequently tested their transfer to novel instances with manipulated features. The authors discovered that pigeons discriminated between the two object classes by using the contrast between animal figures from the background and the orientation axis of the body, but not using features of the face region or body parts. Perhaps further compounding a bias toward local-feature based strategies is the fact that our pseudocategorisation task design required birds to discriminate between S+ and S− image sets without providing an incentive or sufficient viewpoint-invariant information to relate face stimuli to their corresponding real world representations.

For example, Peissig et al. (2019) demonstrated that pigeons' categorisation performance was significantly impaired in a pseudocategorisation task that required birds to peck a key corresponding to one of four viewpoint rotations (−90, 0, 90, and 180°) of four different 3-D geometric objects, relative to a categorisation task that required birds to peck a single viewpoint rotation of only one of the same four objects. Therefore, pigeons in the Pseudocategorisation group only responded based on perceptual groupings of local orientation-dependant features that did not correspond to object identity, whereas the Categorisation group achieved highly accurate responding with access to viewpoint-invariant information on a particular object's identity. Our pigeons' ability to discriminate between S+ and S− line-drawn images of faces was significantly impaired relative to face, scrambled, eye-manipulations, and geometric stimuli. While many of the visual cues indicative of a bird's face were still present in the line drawings, the form of these images was likely an insufficient perceptual cue to relate to the other rewarded face exemplars during discrimination. Therefore, while primates can easily perceive and recognise line-drawn images of faces (Freiwald et al., 2009), birds appear to reduce the stimulus space to a few key dimensions in a pseudocategorisation discrimination paradigm and do not see the correspondence between face drawings, or other sub-categories within the stimulus set and their real world representations. A strategy based on the analysis of local visual features can explain why all 13 of our pigeons were able to learn successfully to discriminate between the S+ and S− stimuli, but did not facilitate the visual perception of face stimuli versus non-face stimuli. As a result, our electrophysiological recordings may be more reflective of the reward prediction or preparatory motor act of pecking, as opposed to the perception of stimuli as examples of face and non-face object categories.

Other lines of evidence indicate that faces do hold relevance for birds. For example, neurobiological and behavioural studies in chicks (Vallortigara, 1992; Vallortigara and Andrew, 1994; Mayer et al., 2016) indicate that the avian right hemisphere plays a critical role in both the perception and recognition of conspecifics as a global configuration of features. Moreover, both pigeons (Patton et al., 2010) and Japanese quail (Domjan and Nash, 1988; Domjan et al., 1989) spontaneously elicit courtship behaviour and preferentially attend to the face region of live and static images of conspecifics indicating that they recognise the visual object as a potential mate. Determining the neural mechanism by which birds perceive and recognise conspecifics faces may require a task design that maximises attention to faces as a global combination of features without the psuedocategorisation of stimuli. An alternative Go/No-Go discrimination task would be one in which pigeons are trained to discriminate between an S+ and S− image set comprising images of two different pigeons faces, respectively, in various viewpoints (Watanabe and Ito, 1990). Pigeons can then be tested in a second phase to discriminate between examples of the original S+ and S− face stimuli that are all manipulated in the same way (e.g., all face stimuli scrambled). Another possible behavioural task design is to remove the S+/S− discrimination entirely and instead employ a passive fixation task in which individual stimuli belonging to separate object categories are all rewarded with grain (Azizi et al., 2019). To maximise attention to the stimuli, pigeons should be required to withhold pecking responses both during a pause period before stimuli are presented and after stimuli appear for a randomly determined duration (e.g., between 2 and 4 s), until a Go stimulus appears in place of the image.

Recent technological advances used in conjunction with the aforementioned behavioural tasks may further ensure that neuronal activity is reflective of visual stimuli. For instance, a system for tracking the gaze of laterally eyed vertebrates has recently been developed (Tyrrell et al., 2014), and could be

used in conjunction with an S+/S− discrimination or passive fixation task while measuring the activity of neuronal populations across the tectofugal visual pathway with electrophysiological recordings or functional magnetic resonance imaging (fMRI: De Groof et al., 2013; Behroozi et al., 2017). The combination of such techniques may enable researchers to present visual stimuli at an appropriate distance to maximise global processing strategies and ensure that subjects view stimuli within their fovea, as is common practise in face perception studies using non-human primates (Tsao et al., 2003; Hung et al., 2015). However, another reason for why we were unable isolate a large proportion of face-selective cells may be that birds also depend on visual cues other than the face region to construct representations of socially significant objects. For example, birds lack the developed facial musculature of primates that is associated with dorsal face-selective patches for the processing of facial expressions and gaze direction in the superior temporal sulcus (STS) of macaques and humans (Moeller et al., 2008; Fisher and Freiwald, 2015). Indeed, there are also large patches of mammalian extrastriate cortex that measure the feature dimensions of body parts that are located directly adjacent to face-selective patches in macaque extrastriate cortex (Pinsk et al., 2009; Bao and Tsao, 2018).

Functional Contributions of the Visual Forebrain to Object Recognition

Recent advances in avian neurobiology have revealed that birds' dorsal telencephalon (comprising the dorsal ventricular ridge and Wulst) shares many genetic and functional similarities with mammalian neocortex, although it does not resemble the neocortex morphologically (Shimizu and Bowers, 1999; Briscoe et al., 2018; Gutiérrez-Ibáñez et al., 2018). For example, the avian dorsal telencephalon receives input from visual, auditory and somatosensory dorsal thalamic neurons, as does the neocortex (Reiner et al., 2004). Briscoe et al. (2018) used RNA sequencing to show that both the bird and crocodilian mesopallium regions share transcription factors regulating genes that control the development of associational neuronal types found in the mammalian neocortex (cells that forward projections only to other telencephalic targets). These cell types are classified as intra-telencephalic neurons (major excitatory cell types found in neocortical layers 2, 3, 5, and 6), and are defined by their functional circuit contributions, rather than on the basis of gross morphology. Such cell types may represent the closest homology with associational networks found in mammalian extrastriate cortex and their expression in the mesopallium suggests that MVL may be the visual nucleus in the tectofugal pathway at which category-level representation of objects emerges. In support of categorical representation in MVL, Marzluff et al. (2012) used PET brain imaging to demonstrate that crows exhibit significant neuronal activation of the anterior mesopallium in response to viewing human faces. Moreover, projections from ENTO to MVL in pigeons are arranged along an anterior–posterior axis (Krützfeldt and Wild, 2005), suggestive of a functional continuation of the parallel visual processing streams for form/colour (anterior) and motion (posterior) at the level of the nRT of the thalamus (Wang et al., 1993), ENTO (Nguyen et al., 2004) and into the telencephalon. Azizi et al. (2019) recently demonstrated that representations of animate (humans and animals) vs. inanimate objects (natural and artificial objects) could be extracted from population responses using a linear discriminant analysis at the level of MVL, but not from ENTO. Interestingly, the sub-category of human faces and body parts was identified to be driving the linear increase in classification performance for animate-category stimuli, indicating that MVL exhibits a population code of features for the representation of biologically relevant object categories. We performed similar multivariate styles of analysis to Azizi et al. (2019) to assess if visual forebrain structures would exhibit a population coding of conspecifics faces, but failed to demonstrate a significant population representation of pigeon faces as a category using RSM (see **Figure 5**), or any relative differences in firing rates elicited by pigeon faces and non-face stimuli between ENTO, MVL, NFL, or TPO.

The absence of any category-selective responses may also be explained by some critical differences in the functional organisation of the tectofugal visual pathway at the level of the association structures. For example, Stacho et al. (2016) determined that MVL, NFL, and TPO were activated equally during form/motion discrimination tasks, and showed no significant differences between hemisphere or anteroposterior position. These findings may reflect a neuronal organisation in birds where parallel processing streams for form/motion are segregated up to primary telencephalic centres (such as ENTO; Nguyen et al., 2004) which subsequently fuse and integrate both form/motion and other multimodal aspects of tectofugal visual information upstream in association forebrain areas to save processing space (Stacho et al., 2016). Our analysis of visual responsivity patterns showed that there were no significant differences in activation between ENTO, NFL, MVL, and TPO, or between hemispheres for each region. While posterior ENTO showed significantly greater visual responsivity than anterior ENTO (see **Table 4**), there was little evidence for anteroposterior differences in activation at the level of MVL. The combined evidence suggests that the association visual forebrain of birds may integrate multimodal sensory representations using mechanisms that differ substantially from mammals. Further electrophysiological and fMRI studies are required to determine what computations ENTO, MVL, NFL, and TPO may contribute to object representation, and further examine their proposed homology with extrastriate visual cortex.

CONCLUSION

We are rapidly approaching a comprehensive understanding of object recognition in macaques, humans, and other mammalian species. How non-mammalian vertebrates solve the problems of object representation without a neocortex is a long-standing problem in evolutionary neuroscience, though multiple lines of accumulating evidence suggest that these abilities arise from circuitry fundamentally similar to extrastriate cortex. While we

found no evidence of face-selective neurons in ENTO, NFL, MVL, or TPO, birds' predisposition to attend to the local features of stimuli in visual discrimination tasks likely influenced our pigeons' perception of objects as a global configuration of feature dimensions. It remains to be determined how the nuclear architecture of the tectofugal visual forebrain constructs stable representations of ethologically relevant percepts, and how the output of such circuits contribute to birds visual behaviour.

DATA AVAILABILITY

The raw data supporting the conclusions of the study will be made available by the authors upon request, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

WC designed the study and performed electrophysiological data collection, MATLAB data analysis, histology, and wrote the manuscript. BP lead MATLAB data analysis and gave invaluable manuscript guidance. MC assisted with the design of the study, wrote all behavioural task programmes to perform the study, read drafts, assisted with the structure of the manuscript, and provided the resources to conduct the study.

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frontiers in Physiology

BRIEF RESEARCH REPORT
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Mapping the Neural Substrates of Recent and Remote Visual Imprinting Memory in the Chick Brain

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Social attachment formed by filial imprinting in newborn chicks undergoes a process of memory consolidation that involves rearrangement of its neural storage substrates. In the first 3 h after imprinting it depends on the integrity of the intermediate medial mesopallium (IMM) and beyond that time on unidentified memory storage structures dubbed S'. To search for the S' memory system in the chick brain, we mapped and compared patterns of activity induced by retrieval of filial attachment memory before and after this critical transition. Chicks were trained in the visual imprinting task, and their memory was reactivated by imprinting stimulus either 1 h (recent memory retrieval) or 24 h (remote memory retrieval) after the completion of training. Patterns of brain activity were mapped by *in situ* hybridization to mRNA of an immediate early gene *c-fos*. We also mapped *c-fos* expression induced by the first presentation of the imprinting stimulus. Memory retrieval triggered massive *c-fos* expression in the chick brain both 1 and 24 h after the end of training. These activity pчatterns mostly coincided with the *c-fos* expression induced by the first presentation of imprinting stimulus. However, in the hippocampus *c-fos* induction was observed only after the first exposure to imprinting stimulus but not after memory retrieval. In the IMM, medio-rostral nidopallium/mesopallium, and hyperpallium densocellulare *c-fos* activation was induced by retrieval of only the remote but not of the recent memory. These *c-fos* mapping data point to the candidate brain structures for systems reorganization of imprinting memory in chicks.

Keywords: chicks, imprinting, learning, memory, retrieval, systems consolidation, *c-fos* expression

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INTRODUCTION

Chicks of precocial birds form strong preference for a moving object that they encounter within the first hours of their life. In the brain of domestic chicks (*Gallus gallus domesticus*), visual imprinting depends on the intermediate medial mesopallium (IMM). Bilateral lesions of the IMM before training prevent learning and the lesions made less than 3 h after the training disrupt the acquired

memory (McCabe et al., 1982). However, there is also an additional memory system (named S') that does not depend on the IMM integrity. In contrast to the IMM-dependent memory, S' system becomes functional 4–6 h after the end of training, and by 26 h it is fully able to sustain the imprinting recall in the absence of the IMM (Cipolla-Neto et al., 1982; Honey et al., 1995).

Despite the existence of S' system was hypothesized long time ago, the neural substrate of this additional memory storage is still unknown. In the present study we addressed this question by comparing neuronal activation induced in the chick brain by the recent (1 h after the end of training) and by the remote (24 h) retrieval of imprinting memory. For this purpose, we used *in situ* hybridization mapping of stimulus-induced expression of an immediate early gene *c-fos* known to be regulated by neuronal activation (Minatohara et al., 2016) and expressed during formation and retrieval of memory in the chick brain (McCabe and Horn, 1994; Suge and McCabe, 2004; Salinska, 2006; Suge et al., 2010; Yamaguchi et al., 2010).

MATERIALS AND METHODS

Chicken embryos of the Ptichnoe strain were obtained from a local supplier on E12-E15 and incubated in darkness until hatching and imprinting (see **Supplementary Figure 1** for the scheme of the experiment). At the age of 24 ± 8 h chicks were placed in a running wheel and exposed to an imprinting object (illuminated rotating box) for 60 min. Species-specific maternal calls were played back during the training. The number of the wheel revolutions toward the training stimulus and in the opposite direction was recorded. After the training chicks were returned to the home boxes and left until the memory retrieval session either 1 h after the end of training (Recent memory retrieval Group, n = 10) or 24 h after the training (Remote memory retrieval Group, n = 10). For memory retrieval, chicks were placed in the running wheel and exposed to the same imprinting stimulus for 20 min. Immediately afterward chicks were sacrificed, and their brains were processed for *in situ* hybridization. Additionally, there were 3 control groups. Chicks of the first exposure group [1st Exp (1st Exposure) Group] (n = 9) were placed in the running wheel and exposed to the imprinting object for 20 min without preceding training, their brains were taken for *in situ* hybridization immediately after this session. Chicks which received training without the retrieval session (Training Group, n = 9) were trained for 60 min and sacrificed 1 h 20 min later. The quiet control chicks (QC Group, n = 8) were kept individually in dark boxes and taken for *in situ* hybridization from there.

C-fos mRNA was detected by *in situ* hybridization on 20 μm cryostat brain sections with the 502 bp digoxigenin-labeled chicken *c-fos* RNA probe synthesized according to the manufacturer's protocol (DIG RNA SP6/T7 Labeling Kit, Roche). The *c-fos* mRNA detection protocol was described elsewhere (Della Ragione et al., 2006). Sections were digitized and quantitative analysis was carried out in six brain regions – the intermediate medial mesopallium (IMM), medio-rostral nidopallium/mesopallium (MNM), medial striatum (MSt), hyperpallium densocellulare (HD), nidopallium dorsocaudal (Ndc), and the hippocampus (Hpc) (Horn et al., 1983; Kuenzel and Masson, 1988; Metzger et al., 1998; Suge and McCabe, 2004; Thode et al., 2005; see **Figure 1A**). Expression density was calculated as the ratio of the number of labeled cells in the selected region to the region area in mm^2. Statistical analysis was carried out using Statistica 6.0. To meet ANOVA assumptions, the data were log-transformed and the between-group differences were estimated using one-way ANOVA. *Post hoc* analysis was performed using the Tukey HSD test.

This study was carried out in accordance with the recommendations of the Directive 2010/63/EU of the European Parliament and of the Council of the European Union issued September 22, 2010, on the protection of animals, used for scientific purposes (Section 27). The protocol was approved by the Ethics committee of the Anokhin Research Institute of Normal Physiology.

RESULTS

Mean number of the wheel revolutions during 20 min sessions was 91.2 ± 32.4 (mean ± SE) for the recent memory retrieval group and 113.0 ± 70.2 for the remote memory retrieval group, while for the first exposure group the mean number of revolutions was 16.6 ± 7.2 which was significantly less than in the retrieval groups [$F(2,24) = 4.23$, $p = 0.028$].

No significant interhemispheric differences were found in *c-fos* mRNA expression in all six analyzed brain regions, therefore the data from the left and right hemispheres were pooled. The ANOVA revealed significant between-group differences for the IMM [$F(4,39) = 6.58$, $p = 0.00038$], MNM [$F(4,25) = 8.97$, $p = 0.00012$] and HD [$F(4,43) = 20.14$, $p = 0.00000$]. Pronounced elevation of *c-fos* expression in these structures was observed in the remote memory retrieval group and in the first exposure group compared to the quiet control and to the training group which received no memory retrieval (**Figures 1A–C**).

The level of *c-fos* expression in the recent memory retrieval group did not differ from the quiet control group and from the training group (**Figures 1A–C**).

Significant group effect was found in the Ndc [$F(4,26) = 6.03$, $p = 0.00143$] and MSt [$F(4,26) = 12.83$, $p = 0.00006$] as well. In these areas both recent and remote memory retrieval induced *c-fos* expression comparable to that in chicks which were presented with the imprinting stimulus for the first time [1st Exp (1st Exposure)] (**Figures 2D,E**). In the hippocampus no induction was observed after the recent and remote memory retrieval while the first exposure to the imprinting stimulus induced strong expression [$F(4,42) = 4.26$, $p = 0.00552$, **Figure 2F**].

DISCUSSION

The aim of the present study was to identify structures of the chick brain that had differential activation during retrieval of

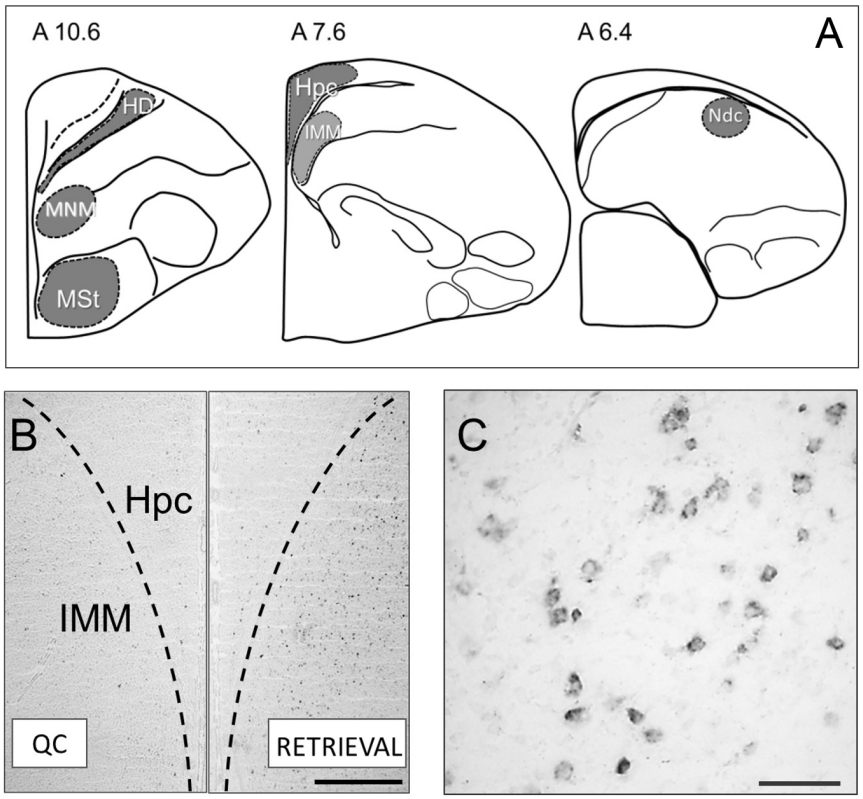

FIGURE 1 | (A) Brain structures used for the *c-fos* expression analysis: HD, Hyperpallium densocellulare; MNM, Medio-rostral nidopallium/mesopallium (Maier and Scheich, 1983); MSt, Medial striatum; Hpc, Hippocampus; IMM, Intermediate medial mesopallium (Horn et al., 1983); Ndc, Nidopallium dorso-caudale (Metzger et al., 1998). **(B)** Micrographs showing representative *c-fos* mRNA staining in the brain of the untrained control chick (left half) and after memory retrieval (right half of the image). Scale bar = 500 μm. **(C)** Micrograph showing cytoplasmic *c-fos* mRNA staining at a higher magnification. Scale bar = 100 μm.

the recent (1 h) and delayed (24 h) visual imprinting memory. In this analysis we relied on the known property of *c-fos* to be a marker of activity-dependent neuronal transcription (Minatohara et al., 2016). During re-exposure to imprinting object this activation may subserve memory reconsolidation (Litvin and Anokhin, 2000; Anokhin et al., 2002) and thus localize memory storage sites.

The weak *c-fos* expression in the quiet control chicks supports our previous observation on its low basal expression in the newborn chicks (Anokhin et al., 1991). The expression in the trained group 80 min after the end of training did not differ from the controls confirming a rapid decay of transiently induced *c-fos* mRNA (**Figure 2**). The highest level of *c-fos* expression was in chicks exposed to the imprinting object for 20 min [1st Exp (1st Exposure) Group]. These results are in line with the data that imprinting-induced *c-fos* expression in the IMM reaches maximum after the 15-min of training and returns to the basal level 75 min after the session completion (Suge et al., 2010).

We found that retrieval of imprinting memory 1 and 24 h after the training induced expression of *c-fos* in several brain regions. The density of *c-fos* positive cells was higher in the remote retrieval group compared with the recent retrieval group in most of the examined structures. After the remote retrieval, *c-fos* expression was significantly increased in the IMM, MNM, MSt, HD, and Ndc as compared with the quiet control group (**Figure 1**).

The expression of *c-fos* in the IMM was induced only by the remote but not the recent memory retrieval (**Figure 2A**). However, electrophysiological recording of the IMM neuronal responses to imprinting stimulus revealed two peaks of high responsiveness – at about 1.75 and 25 h after the onset of training (Horn et al., 2001). These intervals coincide with retrieval sessions in our experiments. Thus, a day after training IMM shows both electrophysiological and *c-fos* neuronal responses which supports the view that IMM participates in the retrieval of imprinting memory at 24 h after the training (Horn, 2004), while the engagement of IMM in retrieval of the recent memory is documented by the electrophysiological (Horn et al., 2001) and the lesion data (McCabe et al., 1982). The dissociation between electrophysiological and *c-fos* data can be due to different aspects of neuronal functions measured by two techniques, *c-fos* being preferentially a plasticity marker.

A similar pattern of differential *c-fos* expression in the recent and remote retrieval was observed in the MNM and HD (**Figures 2B,C**). MNM was defined by learning-induced increase in 2-deoxy-D-glucose uptake, release of glutamate and expression of another immediate early gene ZENK during acoustic imprinting (Maier and Scheich, 1983; Gruss and Braun,

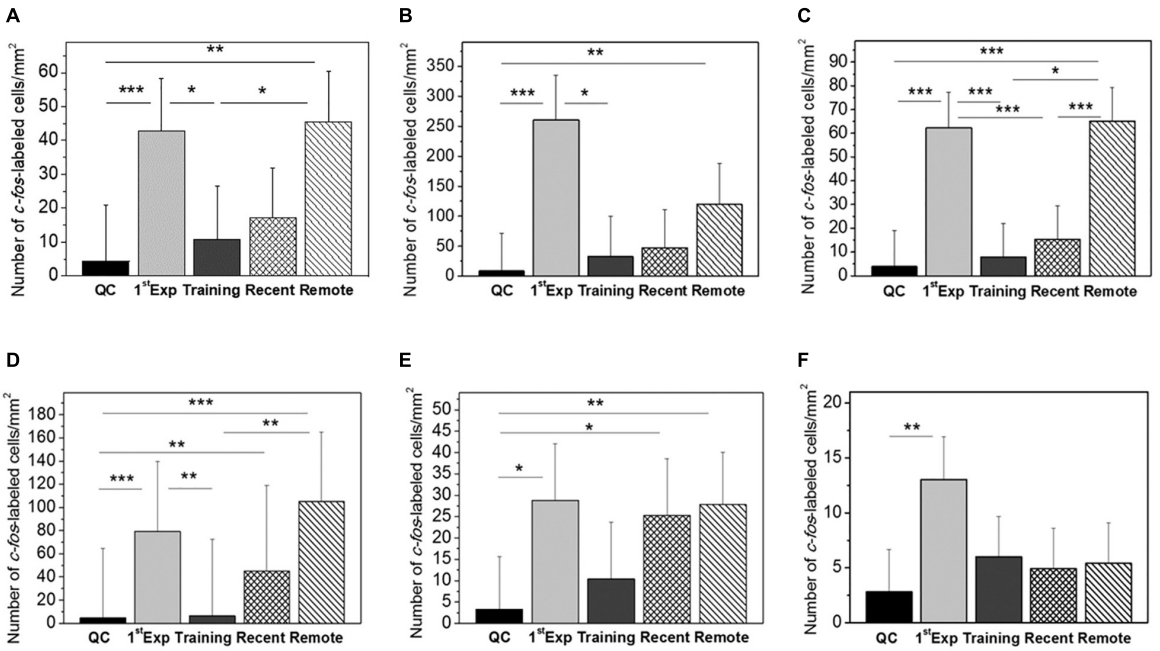

FIGURE 2 | Mean number of *c-fos*-labeled cells in mm^2 in the IMM **(A)**, MNM **(B)**, HD **(C)**, MSt **(D)**, Ndc **(E)**, and Hpc **(F)**. Groups: QC, dark-reared chicks taken from their homeboxes; 1st Exposure, chicks were exposed for 20 min to the imprinting object and the brains taken immediately after the session; Training, brains taken 1 h 20 min after 60-min exposure to the imprinting object; Recent, brains taken immediately after 20-min re-exposure to the imprinting object, the interval between the first (60-min) exposure and the re-exposure 60 min; Remote, brains taken immediately after 20-min re-exposure to the imprinting object, the interval between the first (60-min) exposure and the re-exposure 24 h. Error bars denote 0.95 confidence intervals. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. The between–groups differences were estimated using the one-way ANOVA followed by Tukey HSD applied to log-transformed data.

1996; Bredenkötter and Braun, 1997; Thode et al., 2005). HD core connects the Wulst and IMM and this link is strengthened by imprinting (Nakamori et al., 2013). Selective lesions of the HD impair imprinting (Nakamori et al., 2010). Moreover, c-Fos expression in the HD neurons was activated by the presentation of imprinting stimulus to the P7 day chicks imprinted on the P1 (Nakamori et al., 2010). Our results on the remote retrieval-induced *c-fos* HD expression are in line with these data.

In the MSt and Ndc the *c-fos* expression was increased by both recent and remote memory retrieval (**Figures 2D,E**). Ndc is a nidopallium area discovered by the increased metabolic activity during presentation of the imprinting stimulus to the acoustically or visually imprinted chicks (Bock et al., 1997). 30 min of acoustic imprinting induced expression of the *Arc* gene in the Ndc (Bock et al., 2005), and there was a reduction in spine density in this area after imprinting (Braun et al., 1999). Blockade of NMDA receptors in the Ndc impaired imprinting (Bock et al., 1997). Since Ndc projects to the IMM and is reciprocally connected with the MNM it was suggested that Ndc represents an associative brain region integrating visual and acoustic features of imprinting objects (Braun et al., 1999). MSt belong to the basal ganglia system important for learning and memory in chicks (Gilbert et al., 1991; Csillag, 1999), though kainate lesions of MSt were without effects on chick approaching behavior in the imprinting test (Izawa et al., 2001), which calls for cautionary interpretation of *c-fos* imaging data alone.

Finally, in the hippocampus *c-fos* expression was induced by the first exposure of chicks to the imprinting stimulus but not by the retrieval of imprinting memory (**Figure 2F**). The hippocampus in the chick projects bilaterally to the IMM (Bradley et al., 1985). However, 24 h after imprinting hippocampal neurons were shown to be sensitive only to the distance to the imprinting object but not to the specific object's characteristics (Nicol et al., 1998). Also, 15 min of imprinting training induced c-Fos expression in the hippocampus, but the level of the expression did not correlate with the preference score (Suge and McCabe, 2004). Our data support the view that the hippocampus is recruited during acquisition but not the retrieval of the imprinting memory.

In general, our study revealed a number of brain structures that were activated by the recent and remote retrieval of imprinting memory. They also show that the 24 h memory retrieval induced a broader *c-fos* expression than the retrieval of the 1 h memory. It was previously hypothesized that by the 24 h two parallel systems are supporting the imprinting memory, IMM-based system and the S' system with unknown location (Horn et al., 2001; Horn, 2004). Our *c-fos* mapping data suggest several brain regions which may represent the S' system. Other candidate structures will need to be examined as well, particularly the intermediate hyperpallium apicale which receives direct neural projections from the IMM and plays a critical role

in imprinting retention and recall in chicks (Aoki et al., 2015). As data on the MSt indicate (Izawa et al., 2001) lesion experiments are required to test the role of mapping-identified structures in storage and retrieval of filial attachment memory.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

KA and AT conceived the study and wrote the manuscript. AT and NK designed and performed the experiments and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00351/full#supplementary-material

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REVIEW
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Why Do Birds Flock? A Role for Opioids in the Reinforcement of Gregarious Social Interactions

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The formation of social groups provides safety and opportunities for individuals to develop and practice important social skills. However, joining a social group does not result in any form of obvious, immediate reinforcement (e.g., it does not result in immediate copulation or a food reward), and individuals often remain in social groups despite agonistic responses from conspecifics. Much is known about neural and endocrine mechanisms underlying the motivation to perform mate- or offspring-directed behaviors. In contrast, relatively little is known about mechanisms underlying affiliative behaviors outside of these primary reproductive contexts. Studies on flocking behavior in songbirds are beginning to fill this knowledge gap. Here we review behavioral evidence that supports the hypothesis that non-sexual affiliative, flocking behaviors are both (1) rewarded by positive social interactions with conspecifics, and (2) reinforced because affiliative contact reduces a negative affective state caused by social isolation. We provide evidence from studies in European starlings, *Sturnus vulgaris*, that mu opioid receptors in the medial preoptic nucleus (mPOA) play a central role in both reward and the reduction of a negative affective state induced by social interactions in flocks, and discuss potential roles for nonapeptide/opioid interactions and steroid hormones. Finally, we develop the case that non-sexual affiliative social behaviors may be modified by two complementary output pathways from mPOA, with a projection from mPOA to the periaqueductal gray integrating information during social interactions that reduces negative affect and a projection from mPOA to the ventral tegmental area integrating information leading to social approach and reward.

Keywords: mu opioid receptors, reinforcement, social cohesion, affiliation, songbirds, medial preoptic area, ventral tegmental area, periaqueductal gray

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Many well-studied social behaviors have primary reproductive or survival functions. For example, courtship behaviors are used to attract mates, agonistic behaviors are used to defend breeding and feeding territories, and food-begging calls are used to acquire food. These behaviors are directed toward a specific goal and can be reinforced by immediate, observable outcomes. For example, courtship can be rewarded by copulation, agonistic behaviors can be reinforced

by immediate departure of a rival, and food-begging can be rewarded by receipt of food. However, outside these primary contexts, animals engage in several behaviors for which immediate functions and reinforcing factors are difficult to determine. This includes the formation and maintenance of social groups in gregarious animals.

The formation of social groups has adaptive benefits (e.g., safety and improved foraging efficiency; Powell, 1974; Lazarus, 1979; Sullivan, 1984; Thiollay and Jullien, 1998), and interactions within groups allow animals to develop and practice important social skills that can be used later in goal-directed contexts (Himmler et al., 2013; Vanderschuren and Trezza, 2014; Pellis and Pellis, 2017; Riters et al., 2017). However, joining a group does not result in any form of obvious, immediate reinforcement (e.g., it does not result in copulation or a food reward), and animals at times will remain in social groups even in the face of agonistic interactions with conspecifics. The formation and maintenance of cohesive social groups has evolved several times in vertebrate lineages, suggesting that gregariousness is not only adaptive, but that social grouping is reinforced by some external or internal mechanism at the level of individuals. Much is known about neural and endocrine mechanisms that reinforce mate-, rival-, and offspring-directed behaviors. In contrast, relatively little is known about mechanisms underlying the motivation for animals to affiliate in non-sexual social groups outside of these primary, reproductive contexts. Here, we review studies on flocking in non-reproductive contexts in songbirds that are beginning to fill this knowledge gap.

INTRODUCTION TO NON-REPRODUCTIVE FLOCKING BEHAVIOR

When not mating or defending territories, many animals are solitary; however, there are notable exceptions, with birds ranking as among the most gregarious vertebrates. Birds are well-known for their remarkable flocking behavior. Members of some species spend most of their lives surrounded by flock mates (Goodson and Kingsbury, 2011), while others display predictable seasonal changes in sociality. For example, many birds shift seasonally from pair or solitary living during the breeding season to the formation of flocks for migration or overwintering after the breeding season (e.g., Emlen, 1952a; Feare, 1984; Eens, 1997; Wilson et al., 2016). The factors promoting gregariousness in birds have long been a source of interest. Early ethologists suggested that flocking can result either from non-social factors (e.g., individuals attracted to a common resource such as shade or a food patch) or social factors (i.e., mutual conspecific attraction or an aversion to being alone), with each of these factors likely at play in most flocks (Emlen, 1952a).

The proposed social influences suggest that flocks may result from the integration of both positive reinforcement (i.e., a behavior is strengthened because it leads to a positive outcome) and negative reinforcement (i.e., a behavior is strengthened because it leads to reduction of an aversive state), such that flocking behavior is strengthened because interactions with flock mates both (1) induce a rewarding, positive affective state and (2) reduce a negative affective state caused by social exclusion or isolation, thus creating a complementary system (i.e., positive reinforcement from affiliative interactions and negative reinforcement from termination of isolation).

Flocking Behavior and Positive Reinforcement

At least three lines of research support the proposal that flocking is strengthened by mutual positive associations with flock mates. First, in choice tests zebra finches, *Taeniopygia guttata* prefer to spend more time near larger flocks of conspecifics (consisting of 10 same-sex individuals) compared to smaller flocks (consisting of two same-sex individuals) (Kelly et al., 2011). This finding indicates that in a species that is highly gregarious in nature, the presence of large numbers of conspecifics may be rewarding. Second, isolated European starlings, *Sturnus vulgaris* are willing to work (i.e., trigger sensors) to view images of conspecifics in the absence of any other reward. Furthermore, they respond more to pictures of starlings compared to pictures of landscapes or monkeys (Perret et al., 2015). This demonstrates that conspecific, social stimuli are primary reinforcers in this social species. Third, studies using conditioned place preference (CPP) tests [a common method to assay reward (Carr et al., 1989; Tzschentke, 2007; Trezza et al., 2009; Riters et al., 2013)] show that vocal-social interactions in non-breeding flocks of starlings and zebra finches are associated with a positive affective state (Riters and Stevenson, 2012; Riters et al., 2014; Hahn et al., 2017). In these studies, flocks of male starlings and zebra finches were observed singing in aviaries for 30 min. Each bird was then immediately placed individually into one of two distinctly decorated sides of a conditioning cage for 30 min and afterwards returned to its home aviary. The next day each bird was placed back into the conditioning cage and allowed to move freely between the previously song-paired and the non-song-paired sides of the cage, and the amount of time each individual spent on each side was recorded. The prediction was that if vocal-social interactions in non-breeding flocks are associated with a positive affective state, then birds would learn to associate the positive affective state with the distinctly decorated side of the cage and when given a choice spend most of their time on that side (i.e., they would develop a CPP). Results demonstrated that both male zebra finches and starlings developed a CPP for the chamber that had been paired with production of song in gregarious flocks (Riters and Stevenson, 2012; Riters et al., 2014; Hahn et al., 2017) (**Figure 1**). However, birds did not develop a CPP associated with song produced in mating or agonistic contexts, indicating that the factors that reward goal-directed, immediately reinforced behaviors differ from those underlying affiliative behaviors produced in non-sexual, gregarious contexts.

Although CPP tests are commonly used to examine rewarding properties of drug use, feeding and sexual behaviors, the function of CPPs in wild animals in natural contexts is seldom considered. The finding that vocal-social interactions in gregarious flocks can lead to the development of a CPP suggests

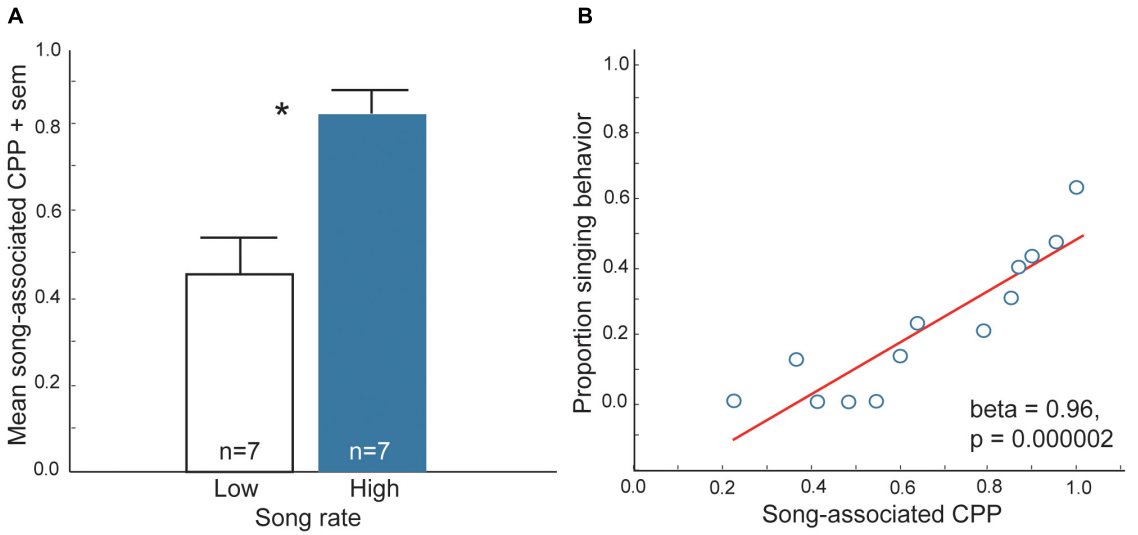

FIGURE 1 | Evidence that vocal-social interactions in starling flocks are associated with a positive affective state. **(A)** Mean time spent on the side of a CPP apparatus that had been paired previously with either low (open bar) or high (filled bar) rates of singing behavior in flocks of male starlings. *$p < 0.05$. **(B)** Correlation between song and CPP in male starlings. *Y*-axis represents the proportion of all vocal behaviors that were songs produced by males during and just prior to being placed in one side of the CPP apparatus (song-paired side). The *X*-axis represents the proportion of time males spent on the previously song-paired side of the apparatus the following day (CPP, considered a reflection of song-associated reward). Each point represents data from a single male. Figures redrawn from Riters and Stevenson (2012).

that a natural function of this type of conditioning may be to strengthen group cohesion through a conditioned preference for a particular flock. Overall, these findings offer support for the hypothesis that social interactions in gregarious contexts can be positively reinforced.

Flocking Behavior and Negative Reinforcement

It is common to observe birds that are separated from flocks appearing to rush to reunite with conspecifics. Early ethologists compared the motivation to flock in gregarious animals to a "hunger, a craving or sensation of discomfort" or a "state of agitation" in the absence of a physical requirement that can only be relieved by reunion with a flock (Trotter, 1916; Craig, 1918; Emlen, 1952a). This suggests that flocking may be strengthened not only because interactions with flock mates induce a positive affective state but also because they reduce a negative affective state. That is, flocking may be negatively reinforced. In gregarious vertebrates, social separation is painful (Macdonald and Leary, 2005; Eisenberger, 2012). For example, separation of young rats from mothers or the removal of guinea pigs, domestic chicks or zebra finches from conspecifics leads to the production of "distress vocalizations" or contact calls that are eliminated by reunion with group mates (Zann, 1985; Hofer et al., 1993; Kyuhou and Gemba, 1998; Warnick et al., 2005). Several studies demonstrate that the same neural systems and modulators that process physical pain also regulate the pain of social separation, and this is proposed to be adaptive given that social disconnection in gregarious species threatens survival (Macdonald and Leary, 2005; Eisenberger, 2012). For example, treatments that reduce physical pain (i.e., induce analgesia) are also found to reduce signs of social pain in animals as well as activity in brain regions underlying physical pain. This includes treatment with opioids such as morphine and even treatment with the common pain reliever acetaminophen (Herman and Panksepp, 1978; Panksepp et al., 1978; Dewall et al., 2010).

The data showing that social and physical pain share underlying mechanisms lead to the prediction that social interactions in flocks may reduce social pain. Some support for this idea comes from a CPP study in male zebra finches. In zebra finches, separation from a flock increased production of distance contact calls, which are triggered by separation from partners or flock mates and reduced when individuals are reunited (Guttinger and Nicolai, 1973; Simpson and Vicario, 1990; Zann, 1996). In contrast to the CPP identified for song in flocks described above, zebra finch contact calls correlated negatively with an individual's affective state measured using CPP (i.e., males that produced high numbers of contact calls avoided the side of the CPP chamber associated with these calls) (Riters and Stevenson, 2012). This indicates that the affective state associated with separation-induced calls is negative.

The idea that social interactions in flocks relieve social pain via mechanisms that also regulate physical pain is supported by a song-associated analgesia study in male starlings (Kelm-Nelson et al., 2012). Singing was recorded in gregarious non-breeding flocks or in a mate-directed context. Each bird was then immediately captured and one foot was submerged in a hot water bath (hot enough to be mildly aversive; some birds retracted the foot immediately, but others did not respond). The amount of time it took for each bird to remove its foot from hot water was noted as a measure of analgesia.

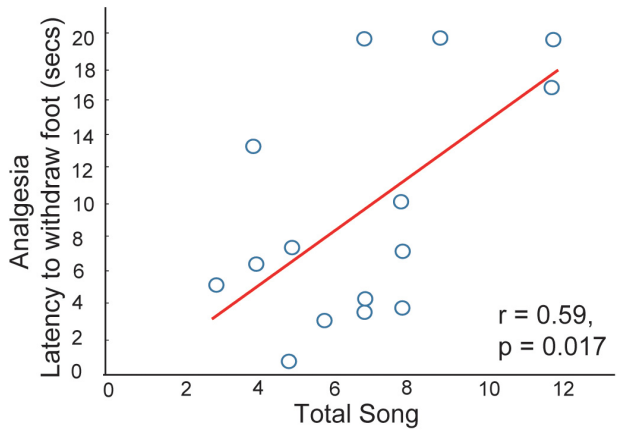

FIGURE 2 | Evidence that vocal-social interactions in starling flocks are associated with pain reduction. Correlation between song production and the latency for a male to withdraw its foot from a hot water bath. Each point represents one individual. Figure redrawn from Kelm-Nelson et al. (2012).

A linear positive correlation was found between this measure of analgesia and the production of song in flocks, indicating that vocal-social interactions in flocks are associated with pain reduction (**Figure 2**). A similar relationship was not found between sexually motivated female-directed song and analgesia, which again suggests that mechanisms mediating mate-directed, immediately reinforced behaviors differ from those underlying affiliative behaviors produced in gregarious contexts. This form of analgesia may function to strengthen group cohesion through the reduction of an aversive state. Thus, these findings offer support for the hypothesis that social interactions in non-sexual, gregarious contexts can be negatively reinforced.

Based on the associations revealed by these behavioral studies between singing in flocks and both positive affect and analgesia, we propose that flocking likely involves a combination of incentives and reinforcement mechanisms. This includes positive reinforcement (i.e., a behavior is strengthened because it leads to a positive outcome) and negative reinforcement (i.e., a behavior is strengthened because it leads to reduction of an aversive state). For example, it may be that an aversion to being alone pushes gregarious individuals to join flocks. Joining a flock may thus be negatively reinforced because it reduces the aversive state associated with isolation. Then, once a bird has joined a flock, behaviors produced within the flock, such as singing, may induce a positive affective state that then functions to positively reinforce associations with the flock.

MECHANISMS UNDERLYING FLOCKING BEHAVIOR

Opioids Modulate the Pain of Being Alone and the Pleasure of Social Contact

Opioid neuropeptides are the neuromodulators that to date have been best studied for their roles in both physical and social pain (Macdonald and Leary, 2005; Eisenberger, 2012). Opioids that bind to mu opioid receptors are also well-known for their rewarding and analgesic properties (Matthes et al., 1996; Trescot et al., 2008; Jhou et al., 2012; Fields and Margolis, 2015). Animals will readily self-administer mu opioid receptor agonists, such as morphine and develop strong CPPs for places associated with mu receptor agonist treatment (Wise, 1989; McBride et al., 1999). Mu receptor agonists also decrease the amount of time rats spend near conspecifics, which has been interpreted to suggest that reward induced by the agonist replaces the need for reward (or relief) that is normally induced by social contact (Herman and Panksepp, 1978; Panksepp et al., 1979). A role for opioids in pain relief induced by social interactions is also supported by past research. For example, reunion of male mice with siblings has been found to induce opioid-dependent analgesia (D'Amato and Pavone, 1993; D'Amato, 1998). Furthermore, the hot water foot dip test of analgesia used in the starling study described above is opioid sensitive (Evrard and Balthazart, 2002; Kelm-Nelson et al., 2012), suggesting that for starlings social contact within flocks (as reflected by flock singing) may release opioids to relieve the pain of isolation.

Data from other species offer more direct support for opioid-mediated social reward and/or reduction of social pain. Mu receptor agonists reduce separation distress vocalizations in rodents and primates; whereas the opioid receptor antagonist naloxone increases these calls (Herman and Panksepp, 1978; Panksepp et al., 1978; Kalin et al., 1988). Distress vocalizations in domestic chicks separated from flock mates were also reduced by systemic treatment with a mu opioid receptor agonist (Warnick et al., 2005). Manipulations of delta and kappa opioid receptors were ineffective, indicating that reduction of social distress induced by social reunion in chicks is mediated selectively by mu receptors. In contrast, peripheral injections of the opioid receptor antagonist naloxone in male zebra finches suppressed "undirected" singing behavior (Dunn and Zann, 1996; Zann, 1996; Khurshid et al., 2010). This is a type of song that is predominantly produced in gregarious flock settings; however, a caveat is that in this study undirected song was produced by birds in isolation rather than in a flock setting. Although differences may be found in birds producing this type of song as part of a gregarious flock, unpublished data in starlings singing in flocks also indicate that mu opioid receptor agonism facilitates singing in gregarious flocks (Riters et al., unpublished data). These findings appear to contradict the studies in rats that show mu agonism decreases time with conspecifics (reviewed above). However, one interpretation is that an optimal level of mu opioid receptor stimulation is needed to facilitate gregariousness, with low doses of MOR agonist stimulating behavior to a point, after which increasing doses suppress behavior (resulting in an inverted-U shaped curve). This idea is supported multiple studies that show inverted-U shaped relationships between mu opioid stimulation and behavior [e.g., for sucrose consumption (Zhang and Kelley, 1997), for self-injection of MOR agonist (Simmons and Self, 2009)]. Thus when taken together, studies to date indicate that opioids that act at mu receptors both reduce vocal behavior indicative of distress and stimulate vocal behavior indicative

of a positive (or less negative) affective state, consistent with a role for opioids in the regulation of flocking through both pain reduction (i.e., negative reinforcement) and pleasure (i.e., positive reinforcement).

A Role for Opioids in the Medial Preoptic Nucleus in Social Interactions in Flocks

There are several brain regions in which the activation of mu opioid receptors can induce reward and analgesia. One such region is the medial preoptic nucleus (mPOA; often referred to as POM in birds) (Tseng et al., 1980; Tseng and Wang, 1992), which has been implicated strongly in the regulation of the affective state associated with birdsong (Riters, 2012; Riters et al., 2017). It is well known across vertebrates that the mPOA stimulates goal-directed, sexually motivated behaviors, including sexually motivated birdsong (Riters and Ball, 1999; Alger and Riters, 2006; Alger et al., 2009). However, this region also appears to play a role in non-sexual vocal-social interactions in flocks of male starlings. In contrast to the inhibition of sexually motivated song by mPOA, lesions of the mPOA tend to promote song in flocks in non-breeding male starlings (Alger and Riters, 2006). In mPOA, opioids that bind to mu receptors inhibit neuronal firing in birds and mammals (Diez-Guerra et al., 1987; Furukawa et al., 1995), suggesting that opioid release associated with vocal-social interactions in flocks may inhibit activity in mPOA to facilitate song in non-breeding flocks.

Opioid measures in mPOA correlate with vocal-social interactions in gregarious flocks. Measures of mu opioid receptor immunolabeling in mPOA correlate positively with vocal-social interactions in gregarious flocks, but only to a point, after which higher rates of song are associated with lower densities of receptor labeling, resulting in inverted-U shaped relationships (Kelm-Nelson and Riters, 2013) (**Figure 3**). While it could be that opioid signaling decreases in birds singing high rates of non-breeding flock song, met-enkephalin labeling in mPOA relates positively to singing behavior even in the birds singing at the highest rates (Riters et al., 2005). Mu opioid receptors down-regulate in response to sustained occupation by enkephalin (Chang et al., 1982; Harrison et al., 1998), suggesting that high levels of opioid release in mPOA associated with the highest rates of vocal-social interaction may cause mu-opioid receptors to down-regulate (explaining the low mu densities in the second half of the curve; **Figure 3**).

Opioids (i.e., enkephalins and other mu receptor agonists) in the mPOA, have been shown in rats to induce analgesia as well as reward (analgesia: Tseng et al., 1980; Tseng and Wang, 1992); reward: Agmo and Gomez, 1991, 1993; Le Merrer et al., 2009). These previous data lead to the hypothesis that opioid release in mPOA caused by joining a flock and interacting socially with flock mates underlies both the reduction in pain (i.e., analgesia) and reward (i.e., song-associated CPP) observed in starling flocks. Consistent with this hypothesis, in male starlings, the affective state associated with vocal-social interactions in flocks (measured using CPP) correlated positively with both preproenkephalin (the precursor of the opioid met-enkephalin) and mu opioid receptor mRNA expression levels in mPOA (Riters et al., 2014). Moreover, preliminary data from a study on the mPOA show that selective downregulation of MOR in this region (induced by siRNA infusion) in male starlings suppresses affiliative song and disrupts song-associated reward, (Riters et al., unpublished results).

THE mPOA ACCESSES BOTH CANONICAL REWARD AND PAIN PATHWAYS

Studies to date highlight opioid activity in mPOA as likely involved in positive and negative reinforcement of song, but this region does not work in isolation. The mPOA receives inputs from multiple brain regions (Chiba and Murata, 1985; Balthazart et al., 1994; Riters and Alger, 2004) and is proposed to integrate information about an individual's internal state with the external environment so that an animal will produce an appropriate motor output (Wood, 1998; Ball and Balthazart, 2004; Hull and Dominguez, 2006; Alger et al., 2009). Of relevance to mechanisms of positive and negative reinforcement are two key output pathways. (1) The mPOA directly accesses the canonical mesolimbic reward pathway via a direct projection to the ventral tegmental area (VTA) (Balthazart et al., 1994; Riters and Alger, 2004), which sends projections to the nucleus accumbens (NAc) (Husband and Shimizu, 2011) (**Figure 4**). This has been the best studied pathway for the regulation of motivated, reward-directed behaviors, yet few studies have focused on the role of this pathway in flocking behavior. (2) The mPOA also gains direct access to a well-studied pain pathway via a projection to the periaqueductal gray (PAG; often referred to as the central gray in papers on birds), a region well-known to regulate pain and aversive emotional states (Behbehani, 1995; Macdonald and Leary, 2005; Lieberman and Eisenberger, 2009; Wright and Panksepp, 2011) (**Figure 4**). This suggests the hypothesis that the mPOA may unite a complementary dual-pathway system to regulate flocking behavior. Below we review data from the few studies involving flocking behavior to date that have examined VTA and PAG. There are certainly other pathways in which opioids modulate pain and pleasure and recent studies identify overlap in circuits regulating pleasure and pain (e.g., Leknes and Tracey, 2008; Mitsi and Zachariou, 2016). We consider the two mPOA output pathways that we highlight here to be a reasonable starting point to begin to reveal mechanisms that reinforce flocking behavior, but additional regions and mechanisms should be considered in future studies.

A Possible Role for the Mesolimbic Reward Pathway in Flocking Behaviors

Dense dopaminergic projections from the VTA to the NAc are among the best studied components of the canonical mesolimbic reward pathway [for recent review see (Baik, 2013)]. Multiple studies demonstrate these dopaminergic projections to be crucial for motivated, approach responses to rewarding stimuli (Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Ikemoto et al., 2015; Volkow et al., 2017). These projections are

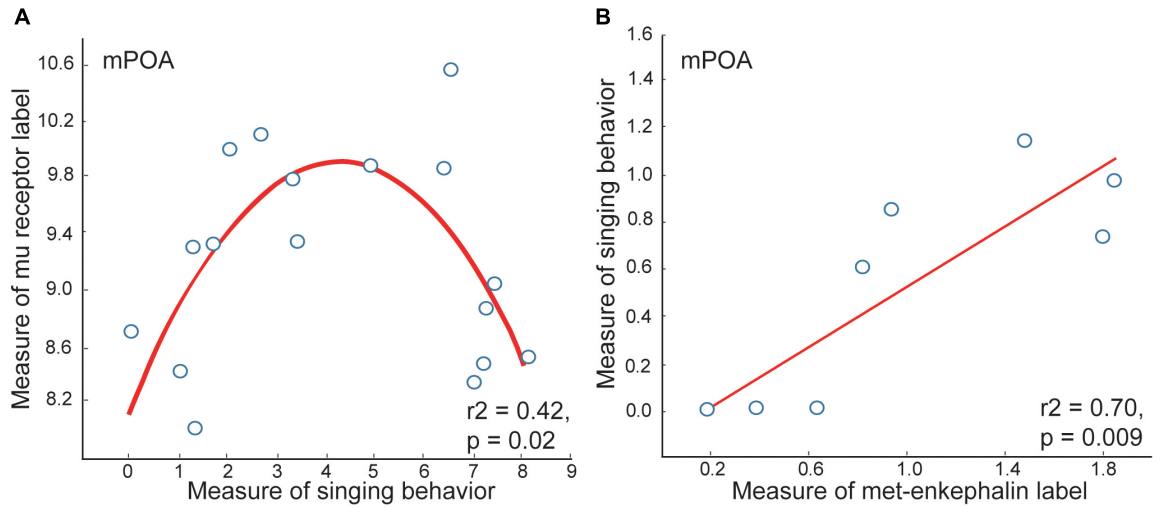

FIGURE 3 | Measures of mu opioid receptor and met-enkephalin immunolabeling in mPOA correlate with vocal-social interactions in starling flocks. **(A)** An inverted-U shaped curve shows the measure of flock song on the *x*-axis and the mean area covered by mu opioid receptor immunolabeling on the *y*-axis. **(B)** A positive linear correlation between flock song on the *y*-axis and the area covered by met-enkephalin immunolabeling. Each point represents one individual. Figures redrawn from Riters et al. (2005) and Kelm-Nelson and Riters (2013).

highly evolutionarily conserved and underlie multiple motivated, reward directed behaviors, with a few studies suggesting this role may extend to flocking behavior. For example, gregarious species of estrildid finch have more neurons labeled for tyrosine hydroxylase (a rate-limiting enzyme in catecholamine synthesis) in VTA than non-gregarious, territorial species (Goodson et al., 2009a). Tyrosine hydroxylase mRNA in VTA also correlates positively with vocal-social interactions in flocks of male starlings (Merullo et al., 2016) (**Figure 5**). These studies are consistent with the possibility that dopaminergic VTA projections modulate the motivation to interact with flock mates. Dopamine is also released in the VTA projection region Area X in male zebra finches singing undirected song (a type of song produced commonly in flocks) (Sasaki et al., 2006). Studies also implicate other neuromodulators, including neurotensin and endocannabinoids, in the VTA in non-sexual, vocal-social interactions in starling flocks. Neurotensin strongly modulates activity of dopamine neurons in the VTA (Steinberg et al., 1995; Kortleven et al., 2012; Stuhrman and Roseberry, 2015) and neurotensin mRNA in VTA correlates positively with singing behavior in starling flocks (Merullo et al., 2016) (**Figure 5**). Endocannabinoid CB_1 receptors also modulate the firing of dopamine neurons in VTA (Merullo et al., 2016), and CB_1 receptor mRNA expression in VTA correlates positively with CPP measures of flock song-associated reward (Merullo et al., 2016; Hahn et al., 2017) (**Figure 5**). In mammals, opioids in the VTA have also been found to indirectly stimulate dopaminergic neurons and motivated approach behaviors, with a similar mechanism identified in songbirds (Gale and Perkel, 2006). However, correlational studies so far do not (Riters et al., 2014), or only weakly (Riters et al., 2005), implicate opioids in the VTA in vocal-social interactions in flocks.

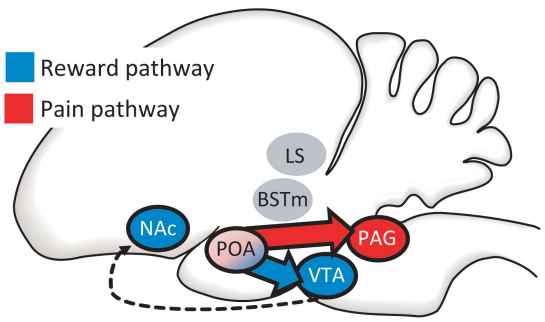

FIGURE 4 | The mPOA directly accesses both canonical reward (in blue) and pain (in red) pathways via projections to (1) the ventral tegmental area [VTA; which then projects to the nucleus accumbens (NAc)] and (2) the periaqueductal gray (PAG). We develop the case that the social pleasure and pain reduction that facilitate and maintain flocking behavior may be modified by these two complementary output pathways from mPOA, with PAG integrating information during social interactions that reduces negative affect and the VTA integrating information leading to social approach and reward. Two additional regions in which opioids are proposed to interact with nonapeptides, the bed nucleus of the stria terminalis (BSTm) and lateral septum (LS) are also shown. The LS, BSTm, POA, PAG, and VTA are all reciprocally connected (connections not shown).

In contrast to the well-studied role of VTA dopamine projections in motivated responses to rewarding stimuli, opioids binding to mu receptors in the NAc are implicated in reward (i.e., the hedonic pleasure induced when a reward is received) (Smith and Berridge, 2007; Berridge, 2009). Although a putative location for the NAc has been identified in birds based on neurochemical and hodological similarity to mammalian NAc (Balint and Csillag, 2007; Balint et al., 2011; Husband and Shimizu, 2011), to date no studies have experimentally examined

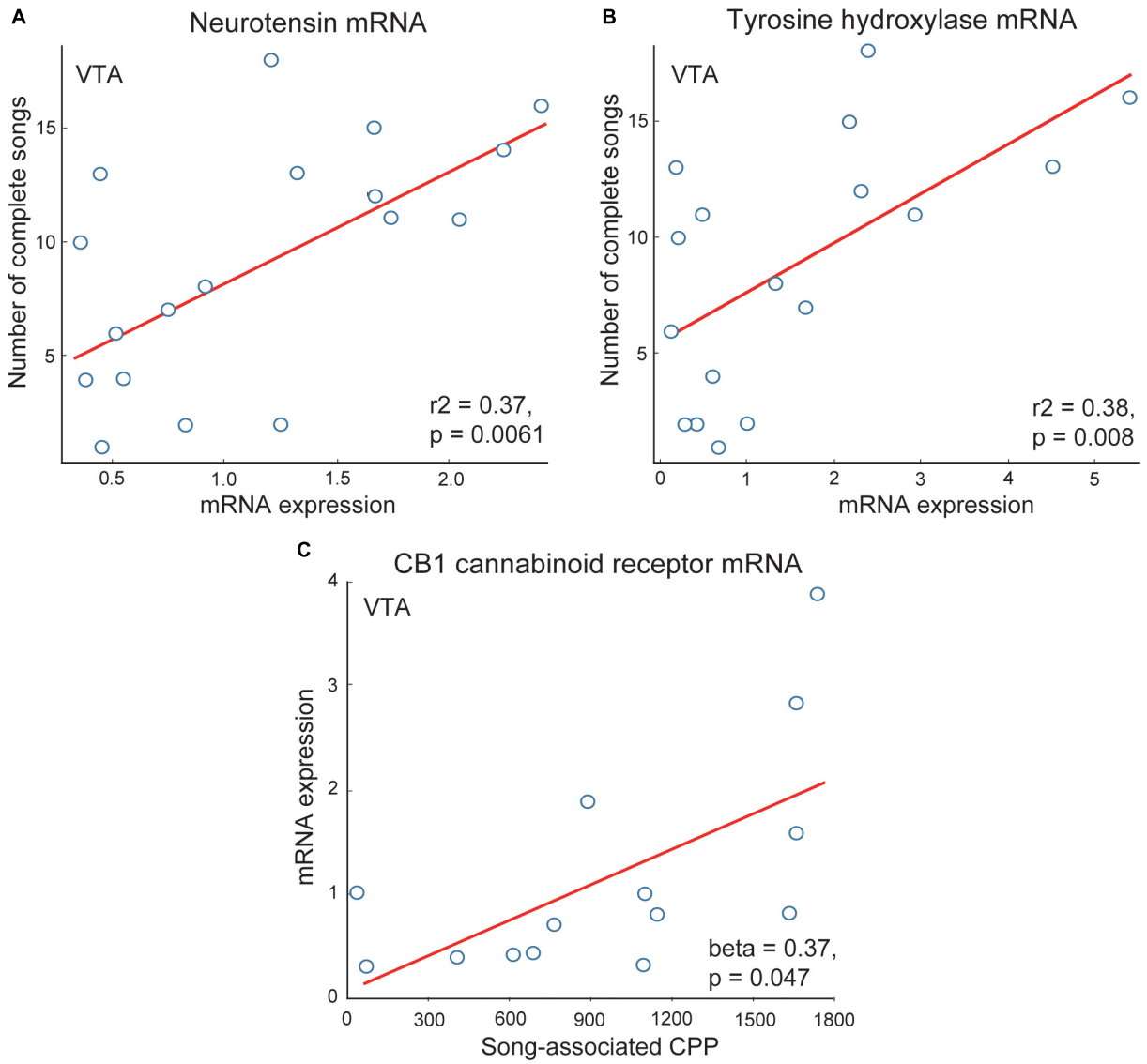

FIGURE 5 | Correlations support roles for neuromodulators in VTA in flocking behavior in starlings. Positive correlations between flock song on the *y*-axis and measures of **(A)** neurotensin and **(B)** tyrosine hydroxylase mRNA in VTA. **(C)** Positive correlation between CB1 cannabinoid receptor mRNA on the *y*-axis and song-associated CPP on the *x*-axis. Each point represents one individual. Figures redrawn from Merullo et al. (2016) and Hahn et al. (2017).

the role of mu opioid receptors in the NAc in social reward in birds. An important next step in this line of research will be to examine the role of mu receptors in NAc in flocking behavior.

A Possible Role for a Negative Reinforcement Pathway in Flocking Behaviors

The PAG has been well studied as a site in which mu opioid receptors act to induce analgesia (Bodnar et al., 1988; Spinella et al., 1999; Wiedenmayer and Barr, 2000; Morgan et al., 2014). The stimulation of mu opioid receptors in PAG also induces CPP (Olmstead and Franklin, 1997). Studies in birds and mammals suggest that the PAG gathers and integrates information about affective state from other brain regions, including mPOA, which it then relays to vocal production areas so that an animal emits a vocal signal reflective of its emotional state (Jurgens and Pratt, 1979; Absil et al., 2001; Gruber-Dujardin, 2010). In mammals, electrical input from mPOA to PAG stimulates vocalizations produced in positive contexts (i.e., calls produced during sexual behavior in guinea pigs or clucking in monkeys) but not distress vocalizations (i.e., isolation distress calls in guinea pigs or shrieks in monkeys) (Kyuhou and Gemba, 1998; Dujardin and Jurgens, 2006). Data also show that enkephalin opioids and stimulation of mu receptors in the PAG suppress negative vocalizations (i.e., hissing in cats) (Shaikh et al., 1991a,b). In starlings, similar to

what was found for mPOA, inverted-U shaped relationships were detected between vocal-social interactions in flocks and measures of mu opioid receptor immunolabeling PAG (Kelm-Nelson and Riters, 2013) (**Figure 6**). Numbers of neurons labeled for the immediate early gene ZENK (also referred to as Egr-1) and numbers of ZENK labeled neurons double labeled for tyrosine hydroxylase were also higher in PAG in male zebra finches producing undirected song in flocks compared to silent males (Lynch et al., 2008). A similar result was observed in Bengalese finches, *Lonchura domestica* producing undirected song when isolated (Matheson and Sakata, 2015). Studies in male starlings also show that the dopamine metabolite DOPAC in PAG correlates positively with singing behavior in gregarious starling flocks (Heimovics et al., 2011) (**Figure 6**). Markers for cannabinoids (i.e., cannabinoid receptors and the cannabinoid transporter FABP7) also correlate negatively with song-associated CPP (Hahn et al., 2017) (**Figure 6**). The function of these relationships must now be tested experimentally using site-specific pharmacological or gene manipulations; however, together with past studies, these correlational data suggest potential roles for the PAG as well as the VTA in vocal-social interactions in flocks and affective state.

The PAG and VTA may modulate motivated behavior by different mechanisms. For example, it has been speculated that infusion of the mu opioid receptor agonist morphine into the PAG induces CPP (Olmstead and Franklin, 1997) by reducing negative affect (rather than by inducing a positive affective state), based on studies showing that a main role for the PAG

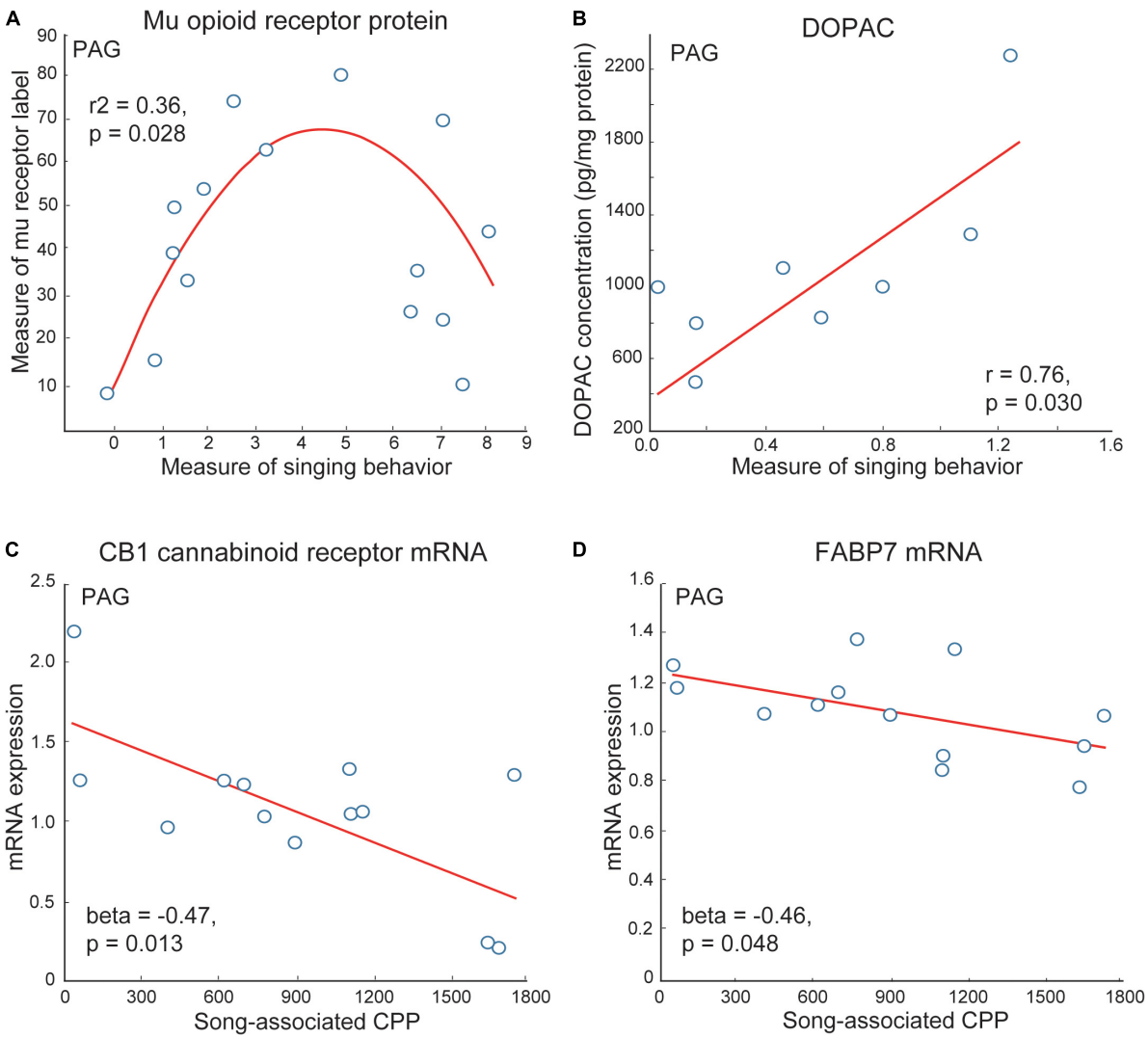

FIGURE 6 | Correlations support roles for neuromodulators in PAG in flocking behavior in starlings. **(A)** An inverted-U shaped curve shows the measure of flock song on the *x*-axis and the mean area covered by mu opioid receptor immunolabeling in PAG on the *y*-axis. **(B)** A positive linear correlation between flock song on the *x*-axis and the dopamine metabolite DOPAC concentrations in PAG on the *y*-axis. Negative correlations between flock song-associated CPP on the *x*-axis and measures of **(C)** CB1 cannabinoid receptor and **(D)** the cannabinoid transporter FABP7 mRNA in PAG. Each point represents one individual. Figures redrawn from Heimovics et al. (2011); Kelm-Nelson and Riters (2013), and Hahn et al. (2017).

is to modulate responses to aversive stimuli (Lieberman and Eisenberger, 2009; Wright and Panksepp, 2011). In contrast, morphine in VTA may induce CPP by increasing positive affect, based on studies showing the VTA to be important for reward and approach (Bozarth and Wise, 1981; Wise and Bozarth, 1987; Berridge and Robinson, 1998; Ikemoto and Panksepp, 1999; Ikemoto et al., 2015; Volkow et al., 2017). This suggests that the pleasure and pain that facilitate flocking behavior may be modified by these two complementary output pathways from mPOA, with PAG integrating information during social interactions that leads to pain reduction and the VTA integrating information leading to social approach and reward.

INTEGRATION WITH PRIOR STUDIES ON "NONAPEPTIDES" AND FLOCKING BEHAVIOR

This review is focused on opioids and the reinforcement of flocking behavior; however, a major focus of current research on group living, including flocking in birds, is on neuropeptides in the vasopressin/oxytocin, "nonapeptide" family (homologs to avian vasotocin/mesotocin) (Goodson, 2008, 2012; Goodson et al., 2009b, 2012a; Goodson and Kingsbury, 2011; Ondrasek et al., 2018; Beery, 2019). Opioids have long been known to alter nonapeptide release (Bicknell and Leng, 1982; Brown et al., 2000), and nonapeptides influence opioid activity to modulate behavioral responses to social and painful stimuli (including the pain of social separation) (Csiffary et al., 1992; Yang et al., 2007, 2011a,b; Moaddab et al., 2015; Amini-Khoei et al., 2017). For example, oxytocin increases tissue sensitivity to opioids (Meguro et al., 2018), and enhances mu opioid receptor agonist induced CPP and analgesia (Moaddab et al., 2015; Meguro et al., 2018). It has been proposed that oxytocin may increase the salience of stimuli that release opioids (Moaddab et al., 2015). This suggests that if vocal-social interactions in flocks release opioids, oxytocin may enhance the reinforcing effects of these social interactions.

Despite the extensive evidence for mechanistic overlap, roles for opioids and nonapeptides in social behavior are not commonly considered together, and it has been suggested that researchers may be ignoring critical, in some cases dominant, input from opioids (Nelson and Panksepp, 1998; Depue and Morrone-Strupinsky, 2005) and overgeneralizing roles for nonapeptides in social behavior (Insel and Shapiro, 1992; McCall and Singer, 2012). Here we provide an overview of studies on nonapeptides and flocking in birds and consider ways in which these peptides may interact with opioids to reinforce flocking behavior.

Like opioids, nonapeptides induce analgesia in mammals (Yang et al., 2007, 2011a,b; Xin et al., 2017). The nonapeptide oxytocin also reduces separation distress vocalizations (Insel and Winslow, 1991; Panksepp, 1992) and induces conditioned social preferences (Kent et al., 2013; Kosaki and Watanabe, 2016). However, these studies also show that oxytocin results in either a modest or no CPP in the absence of a social partner, which may reflect a selective role for nonapeptides in social, not general, reward. In songbirds, nonapeptides are also implicated in positive responses to social, flock-related stimuli. A strong body of research demonstrates that vasotocin-containing neurons in the bed nucleus of the stria terminalis (BSTm) increase gregariousness (i.e., preferences for a large versus a small flock) and reduce anxiety behavior (Kelly et al., 2011). Measures of ZENK in the BSTm in starlings correlate positively with song in gregarious flocks, but not sexually motivated, male song (Heimovics and Riters, 2007). Neurons positive for the immediate early gene c-fos in BSTm that were activated in response to positive social stimuli (i.e., the presence of conspecifics in gregarious finch species) were also found to be vasotocin-positive and proposed to play a role in positively (but not negatively) valenced responses to social stimuli (Goodson and Wang, 2006). The neurons originating in BSTm project to the lateral septum (LS), which is a site in which vasotocin-like receptors promote gregariousness (Kelly et al., 2011). Studies also implicate mesotocin-like receptors in LS in gregariousness (Goodson et al., 2009b, 2012b; Ondrasek et al., 2018), and a study in female zebra finches shows that mesotocin-like receptor antagonist infused directly into LS reduced gregariousness (Goodson et al., 2009b). Together these data provide support for causal roles for nonapeptide projections from BSTm to LS in flocking.

Opioids in the BSTm are also implicated in flocking behavior. In starlings the relationship between vocal-social interactions in flocks and mu opioid receptor labeling in BSTm was curvilinear (i.e., inverted-U shaped), similar to the relationships identified in the mPOA and PAG (Kelm-Nelson and Riters, 2013). This suggests the hypothesis that regions in which nonapeptides and/or opioids modulate gregariousness are part of a network that controls flocking behavior. In support of this, the BSTm and LS (i.e., brain areas in which nonapeptides are implicated in flocking behavior) are reciprocally connected to the mPOA as well as proposed output regions underlying positive and negative reinforcement (i.e., the VTA and PAG) (Riters and Alger, 2004; Goodson, 2005). Electrical input from BSTm to PAG evokes both aversive (i.e., shrieking) and non-aversive (i.e., chattering) vocalizations in monkeys, with injections of the opioid receptor antagonist into PAG reducing the threshold to produce aversive but not, non-aversive calls (Jurgens and Lu, 1993). Met-enkephalin in BSTm also suppresses negative vocal behavior (i.e., hissing) in cats (Brutus et al., 1988). Recent data showing dense concentrations of mesotocin- and vasotocin-like receptors in mPOA in starlings (females in this study) captured in winter when they flock (Ondrasek et al., 2018), suggest that a role for vasotocin in mPOA in seasonal changes in flocking is worth exploring. Studies are now needed to explore roles for opioid/nonapeptide interactions in an expanded neural circuitry that includes mPOA, PAG, VTA, BSTm, and LS.

A Possible Role for Steroid Hormones in Flocking Behavior

As introduced earlier, many songbirds shift seasonally from pair or solitary living during the breeding season to the formation of flocks after the breeding season. In an early review of flocking behavior, Emlen (1952a) suggested that seasonal increases in

steroid hormone concentrations may disrupt a default state of gregariousness by facilitating behaviors disruptive to social cohesion (i.e., agonistic and sexual behaviors) (Emlen, 1952a). Consistent with this idea, in many temperate zone breeding songbirds, gonadal steroid concentrations are low outside the breeding season when birds often congregate in flocks (e.g., Wingfield and Farner, 1978; Dawson, 1983; Van Duyse et al., 2003). Low sex steroid hormones outside the breeding season in male songbirds are associated with the production of relatively shorter and less stereotyped songs (Smith et al., 1995, 1997; Riters et al., 2000; Alger et al., 2016). These songs are less attractive to females and less threatening to male competitors compared to longer, more stereotyped songs that are produced in primary reproductive contexts (Searcy and Yasukawa, 1996; Woolley and Doupe, 2008). Thus, social tolerance may be promoted in flocks through the de-emphasis of song features that induce sexual and agonistic responses.

As concentrations of testosterone in males and estradiol in females rise at the onset of the breeding season, flocks disperse and former flock mates begin to aggressively defend breeding territories and to compete for mates, singing long, stereotyped songs that repel conspecifics and attract mates (Wingfield and Farner, 1978; Dawson, 1983; Smith et al., 1995, 1997; Searcy and Yasukawa, 1996; Riters et al., 2000; Van Duyse et al., 2003; Alger et al., 2016). In some species, treating wintering birds with testosterone increases the frequency of aggressive interactions in flocks and can lead to increased spacing among flock individuals (Emlen and Lorenz, 1942; Baptista et al., 1987; Archawaranon et al., 1991). Furthermore, the mPOA has been identified as a central site in birds and mammals in which testosterone acts to promote agonistic and sexual motivation (Schlinger and Callard, 1989; Watson and Adkins-Regan, 1989a,b; Balthazart et al., 1990; Riters et al., 1998; Coolen and Wood, 1999; Hull et al., 1999; Ball and Balthazart, 2004; Balthazart and Ball, 2007), as well as the production of sexually motivated songs in canaries, *Serinus canaria* (Alward et al., 2013, 2018). Thus testosterone may act in mPOA to dissociate flocks by promoting aggressive and sexual behaviors among flock members.

In many vertebrates, opioids in the mPOA and/or their receptors change seasonally and are regulated by steroid hormones (Watson et al., 1986; Hughes et al., 1990; Mateo et al., 1992; Hammer et al., 1994; Eckersell et al., 1998; Holland et al., 1998; Scott et al., 2008; Woods et al., 2010; Spool et al., 2016). [Nonapeptides and/or their receptors also change seasonally and are regulated by steroid hormones, but we limit our discussion here to opioids because they are the main focus of this review and there are several papers that detail region- and species-differences in steroid effects on nonapeptides (e.g., DeVries et al., 1985; Goodson and Bass, 2001; Panzica et al., 2002; Plumari et al., 2004; Goodson and Thompson, 2010)]. One possibility is that steroid-dependent changes in opioids (and nonapeptides) modify flocking seasonally. Results of studies in starlings are consistent with this hypothesis. Treatment of castrated male starlings with testosterone alters opioid markers in a complex fashion, resulting in increased mu-opioid receptor and preproenkephalin mRNA and steroid-related mRNA (i.e., androgen receptors and aromatase) in mPOA relative to controls, but also reducing numbers of immunolabeled mu opioid receptor cell bodies in the rostral portion of mPOA (**Figure 7**) (Spool et al., 2016, 2018b), a subregion thought to modulate appetitive sexual behavior (Riters and Ball, 1999; Balthazart and Ball, 2007). In female starlings, individuals with elevated concentrations of estradiol tend to have higher preproenkephalin mRNA in mPOA relative to females with low concentrations of estradiol (Spool et al., 2018a). Additionally, in free-living songbirds mu opioid receptor mRNA in the mPOA changes seasonally, peaking during the breeding season (Woods et al., 2010). The results of these studies suggest that seasonal, steroid-dependent changes in opioid activity in mPOA may modulate seasonal changes in gregariousness such as those observed in European starlings.

A study in house sparrows, *Passer domesticus*, another seasonally gregarious songbird offers additional indirect evidence that changes in seasonal flocking behavior are linked to opioid release. In this study testosterone treatment was found to induce analgesia as measured using the opioid-sensitive hot water foot withdrawal test of analgesia described above in starlings (Hau et al., 2004). This suggests that seasonal changes in testosterone may alter opioid release, or tissue sensitivity to opioids, to reduce aversive states induced by solitude at times when it is adaptive for individuals to remove themselves from social flocks. Given the overlap in the role of opioids in both physical and social pain, it may be that a testosterone-induced increase in opioid release or receptor numbers that occurs during the breeding season replaces the need for pain relief that is induced by social interactions in flocks in the non-breeding season.

Although there is much evidence to support a role for steroid hormones in shifting animals from a prosocial, tolerant state to an agonistic, intolerant state, the relationship between steroid hormones and flocking behavior in birds is complex. For example, in many species some level of aggression persists in non-breeding flocks despite low concentrations of circulating steroids, usually as the result of competition over food and roosting resources (Sabine, 1949; Lockie, 1956; Pinxten et al., 2000; Smith et al., 2005), and the flock remains together despite these conflicts. This type of aggression has been found to be regulated by brain site-specific *de novo* steroid synthesis (Heimovics et al., 2015a,b), yet the extent to which local neurosteroid synthesis modulates flocking behavior has not been studied. Furthermore, some seasonally breeding birds mate in colonial settings and exhibit social tolerance even while sex steroid hormones are elevated. Animals in these breeding groups typically defend small spaces around nest sites [e.g., zebra finches (Zann, 1996), magellanic penguins, *Spheniscus magellanicus* (Stokes and Boersma, 2000), black skimmers, *Rynchops niger* (Burger, 1981), cliff swallows, *Petrochelidon pyrrhonota* (Emlen, 1952b)]. Larger colony sizes (i.e., greater numbers of potential competitors) in cliff swallows are associated with greater circulating concentrations of testosterone in both males and females (Smith et al., 2005). Furthermore, in male starlings testosterone was higher in males nesting in a dense colony compared to males nesting at more dispersed sites (Ball and Wingfield, 1987). Thus in many species increases in sex steroid hormones are associated with flock dissociation at the beginning of the breeding season, yet in other species

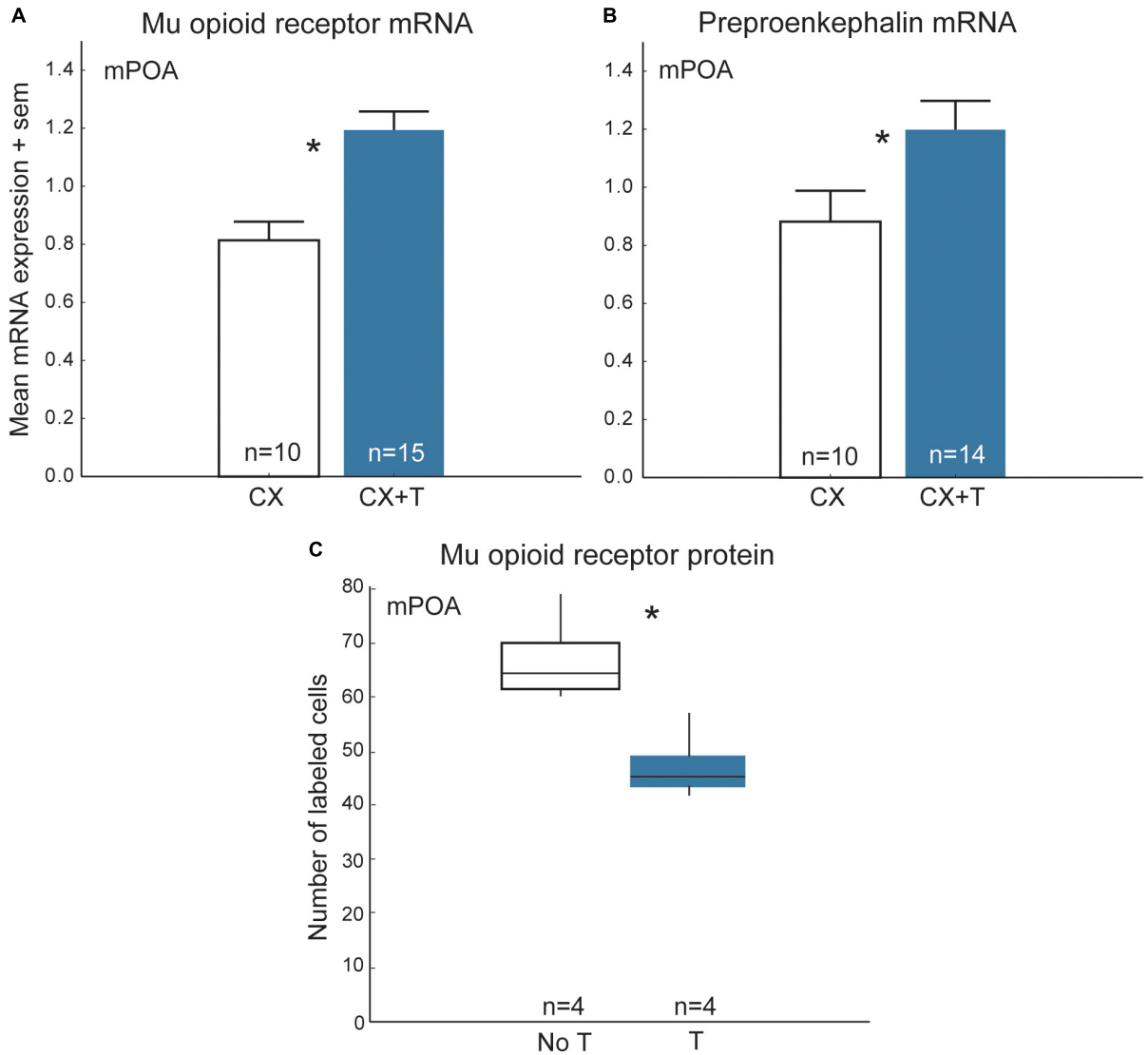

FIGURE 7 | Steroid-dependent changes in opioids in mPOA may modify flocking behavior seasonally in starlings. Mean + sem mRNA expression measures in mPOA for **(A)** mu opioid receptors and **(B)** preproenkephalin in castrated (CX; open bars) and castrated males treated with testosterone (CX + T; filled bars). **(C)** Numbers of cells in mPOA immunolabeled for mu opioid receptor protein in males with naturally low testosterone that were not treated with testosterone (No T) and males that were treated with testosterone (T). The middle lines in the boxes are medians, the outer edges of the boxes are 1st and 3rd quartiles (i.e., the interquartile range). The whiskers represents data within 1.5 * the interquartile range (i.e., 1.5 * the width of the box). Figures redrawn from Spool et al. (2016, 2018b).

they promote small-scale territoriality and resource guarding within colonial groups without causing flocks to dissociate. Season and species-specific differences in steroid effects on modulators, such as opioids or nonapeptides, in brain regions involved in flocking may reconcile these variable findings across species and seasons.

SYNTHESIS, IMPLICATIONS, AND CONCLUSION

We propose that studies of songbirds reveal a novel network model for the integration of positive and negative reinforcement processes in non-sexual affiliative social behavior. Most studies on affiliative behavior focus on the positive affective state induced by social contact that rewards individuals interacting together. However, this review highlights that in social animals, affiliative contact is also reinforced because it reduces a negative affective state caused by social exclusion or isolation, thus creating a complementary system (i.e., positive reinforcement from affiliative interactions and negative reinforcement from termination of isolation). In this review we build the case that both of these mechanisms are central to flock formation and maintenance and propose that mu opioid receptor activity in the mPOA may modulate a positive state induced by flocking, via a projection to VTA, and may reduce a negative affective

state resulting from social separation, via a projection to PAG. Neural systems that underlie important social behaviors are evolutionarily conserved (Panksepp, 2005, 2016; O'Connell and Hofmann, 2011). This suggests that a molecular/genetic substrate that existed in a common ancestor has been conserved to provide a foundation for the generation of novel social behaviors across vertebrates, and to fine-tune social behaviors to match the ecological needs of individual species. Thus studies of songbird flocking may advance the understanding of how the molecular substrates underlying social reinforcement have evolved across vertebrates, including humans.

In humans, profound deficits in gregarious social interactions (i.e., playful, non-sexual social interactions) are associated with mental disorders, including depression and autism spectrum disorders. Although several animal models can be used to study goal-directed (i.e., mate- or rival-directed) behaviors, songbirds are one of the only experimental systems to model aspects of learned vocal communication in a non-sexual, yet affiliative context in adults. A survey of studies on humans reveal parallels to songbird studies. For example, in human studies vocal behaviors [i.e., undirected swearing, affiliative social laughter, and vocal repetition or rhythmic respiration (e.g., during meditation)] induce analgesia and/or are associated with a feeling of well-being (Stephens et al., 2009; Dunbar et al., 2012; Ahmed et al., 2014), similar to what has been observed for vocal behaviors produced in flocks in songbirds. Furthermore, in humans both reward and pain neural networks are implicated in social reward and the pain of social rejection (Macdonald and Leary, 2005; Lieberman and Eisenberger, 2009; Eisenberger, 2012), similar to evidence we review here in songbirds. Studies of flocking behavior in birds thus have the potential to provide insight into mechanisms by which behaviors that involve vocal-motor-respiratory stimulation (e.g., controlled breathing, ohms during meditation, swearing and laughter) may naturally promote opioid or nonapeptide release and positive (or less negative) affect in humans. Information provided by songbirds about basic mechanisms underlying affiliative social interactions may also provide important insights into mechanisms underlying positive social interactions that are disrupted by mental illness in humans.

AUTHOR CONTRIBUTIONS

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BRIEF RESEARCH REPORT
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Embryonic Exposure to Valproic Acid Affects Social Predispositions for Dynamic Cues of Animate Motion in Newly-Hatched Chicks

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Early predispositions to preferentially orient toward cues associated with social partners have been documented in several vertebrate species including human neonates and domestic chicks. Human newborns at high familiar risk of Autism Spectrum Disorder (ASD) show differences in their attention toward these predisposed stimuli, suggesting potential impairments in the social-orienting mechanisms in ASD. Using embryonic exposure to valproic acid (VPA) we modeled ASD behavioral deficits in domestic chicks. To investigate social predispositions toward animate motion in domestic chicks, we focused on self-propulsion, using two video-animations representing a simple red circle moving at constant speed (speed-constant) or one that was changing its speed (accelerating and decelerating; speed-change). Using a spontaneous choice test for the two stimuli, we compared spontaneous preferences for stimuli that autonomously change speed between VPA- and vehicle-injected chicks. We found that the preference for speed changes was abolished in VPA-injected chicks compared to vehicle-injected controls. These results add to previous findings indicating similar impairments for static social stimuli and suggest a specific effect of VPA on the development of mechanisms that enhance orienting toward animate stimuli. These findings strengthen the hypothesis of an early impairment of predispositions in the early development of ASD. Hence, early predispositions are a potentially useful tool to detect early ASD symptoms in human neonates and to investigate the molecular and neurobiological mechanisms underlying the onset of this neurodevelopmental disorder.

Keywords: valproic acid, social predispositions, newly-hatched chick, autism spectrum disorder, animacy, *Gallus gallus*

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INTRODUCTION

Neonates of some vertebrate species orient their first approach responses toward objects that exhibit features present in social partners and caregivers: face-like configuration, biological motion and self-propulsion. Comparative research on human infants and newly hatched domestic chicks (*Gallus gallus*) found striking similarities in the static and dynamic visual cues that attract attention of these different species soon after birth (Di Giorgio et al., 2017a). Among dynamic cues, point-light displays depicting biological motion are preferred by neonates of both species to the same configuration of dots rigidly rotating or moving randomly (Vallortigara and Regolin, 2006;

Simion et al., 2008). Chicks also seem to have a spontaneous preference for objects autonomously starting to move over objects set in motion after a collision (Mascalzoni et al., 2010) and for objects autonomously changing their speed over constant moving ones (Rosa-Salva et al., 2016). Similarly, human neonates exhibit a looking preference for self-propelled objects autonomously starting from rest (Di Giorgio et al., 2017b).

Alterations in social predispositions appear to be linked to Autistic Spectrum Disorders (ASD) a complex group of neurodevelopmental disabilities characterized by important deficits in the domain of social cognition (Sacrey et al., 2015). Impairments in face discrimination and recognition have been widely observed in ASD individuals (Dawson et al., 2005). Young children with ASD show altered processing of stimuli depicting biological motion (Freitag et al., 2008; Klin et al., 2009) and difficulties in spontaneous categorization of self-propelled motion as animate (Rutherford et al., 2006). Neonates at high familiar risk of ASD show significant differences compared to low-risk neonates in the preference for a face-like stimulus and for biological motion, suggesting an impairment in the development of the predisposed mechanisms for detecting animate beings (Di Giorgio et al., 2016). Observing the same impairment for both static and dynamic stimuli in a different species would argue in favor of a common developmental origin of these predispositions.

Valproic acid is an anticonvulsant and a mood stabilizer widely used to treat epilepsy, migraine and bipolar disorder (Johannessen and Johannessen, 2003). In humans, prenatal exposure to VPA has been shown to increase the risk of developing ASD (Christensen et al., 2013). Embryonic exposure to VPA has been widely used to model the ASD syndrome in rodents (see for a review Nicolini and Fahnestock, 2018). Embryonic exposure to VPA has been shown to induce impairments in chicks' aggregative behavior (Nishigori et al., 2013) and in their early predisposition for static stimuli (Sgadò et al., 2018).

To further study the effect of VPA on early predispositions, and to investigate whether the impairment for static cues is accompanied by impairment in predispositions for dynamic cues, we compared the spontaneous preference for self-propelled stimuli in VPA- and vehicle-injected chicks.

MATERIALS AND METHODS

Embryonic Injections

Fertilized eggs of domestic chicks (*Gallus gallus*) of the Ross 308 (Aviagen) strain were obtained from a local commercial hatchery (Agricola Berica, Montegalda, Italy) and incubated at 37.7°C and 60% of relative humidity in the darkness. The first day of incubation was considered embryonic day 0 (E0). At E14, fertilized eggs were selected by candling before injection. Embryo injection was performed according to previous reports (Nishigori et al., 2013; Sgadò et al., 2018). Briefly, a small hole was made on the eggshell above the air sac, and 35 μmoles of VPA (Sodium Valproate, Sigma-Aldrich) dissolved in double distilled injectable water were administered to each fertilized egg, in a volume of 200 μl. Age-matched control eggs were injected using the same procedure with 200 μl of vehicle (double distilled injectable water). After sealing the hole with paper tape, eggs were placed back in the incubator (FIEM srl, Italy). Previous reports have analyzed the effect of different doses and time of administration of VPA on embryonic development in different vertebrate species (see for a review Roullet et al., 2013; Ranger and Ellenbroek, 2016). The typical dose and time of administration in rodents is 200–500 mg/kg in acute, single dose administration between E12 and E14. In domestic chicks, administration of 35 μmoles/egg (corresponding to approximately 100 mg/kg) has been tested between E10 and E14 with differential effects on hatching rate, showing a dramatic decrease of hatchings at E10 and a significant decrease of hatchings at E12 but no significant effect at E14 (Nishigori et al., 2013). Administration of 35 μmoles/egg at E14 induced social deficits without affecting hatchability, motor behavior and imprinting abilities (Nishigori et al., 2013; Sgadò et al., 2018).

During incubation and hatching, eggs and chicks were maintained in complete darkness, preventing any visual experience prior to the test. Controlling the visual experience during pre- and post-natal development enable to exclude any interference of visual stimuli in the expression of predispositions toward animacy cues, and to demonstrate the innate nature of these mechanisms. Each chick was tested only once.

Apparatus, Stimuli and Test

We used the same procedure previously described to assess chicks' predispositions for speed-changes. Briefly, carefully avoiding any other visual experience, the day of hatching chicks were individually placed in the center of the test apparatus, a corridor (85 × 30 × 30 cm), open at the two ends where two video screens were displaying the experimental stimuli. The corridor was divided in three sectors: a central sector (45 cm long) delimited by two steps, that the animals had to climb to enter the two choice sectors (each 20 cm long) immediately adjacent to the two screens. Stimuli were two video-animations representing the movement of a simple red circle. In one video the object was moving at constant speed (speed-constant) and in the other one it was changing its speed (accelerating and decelerating; speed-change). A spontaneous choice test of 6 min was performed for the two stimuli. Chicks' preference for the speed-change stimulus was measured by the ratio of time (in seconds) spent in the choice sector near the speed-change stimulus divided by the cumulative time spent in either of the choice sectors (preference). Chicks remaining in the central sector were not included in the analyses. Values of this ratio could range from 0 (full preference for the speed-constant), to 1 (full preference for the speed-change), whereas 0.5 represented no preference. For more detailed information on the procedure, see Rosa-Salva et al. (2016). Chicks' level of motility was measured by evaluating the latency (in seconds) to first approach, irrespective of the stimulus approached. The tests were performed manually and scored online. To evaluate reliability of scoring and potential biases, 10% of all subjects were scored again offline by a second experimenter blind to the treatment group and right/left position of the two stimuli.

Overall, we blindly coded videos of 10 animals randomly chosen from both treatment groups. We obtained a Pearson's correlation of 1.000, $p < 0.001$ between the preference scores calculated using our original data and the blind coding. For the present study 51 VPA-injected (males = 27) and 52 vehicle-injected (males = 26) chicks were tested.

Data Analysis

Effects of Treatment (VPA and vehicle injection) and Sex (male, female) on the preference for the speed-change stimulus were assessed by a multifactorial analysis of variance (ANOVA) on the dependent variable preference score. One-sample two-tailed *t*-tests were run to test significant departures from chance level (0.5) of the preference score, separately for the two groups. The number of chicks that first approached the speed-change or the speed-constant stimulus in the two treatment groups was compared using the chi-square test of independence. Effects of Treatment and Sex on latency to first approach were assessed by an ANOVA on the latency to first approach one of the stimuli. All statistical analyses were performed with IBM SPSS Statistic for Windows (**RRID:SCR_002865**). Alpha was set to 0.05 for all the tests. The dataset generated for this study is available in **Supplementary Table S1** of the **Supplementary Material**.

RESULTS

The average egg hatchability was 75%. Results of the ANOVA on the preference for the speed-change stimulus showed a significant effect of Treatment [$F_{(1,99)} = 4.296$, $p = 0.041$; **Figure 1A**], and no significant effect of Sex [$F_{(1,99)} = 0.0001$, $p = 0.992$] nor any significant interaction [Treatment × Sex: $F_{(1,99)} = 0.151$, $p = 0.698$]. In the control group (vehicle-injected), the preference for approaching the speed-change stimulus was similar to what previously observed, and the preference scores were significantly higher than chance level [$t_{(51)} = 2.365$, $p = 0.011$; $M = 0.673$, $SEM = 0.066$, **Figure 1A**]. On the contrary, VPA exposure significantly reduced the preference for the speed-change stimulus: the preference scores for approaching the speed change stimulus did not differ from chance level [$t_{(50)} = -0.406$, $p = 0.686$; $M = 0.472$, $SEM = 0.696$, **Figure 1A**]. A significant difference between the two groups was found also in the number of chicks that first approached the speed-change stimulus ($\chi^2 = 4.314$, $p = 0.047$). While in the vehicle-injected group a significantly higher number of chicks first approached the speed-change stimulus ($\chi^2 = 6.231$, $p = 0.018$; speed-change $N = 35$, speed-constant $N = 17$), in the VPA-treated group no significant difference was found in the number of chicks that approached the two stimuli ($\chi^2 = 0.176$, $p = 0.78$; speed-change $N = 24$, speed-constant $N = 27$).

To evaluate motility, we measured the latency to the first approach, independent of the stimulus, and found no significant effects of Treatment [$F_{(1,99)} = 2.672$, $p = 0.105$; **Figure 1B**], Sex [$F_{(1,99)} = 1.124$, $p = 0.292$], nor any interaction [$F_{(1,99)} = 0.000$, $p = 0.99$].

DISCUSSION

We investigated unlearned predispositions to orient toward animate motion cues in VPA-injected chicks compared to vehicle-injected controls, using a choice preference test between a speed-change and a constant moving stimulus. We showed a detrimental effect of VPA on the typical spontaneous preference for the speed-change stimuli conveying animacy cues (Rosa-Salva et al., 2016). These results are in line with previous studies investigating static cues to animacy (such as the head and neck region of the mother hen, Sgadò et al., 2018) and our hypothesis of a disruption of unlearned predispositions in animal models of ASD.

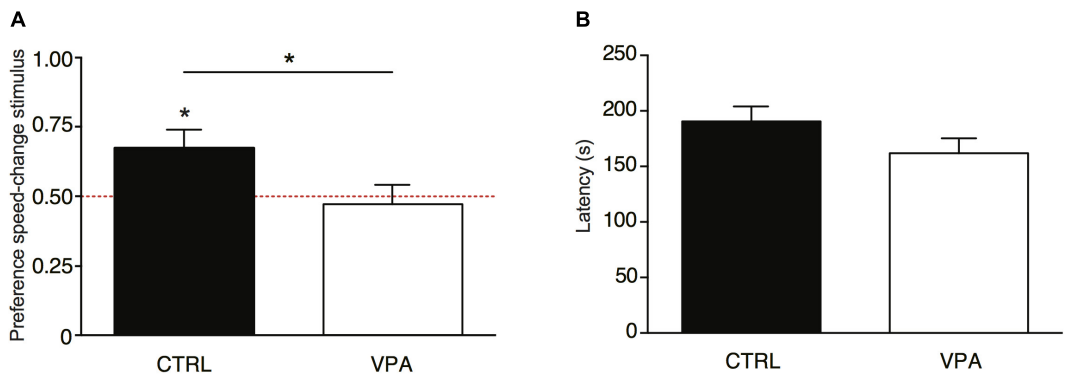

FIGURE 1 | (A) Social preference responses for the speed-change stimulus shown as the ratio of time (in seconds) spent in the choice sector near the speed-change stimulus divided by the cumulative time spent in either of the sectors (see section "Materials And Methods" for details). Analysis of variance of social preference scores using Treatment and Sex as between-subjects factors, revealed a significant main effect of Treatment (line with *), with no other main effects or interactions. Preference scores were significantly different from chance level for vehicle-injected chicks (control group), but not for VPA-treated chicks. Asterisks on top of bars indicate significant departures from chance level, marked by the red line at 0.5. **(B)** Latency to first approach assessed as a measure of motility. Analysis of variance on latency to first approach using Treatment and Sex as between-subject factors showed no significant main effects of Treatment, Sex or interaction Treatment × Sex. Data represent mean ± SEM, *$p < 0.05$.

In phylogenetically distant species of vertebrates, such as domestic chicks and humans, similar mechanisms have been described to drive early approach responses toward static and dynamic cues typically associated with animate figures. The adaptive function of early predispositions has been hypothesized to be in directing attention toward highly important animate stimuli, enabling future learning through experience and enhancing social interactions (Johnson et al., 2015; Di Giorgio et al., 2017a; Powell et al., 2018). In chicks, predispositions are likely to orient the young animal toward the mother hen (or other brood mates), directing subsequent filial imprinting responses toward animate stimuli (Miura and Matsushima, 2016). In human newborns, subcortical fast and automatic mechanisms have been hypothesized to underlie these social predispositions, directing attention toward animate entities to create an early social bond with the caretakers and social companions (Tomalski et al., 2009; Johnson et al., 2015; Di Giorgio et al., 2017a). Subsequently, experience may modulate and specialize more sophisticated mechanisms devoted to the processing of social stimuli (Johnson et al., 2015; Versace et al., 2016, 2018).

Several accounts suggest that abnormalities in this early social-orienting system may lead to deficits in social stimuli processing, limiting attention to salient social stimuli, decreasing their reward value and resulting in the atypical social behavior associated with ASD.

To investigate the contribution of these social-orienting mechanisms in atypical social behavior related to ASD, we modeled ASD-like social impairments in domestic chicks using embryonic exposure to VPA. We then measured preference responses to different social stimuli, either stationary (the face-like configuration visible in a stuffed hen, Sgadò et al., 2018) or dynamic (speed-changes, this work), in visually-naiïve VPA-injected and vehicle-injected domestic chicks.

In this study, we have investigated social predispositions toward animate motion, focusing on the predisposition to approach objects that appear self-propelled due to an "internal energy source" that produces changes of speed. Using behavioral responses to visual stimuli, we have documented the absence of the typical predisposed preferences for animacy stimuli in domestic chicks, as a consequence of embryonic VPA exposure. This drug has been used to model ASD core deficits in other vertebrate species (Ranger and Ellenbroek, 2016) although chicks are the first precocial species in which its effect on social behavior has been investigated (Nishigori et al., 2013; Sgadò et al., 2018). Precocial species, like domestic chicks, are characterized by the early maturation of the motor and sensory system, that allows to perform behavioral tests soon after birth, before gaining any social experience. Our findings, hence, open new possibilities to tackle the early onset of predispositions relevant for social life, focusing on dynamic cues.

Moreover, these findings extend previous literature reporting impairments in the preference response for static, face-like configurations of the stuffed hen stimulus (Sgadò et al., 2018). The observation of a parallel impairment in social predispositions for both static and dynamic cues in different species suggests a common developmental origin of this social-orienting system. Since the neuroanatomical substrates of predispositions for approaching static and dynamic stimuli are at least partially different (Mayer et al., 2016a,b, 2017; Lorenzi et al., 2017), observing here the impairment of both classes of predispositions suggests the existence of a common mechanism.

Our work on VPA-mediated impairment of early predispositions, together with the deficits documented in human neonates at high risk of ASD (Di Giorgio et al., 2016), supports the hypothesis of early social orienting mechanisms shared across species whose impairment or delay might have a pivotal role in the pathogenesis of autism.

Future studies should capitalize on these findings to investigate the molecular and neurobiological mechanisms underlying those ASD early symptoms that are associated with predisposed orienting mechanisms toward social stimuli.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Italian and European Union laws for the ethical treatment of animals. The protocol was approved by the Ethical Committee of the University of Trento and licensed by the Italian Health Ministry (permit number 986/2016-PR).

AUTHOR CONTRIBUTIONS

PS, EV, OR-S, and GV conceived and designed the experiments. EL and AP conducted the experiments. PS, EV, and OR-S developed the behavioral paradigms. EL, AP, PS, OR-S, and EV analyzed the data. EL and PS drafted the manuscript. All the authors wrote the manuscript and approved the final version for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00501/full#supplementary-material

TABLE S1 | Dataset table. List of all data generated and analyzed in this study.

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REVIEW
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Visual Imprinting in Birds: Behavior, Models, and Neural Mechanisms

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Filial imprinting is a process, readily observed in precocial birds, whereby a social attachment is established between a young animal and an object that is typically (although not necessarily) a parent. During a perinatal sensitive period, the young animal learns characteristics of the object (the imprinting stimulus) simply by being exposed to it and will subsequently recognize and selectively approach this stimulus. Imprinting can thus establish a filial bond with an individual adult: a form of social cohesion that may be crucial for survival. Behavioral predispositions can act together with the learning process of imprinting in the formation, maintenance, and modification of the filial bond. Memory of the imprinting stimulus, as well as being necessary for social recognition, is also used adaptively in perceptual classification of sensory signals. Abstract features of an imprinting stimulus, such as similarity or difference between stimulus components, can also be recognized. Studies of domestic chicks have elucidated the neural basis of much of the above behavior. This article discusses (1) principal behavioral characteristics of filial imprinting and related predispositions, (2) theoretical models that have been developed to account for this behavior, and (3) physiological results elucidating the underlying neural mechanisms. Interactions between these different levels of analysis have resulted in advancement of all of them. Taken together, the different approaches have helped define strategies for investigating mechanisms of learning, memory, and perception.

Keywords: learning, memory, domestic chick, recognition, neural networks, perceptual learning

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INTRODUCTION

Filial imprinting has been recognized since antiquity and its behavioral characteristics reviewed extensively (Heinroth, 1911; Lorenz, 1935, 1937; Bateson, 1966; Sluckin, 1972; Hess, 1973; Bolhuis, 1991). It is readily observed in the young of many precocial species (i.e., where neonates are relatively mature and capable of locomotion in the immediate postnatal period) and most of the available information comes from newly hatched galliform birds such as chickens, ducks, and quail. Filial imprinting involves the young animal following a conspicuous stimulus, learning the stimulus' characteristics, and consequently restricting its social preferences toward that stimulus. The consequences of filial imprinting can last well into later life and the phenomenon is generally adaptive, biasing the young animal's behavior toward the protection of parents or other conspecifics. Sexual imprinting, whereby an animal's sexual preferences are influenced by its previous experience, typically occurs around the time the animal assumes its adult appearance but may be influenced by experience in infancy. Thus, filial and sexual imprinting, though demonstrably distinct in terms of behavior, are interrelated (cf. Bolhuis et al., 1989).

Although imprinting occurs in a variety of sensory modalities, this article is mainly concerned with visual filial imprinting, which will be referred to simply as "imprinting" unless the term needs to be qualified to avoid ambiguity. Three interlinked perspectives will be considered. First, behavioral observations and experiments; second, theoretical models that have arisen from the behavioral work; and third, physiological experiments stimulated by the other two lines of research, which in principle permit theoretical predictions to be tested. The review discusses the contribution of imprinting to social cohesion in precocial avian species. It then describes theoretical models designed to account for key behavioral characteristics of imprinting. Finally, physiological data pertaining to mechanisms underlying the behavior are discussed. Such data may be used to test the models which, if validated, have the potential to be an explanatory link between the behavior and its underlying neural mechanisms.

THE SENSITIVE PERIOD FOR IMPRINTING

Imprinting typically occurs within a perinatal sensitive period, which typically lasts for several days but which is very variable in duration (Bateson, 1966; Sluckin, 1972; Bolhuis, 1991). There is evidence that the beginning of the sensitive period depends, at least in part, on factors that are independent of sensory input (see also Section "The Sensitive Period for Imprinting" below). For example, sensitivity of ducklings to an imprinting stimulus was found by Gottlieb (1961) to be strongly associated with time since the start of embryonic development. It is also possible that time after hatching, and thus possibly the experience of hatching, contributes to the onset of the sensitive period (see also Williams, 1972; Hess, 1973; Landsberg, 1976). The degree of control exerted by developmental processes over the end of the sensitive period is less clear, but there is abundant evidence that imprinting itself can terminate the sensitive period. This is not to say that there is not an ontological termination of the sensitive period, since the end of the sensitive period is revealed behaviorally: the animal selectively approaches familiar objects and avoids novel objects as result of the social preferences acquired through imprinting. Such behavior does not necessarily reflect the ability to learn about an imprinting stimulus. The neural plasticity that is necessary for imprinting may, therefore, outlast the sensitive period (cf. Bateson, 1979), and there is evidence that this is indeed the case. For example, chicks that are already imprinted to an object and which avoid a second object, indicating behaviorally the end of the sensitive period, can eventually be imprinted to that second object (Salzen and Meyer, 1968; Cherfas and Scott, 1981; Bolhuis and Bateson, 1990).

Predispositions

It would be surprising if all novel objects encountered by a naïve young animal were equally attractive and there is much evidence that different objects elicit different types and degrees of behavior in dark-reared chicks. For example, visually naïve chicks differentially approach objects of different colors (Bateson and Jaeckel, 1976). On finding a region in the chick forebrain (the IMM) that is critical for imprinting (see below), Horn and McCabe (1984) surveyed the results of several experiments in which this region had been ablated, and found that lesions to the IMM were very effective in eliminating preferences acquired through imprinting when the training stimulus was an artificial object such as a colored box or cylinder. However, the lesions were only partially effective when the training stimulus was a naturalistic object (the stuffed skin of an adult jungle fowl, resembling the presumed ancestral form). Subsequent experiments led to the conclusion that certain features of an imprinting stimulus elicit approach activity that does not depend on prior exposure to that particular stimulus. There is thus a predisposition to approach an object bearing these features. Dismantling the jungle fowl model and presenting its component parts in various positions and orientations implicated features in the head and/or neck as the critical targets of the predisposition (Johnson and Horn, 1988). Such features were later identified more precisely as the naturally occurring configuration of the eyes and mouth (Rosa-Salva et al., 2009). The predisposition is evidently triggered by mild interventions such as handling or exposure to light, as well as exposure to an imprinting stimulus (Johnson et al., 1985). The object of the predisposition is not restricted to conspecifics or congeners, since visually naïve chicks with the predisposition will preferentially approach a stuffed duck or polecat (Johnson and Horn, 1988). The predisposition evidently biases chicks' approach behavior toward certain types of naturalistic stimulus, whereupon imprinting is available to establish a filial bond that is specific to the stimulus through learning. The relatively wide range of objects which the chick is predisposed to approach could be adaptive if the predisposition is activated only in a mildly stressful situation, such as social isolation, in an environment where there are more conspecifics than predators. The predisposition might then increase the probability of imprinting to a protective adult, albeit one that is not necessarily a close relative. Further predispositions have been described in newly hatched chicks, which suggest predilections for predictors of animacy, such as biological motion (Vallortigara et al., 2005), self-propulsion (Mascalzoni et al., 2010), the ability spontaneously to accelerate or decelerate (Rosa-Salva et al., 2016), alignment of an object's major axis with its direction of motion (Rosa-Salva et al., 2018), and rotation (Rosa-Salva et al., 2018). Such stimuli tend to capture the animal's attention and elicit approach behavior. The extent to which they lead to filial attachments by means of imprinting as opposed to predatory behavior, for example, remains to be determined.

Johnson and Bolhuis (1991) classified predispositions into general and specific, according to whether they are triggered by simple properties of a stimulus such as color (cf. Bateson and Jaeckel, 1976), or more complex combinations of features such as components of a face or biological motion as described above. Physiological experiments (see below) are helping to characterize these predispositions in terms of neural mechanisms. There is a striking similarity between chicks' predisposition to approach faces and a predilection for face-like patterns in human neonates (see Di Giorgio et al., 2017 for review).

Transient Preference for Novelty During Imprinting

A domestic chick's preference for an imprinting stimulus during exposure to the stimulus (referred to as "training" in an experimental context) typically increases with duration of exposure (Bateson and Jaeckel, 1974; Zajonc et al., 1975). The temporal pattern of this increase in preference need not be linear. Under controlled training conditions and low variation in chicks' rate of approach, a transient preference for novelty was found to emerge before a strong preference for the training stimulus became established (Bateson and Jaeckel, 1976). These authors suggested that the transient reversal of preference results from a tendency to prefer slight novelty once they have become familiar with a training stimulus after a brief encounter (about 15 min in the experiments in question). This might be adaptive if the chicks were thereby prompted to explore slightly novel stimuli, such as different views of an imprinting object, while many features of the stimuli, such as color, appear to remain relatively constant. Under natural circumstances, when the imprinting stimulus is a mother hen, a chick might first become attracted to one view of the hen and later prefer a different view. A progressive series of such events could cause the chick to become familiar with many views of the hen, aiding recognition of the hen from several viewing angles. This idea was tested by Jackson and Bateson (1974), who trained chicks with either a red or a yellow stimulus and then allowed the chicks to choose a stimulus of either color by pressing a pedal. Consistent with the prediction, after 15 min exposure, the chicks actively worked to obtain exposure to the novel color. After 30 min exposure, a similar but weaker trend was observed and after 60 min, chicks chose the familiar color. In natural conditions, this type of behavior might be expected to bias chicks' behavior toward a slightly novel view of a mother hen, thus obtaining information about different views of her, facilitating recognition of the mother from different viewpoints in different viewing conditions. This hypothesis is supported by the results of Honey and Bateson (1996), which imprinted chicks on the side and back views of a hen in rapid temporal succession and found that the chicks took longer to learn the difference between these two views than chicks trained on the two views separated by much longer intervals. The results thus suggest that a stronger perceptual link is formed between two stimuli, the more rapidly one stimulus is presented after the other.

Classification Together of Temporally Juxtaposed Stimuli

It is noteworthy that a theoretical model predicting a temporary preference for slight novelty (Bateson, 1973, 1974; see below) implies a time-dependent perceptual modification, which determines whether a chick classifies any particular stimulus together with the familiar training stimulus. The possibility of classification together was also raised by behavioral experiments in which two visual imprinting stimuli were shown to chicks according to different schedules. Chantrey (1974) trained chicks by exposing them to two visual imprinting stimuli, presented alternately. He found that rapid alternation with a short inter-onset time (e.g., 7 s exposure to stimulus A, 8 s exposure to no stimulus, 7 s exposure to stimulus B, etc.) had a different effect from a longer inter-onset time (e.g., 30 min exposure interspersed with 30 min exposure to no stimulus). Total amount of exposure to each stimulus was kept constant. The difference lay in the ease with which the chicks could subsequently learn to distinguish stimulus A from stimulus B in an operant training procedure when one of these stimuli was associated with food reward. With shorter inter-onset times, the discrimination was learned more slowly. This result prompted the hypothesis that rapid alternate exposure to the two stimuli caused them to be classified together. In support of this interpretation, Chantrey (1976) also found that rapidly alternating exposure of chicks to two different colored stimuli led to similar behavior toward the two stimuli, whereas a longer inter-onset time caused the two stimuli to elicit different behaviors; see also Honey and Bateson (1996), discussed above. If the interpretation of classification together is correct, rapidly alternating views of different parts of a mother hen during imprinting might cause a chick to classify these different views together, so that the hen was approached subsequently irrespective of which view was momentarily presented to the chick. It is noteworthy that such a process could in principle be facilitated by preference for slight novelty during imprinting (see above).

Imprinting to Several Objects

During infancy, a young animal typically encounters a wide range of stimuli, raising the question of how stimuli that are appropriate for filial bonding may be distinguished from those that are not, including those which may actually be harmful. Available possibilities include predispositions to cleave to appropriate objects (e.g., parents), familiarity as a result of prolonged exposure to these objects and reinforcement of behavior that brings the infant into close contact with them by such factors as warmth and somatosensory stimulation. Given that a parent is often the first object to be seen after hatching, one might also suppose that order of exposure is important in molding a young animal's subsequent filial behavior. Salzen and Meyer (1968) imprinted chicks, first with either a green or a blue ball and later with the alternative object, i.e., either a green ball followed by a blue ball or vice versa. The chicks were given repeated test choices between the two objects. A strong preference was readily acquired for the first object encountered and the preference was later reversed after prolonged exposure to the alternative object. Imprinting can therefore be reversed by sequential exposure to two stimuli. The question was reexamined by Cherfas and Scott (1981), who also found a reversal of preference, but additionally found reemergence of a preference for the first stimulus if chicks were isolated for 3 days after exposure to the second stimulus. It was unclear whether the reemergence of the original preference was due to forgetting of both stimuli and a predisposed bias toward one of them. Bolhuis and Bateson (1990) addressed this issue using two disparate training stimuli and found that a preference for the stimulus of first exposure eventually recurred irrespective of the order in which the two stimuli were presented. There is therefore a primacy effect: the first filial attachment can

be over-ridden but there is a tendency to revert to this original attachment with time. Notwithstanding this reversion, it is still possible after secondary imprinting to imprint to a third stimulus (Devos and Vankampen, 1993), consistent with the idea that imprinting can update representations in the course of establishing a filial bond.

Relational Concept Learning

Exposure of young ducklings to a moving visual stimulus for about 30 min results in a predicable filial attachment to the stimulus (Martinho and Kacelnik, 2016). The ducklings clearly recognized the stimulus after exposure, but the question arises as to what type of information contributes to recognition. One possibility is simple morphological template matching between the training stimulus and what is learned during training. There is, however, the alternative possibility that abstract features of the stimulus are stored and used for recognition. Such a process might offer the advantage of economy of coding: rather than a point-by-point representation of the stimulus, more general features such as size, color, and symmetry might efficiently be encoded as critical features of the stimulus. Martinho and Kacelnik (2016) trained ducklings with an imprinting stimulus comprising two halves, possessing either the same or different forms, or the same or different colors. The ducklings were then tested to find out whether they had acquired a preference for "sameness" or "difference" by giving a duckling a choice between two novel stimuli, one comprising two parts that were the same and the other in which the two parts were different. The results indeed indicate that the relational properties of the stimuli, i.e., "sameness" or "difference," can be encoded and used as a basis for discrimination.

MODELS OF IMPRINTING

Model 1

Transient reversal of preference during imprinting was explored in a theoretical model by Bateson (1973, 1974). The model is highly simplified but shows how two processes that simultaneously progress with time during training can act together to produce a temporary preference for slight novelty. In its simplest form, the model assumes that a naïve chick is equally responsive to all objects, even two stimuli that are so disparate as to be at opposite ends of the stimulus continuum. Exposure to one such object (the training object) increases a chick's responsiveness to that object as training progresses. Also increasing with exposure time is the chick's responsiveness to a stimulus that is perceived as slightly different from the training stimulus and which, as such, is maximally attractive. Finally, the difference between two stimuli that is required for the stimuli to be perceived as different decreases with exposure time (**Figure 1**). With appropriate choice of parameters, the model predicts: (1) a transient preference for slight novelty as training progresses. It also predicts (2) that when an alternative stimulus is used in a choice test with the training stimulus, the more similar the stimuli in the test, the later the transient preference for novelty will occur. The first prediction was consistent with the results of Bateson and Jaeckel (1976) in a direct test of the prediction; the second prediction has yet to be rigorously tested owing to the difficulty of obtaining stimuli with suitable levels of disparity from one another.

The model thus accounts for the observation that a training stimulus becomes more attractive as it becomes familiar, but also for the finding that a slightly different stimulus can transiently

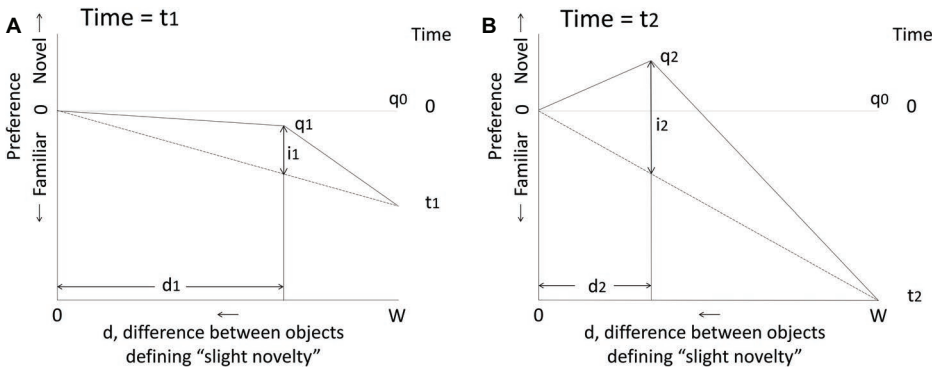

FIGURE 1 | Predictions of the simplest form of the model of Bateson (1973, 1974). On both graphs, the vertical axis represents the chick's preference during imprinting to a training object and the horizontal axis represents the difference *d* between the training object and any other object that is perceived as "slightly novel"; "slight novelty" corresponds to a level of novelty that is maximally attractive. *d* ranges between zero and *W*, the width of the stimulus continuum. Before imprinting starts, the model has no preference for any object: all are equally novel, equally attractive and $d = W$. After imprinting has started, preference for the training object increases linearly with time (*t*). *d* decreases linearly with time, as the chick becomes more familiar with the training object and is able to discriminate it better from a novel object. Point *q* represents the chick's state as *d* changes with time. The attractiveness of "slight novelty" is given by *i*, which also increases linearly with time until discrimination is so good that *d* goes to zero and "slight novelty" ceases to exist. Thereafter, preference for familiar continues to increase unopposed to some limiting value. **(A)** State q_1 at time t_1. A preference for the now familiar training object has been acquired and the influence of "slight novelty," given by i_1, is not yet strong enough to oppose the acquired preference for familiar; there is therefore a net preference for the training object relative to all others, including the maximally attractive "slightly novel" object. The position q_1 is defined by d_1 and i_1. **(B)** State q_2 at time t_2. Preference for the familiar training object over any other object has increased further, but the preference for "slight novelty" has increased sufficiently to give a net preference for "slight novelty" over the training object. At some further time, *d* will decrease to zero and preference for the familiar training object is thereafter unopposed. See Bateson (1973, 1974) for further details, including adjustments to account for real-world complications such as biases in the preferences of naïve chicks.

become more attractive still. A further feature of the model is that the threshold for perception of slight novelty decreases as training progresses; this seems plausible given that more time spent observing the training stimulus gives more opportunity to learn about it. By implication, two stimuli differing by less than this threshold would elicit the same level of response and effectively be classified together by the chick.

Model 2

The model of O'Reilly and Johnson (1994) is a neural network comprising an input layer (layer 0, corresponding to the hyperpallial visual projection area of the forebrain, receiving visual input from the lateral geniculate nucleus of the thalamus) and two further layers (1 and 2), corresponding to different components of the IMM (cf. Section "Transient preference for novelty during imprinting"). Layer 0, which contains units with properties of simple and complex visual cortical cells as found in mammals (see e.g., Douglas and Martin, 2004), sends converging excitatory inputs to layer 1, which in turn sends converging excitatory inputs to layer 2 (**Figure 2**). The effect of this cascading configuration is to preserve the features of the visual imprinting stimulus but as a representation that is invariant with respect to retinal position. The inputs to layers 1 and 2 bear modifiable synapses that obey a Hebbian rule (Hebb, 1949), namely that conjoint pre- and post-synaptic activity strengthens the synapse such that coincident inputs on a post-synaptic cell are strengthened. There is reciprocal excitatory feedback from layer 2 to layer 1 and lateral inhibition between neighboring units in layer 2. Neurons in the model exhibit hysteresis, namely persistence of activity after activation. The properties of Hebbian plasticity and hysteresis, with suitable parameters, convey biologically realistic properties on the model and make the following predictions:

1. There is a translation-invariate representation of the training stimulus within the IMM.
2. Selective modification of connections leading to discrimination between familiar and unfamiliar stimuli.
3. A sensitive period for learning that terminates once learning has progressed to a certain level.
4. Limited reversibility of imprinting on exposure to a second training stimulus.
5. Residual recognition of the first training stimulus after training with a second training stimulus.
6. Generalization between a training stimulus and other, similar stimuli.
7. The inability to discriminate between two different training stimuli if they are present in close temporal contiguity, termed "temporal blending" by O'Reilly and Johnson (1994).
8. For given high level of temporal contiguity with which two training stimuli are presented, the more similar the stimuli, the lower the level of temporal blending. The opposite is the case if there is more delay between alternate stimulus presentations.

Properties (1)–(7) have been demonstrated in behavioral experiments (cf. Bateson, 1966; Sluckin, 1972; Horn, 1985; Bolhuis, 1991). Property (8) may seem counter-intuitive: one might expect similar stimuli to be consistently highly prone to temporal blending when presented serially with a very short delay. Indeed, Chantrey (1974) and Honey and Bateson (1996) found evidence for temporal blending in chicks trained with alternate serial presentation with two imprinting stimuli. However, the effect was not found with a different pair of training stimuli and different experimental conditions (Stewart et al., 1977), possibly due to conflicts implied in the simulation.

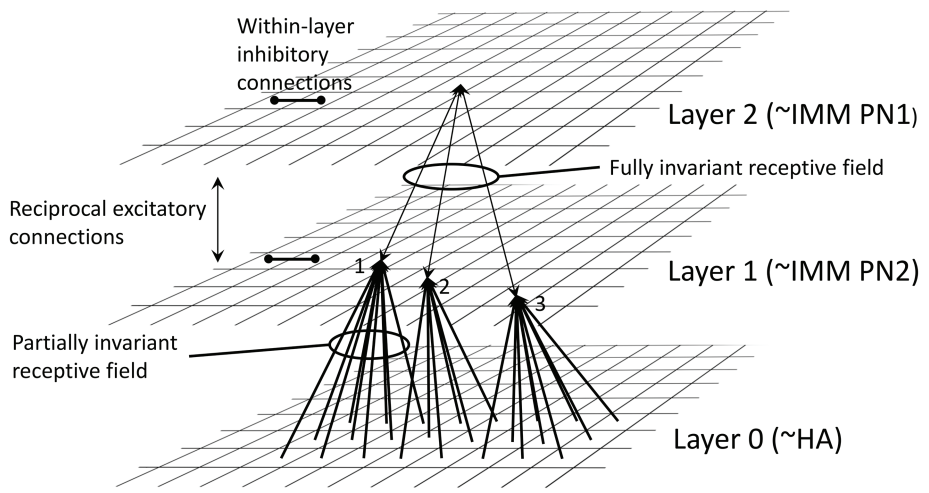

FIGURE 2 | Architecture of the neural net model of O'Reilly and Johnson (1994), redrawn from their paper. Layer 0 is suggested to correspond to the visual wulst, including the hyperpallium apicale (HA). Connections from units in layer 0 converge *via* Hebbian synapses onto units in Layer 1, suggested to correspond to part of the IMM. There is further projection *via* Hebbian synapses to Layer 2, also in the IMM. Two types of projection neuron (PN) are proposed: some axons from PN1 neurons are suggested to project out of the IMM, and PN2 neurons are suggested to be intrinsic to the IMM. Reciprocal excitatory connections combined with hysteresis render the receptive fields in Layer 2 fully position-invariant. Mutual inhibition between units within Layers 1 and 2 is also a necessary feature of the model. See O'Reilly and Johnson (1994) for details.

Model 3

Bateson and Horn (1994) describe a neural network implementation of a model of imprinting developed previously (Bateson, 1981, 1991; Horn, 1985). The model possesses three layers: analysis, recognition, and executive, each layer containing a set of modules acting in parallel (**Figure 3**). Analysis modules respond selectively to particular aspects of a stimulus such as size, shape, and color and evidently have acquired the position-invariance such as can be achieved by the process incorporated into the model of O'Reilly and Johnson (1994). In the naïve state, each analysis module is connected to each module in the recognition layer by links that can be modified in an activity-dependent manner. Each recognition module is in turn linked to an executive module by modifiable convergent connections. An executive module controls filial approach behavior toward the stimulus, as either approach or withdrawal behavior. As well as information about an imprinting stimulus flowing from analysis to recognition to executive through modifiable links, there is a direct pathway from analysis to executive, by-passing recognition and also containing modifiable links.

The rule by which a modifiable link between modules can be strengthened is Hebbian (Hebb, 1949), in that strengthening occurs if both input and recipient components of the link are simultaneously active. A link is weakened if the recipient component is active and the input is inactive, a principle arising from studies of activity-dependent plasticity in developing sensory systems (Stent, 1973). When the recipient component is inactive, the strength of the link does not change, irrespective of the state of the input. Input to a recognition module from an analysis module results in: (1) activation of an intrinsic excitatory unit, which in turn activates an output unit projecting to an executive module, all *via* modifiable links; (2) activation of a unit that inhibits the output unit (**Figure 3**), non-modifiably. Modules within a layer inhibit each other reciprocally via non-modifiable links. When the animal is in the naïve state, the activity of each recognition module fluctuates spontaneously.

With suitable choice of parameters, the model reproduces a considerable number of behavioral results, in particular:

1. acquisition of preference for an imprinting stimulus, including where stimuli differ in their attractiveness before training;
2. acquisition of a preference for inconspicuous details of a stimulus when paired with conspicuous stimulus details, as happens when imprinting leads to recognition of individual animals (Johnson and Horn, 1986b);
3. stimulus generalization after imprinting (Jackson, 1974; Bolhuis and Horn, 1992);
4. a sensitive period for imprinting that is closed by imprinting itself (cf. Bolhuis, 1991);
5. classification of a stimulus on the basis of only a subset of its features, and when the contents of the subset changes – a so-called "polymorphous category" (cf. Von Fersen and Lea, 1990);
6. classification together of different stimuli, either when the stimuli are presented simultaneously (Bateson and Chantrey, 1972; Chantrey, 1972) or in a rapid temporal sequence (Chantrey, 1974);

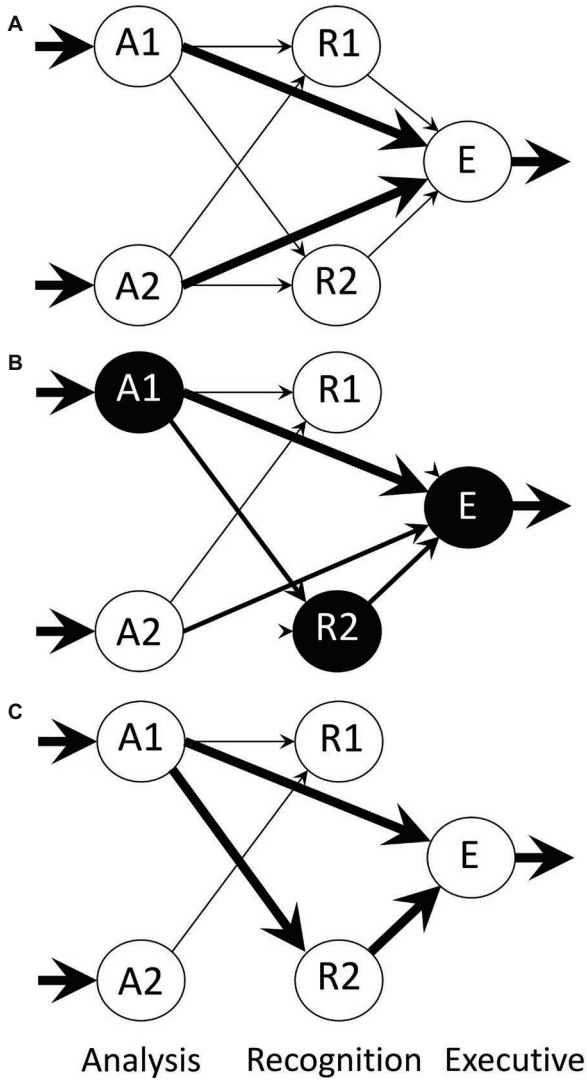

FIGURE 3 | Architecture of the neural net model of Bateson and Horn (1994), from an illustration by Bateson (2015). The model comprises three layers: A (analysis), R (recognition) and E (executive). The A and R layers contain a number of modules, only two of which are shown here. Each A module relays processed sensory input bearing information about the training object, such as size, shape, color, etc., the arrows indicate flow of information, and the thicknesses of the arrows represent connection strength of the Hebbian synapses at the head of each arrow. Within each of the A and R layers, there is reciprocal inhibition *via* non-modifiable connections between modules (not shown). There are also direct Hebbian links from analysis to executive, by-passing the R layer, permitting a predisposition to be expressed and allowing simple conditioning outside the R layer. Modules are spontaneously and variably active and mutual inhibition within a layer permits the representation of an imprinting stimulus to be encoded by strengthening the more active pathways. The activity of these pathways weakens alternative inactive pathways. **(A)** Initial state of the network before training. **(B)** Network during training with an imprinting stimulus that activates analysis module A1. Recognition module R2 happens to be highly active when input from A1 first arrives and consequently 'captures' that input while suppressing other recognition modules. **(C)** Network after training is complete.

7. the updating of a stimulus representation when the stimulus gradually changes and certain of its features disappear with time (Bateson, 1979);

8. the persistence of the ability to learn a task requiring discrimination between two visual stimuli after ablation of a brain region (the IMM) that is necessary for imprinting (Johnson and Horn, 1986a,b);
9. the formation of associative links between different stimuli by conditioning outwith the IMM (McCabe et al., 1982);
10. the expression of predispositions to approach certain classes of stimulus.

Estimation of the model's parameter values has been attempted. This of course makes the assumption, yet to be comprehensively tested, that the model is physiologically valid. The modifiable links in the model can be strengthened by use or weakened by inhibition from another pathway. Strengthening was set in the model to four times as strong as weakening. Griffiths (1998) trained chicks for 120 min with stimulus A. A control group received no further training while an experimental group received a further 180 min of training with stimulus B. Chicks were then tested by being given a choice between either A and B or A and C, which had not previously been seen. Using the control chicks as a standard for comparison, further training with B reduced preference for A against C, assumed to be due to weakening of links encoding A by exposure to C. Further training with B caused a greater reduction of preference for the red triangle against B, assumed to be due to weakening of links encoding A plus strengthening links for B. Notwithstanding all the assumptions, the strengthening to weakening ratio thus determined was estimated as 4.3:1, corresponding closely the value of 4 assumed in the model.

PHYSIOLOGICAL MECHANISMS

Memory for the Imprinting Stimulus

Bateson, Horn, and Rose conducted a series of experiments on domestic chicks to determine whether neural changes could be detected that were specifically related to the learning that occurred in the course of imprinting. Evidence for such a change was found in the forebrain (reviewed in Horn et al., 1973) and further localized by Horn et al. (1979) to a restricted part of the forebrain roof, the intermediate and medial mesopallium (IMM), known as the IMHV before revision of avian brain nomenclature by Reiner et al. (2004). The position of the IMM is shown in **Figure 4**.

Horn (1985) gives a detailed account of the evidence that the IMM is a site of memory for features of a visual imprinting stimulus. This evidence includes lesion studies indicating that the IMM is necessary for both acquisition (McCabe et al., 1981) and retention (McCabe et al., 1982) of a preference through imprinting, and that an increase in the area of apposition of spine synapses was observed after imprinting training (Bradley et al., 1981; Horn et al., 1985); this morphological change was lateralized to the left side of the IMM. The preferential involvement of the left side of the IMM in learning-related changes after imprinting has been a common occurrence over many studies and is consistent with hemispheric asymmetries found in lesion studies of the IMM (Horn, 1985; McCabe, 1991; Solomonia and McCabe, 2015).

Since spine synapses are often excitatory (see e.g., Nafstad, 1967; Errington et al., 1987), receptors for the excitatory neurotransmitter L-glutamate were studied in the IMM, and a localized learning-related increase in numbers of N-methyl-D-aspartate (NMDA) receptors in the left IMM was found after imprinting training (McCabe and Horn, 1988, 1991); see McCabe (2013) and Margvelani et al. (2018) for a discussion of how one might infer that a neural change observed after training is associated with learning and/or memory. NMDA receptors in the IMM are evidently necessary for learning, since local injection of the competitive NMDA receptor blocker D-AP5 at an estimated concentration specific for NMDA receptor blockade prevented imprinting without detectable effect on visuomotor capabilities (McCabe et al., 1992). Calcium-dependent, potassium-stimulated release of L-glutamate from the IMM also rose after imprinting training, although not in a manner specifically related to learning (Meredith et al., 2004). Learning-related phosphorylation of a

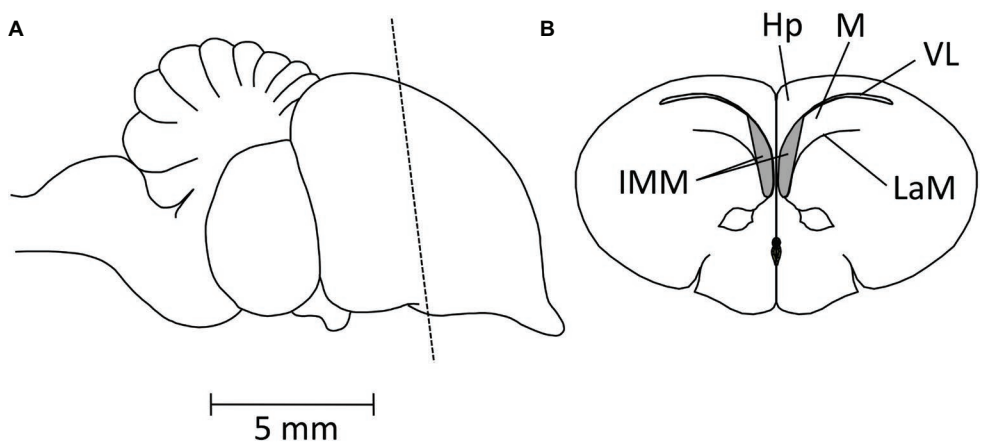

FIGURE 4 | Diagrams showing the position of the IMM. **(A)** Side view of chick brain, anterior pole to the right. The broken line indicates the plane of the coronal section shown in **(B)**. IMM, intermediate and medial mesopallium; Hp, hippocampus; M, mesopallium; VL, lateral ventricle; LaM, medullary lamina.

glutamate receptor, namely the GluA1 subunit of the ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor has, however, been detected (Solomonia et al., 2013).

Immunocytochemical labeling of the activity marker c-fos protein identified a population of neurons in the IMM that were specifically activated when a chick learned about an imprinting stimulus (McCabe and Horn, 1994). Almost all these neurons were immunopositive for taurine (Potter et al., 1998), the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and the calcium-binding protein parvalbumin, but not for the calcium-binding protein calbindin (Ambalavanar et al., 1999). An sub-population of IMM neurons associated with imprinting memory has thus been identified. Interestingly, imprinting also gives rise to presumed synaptic release of both GABA and taurine from the IMM (McCabe et al., 2001; Meredith et al., 2004).

Learning-related changes in presumed synaptic physiology in the IMM following imprinting were classified by Solomonia and McCabe (2015) into early, intermediate and late according to whether they occurred up to approximately 7 h, 7–15 h or > 15 h, respectively, after training with an imprinting stimulus. Early changes include enhanced calcium-dependent release of GABA and taurine (McCabe et al., 2001), possibly under the control of phosphorylated myristoylated alanine-rich C-kinase substrate (MARCKS protein) (Sheu et al., 1993; Van der Zee et al., 1995; Solomonia et al., 2003, 2008), and released from inhibitory neurons that are immunopositive for parvalbumin and protein kinase C-gamma but not calbindin (Ambalavanar et al., 1993, 1999). In this early period, there is also up-regulation of autophosphorylated calcium/calmodulin-dependent protein kinase II (CaMKII) (Solomonia et al., 2005), responsible for increased phosphorylation of Serine 831 in the GluA1 subunit of the AMPA glutamate receptor. Calcium-dependent release of L-glutamate in the IMM is also increased in this early period, but not quantitatively correlated with the strength of learning. Therefore, any learning-related modulation of glutamatergic activity is likely to be by modification of AMPA receptors. Calcium-dependent release of GABA and taurine continue to be enhanced in the intermediate period (7–15 h after the end of training), but accompanied by a non-specific down-regulation of the gamma-4 subtype of GABA receptor (Harvey et al., 1998). An up-regulation of the NMDA subtype of glutamate receptor that is correlated with preference score is also observed, restricted to the left IMM (McCabe and Horn, 1988). In the late period (>15 h after the end of training), there is evidence of trophic changes and synaptic stabilization approximately in proportion to the amount learned by the chicks, namely up-regulation of neural cell adhesion molecules (Solomonia et al., 1998), clathrin (Solomonia et al., 1997) and amyloid precursor protein (Solomonia et al., 2003) and cognin/brain spectrin (Meparishvili et al., 2015). Other learning-related changes at this time are suggestive of membrane and cytoskeletal stabilization, implicating alpha-fodrin (Solomonia et al., 2011) and MARCKS (Solomonia et al., 2003, 2008).

Learning-related changes in the IMM have been found in mitochondrial proteins: subunits I and II of cytochrome c oxidase, a critical enzyme in oxidative metabolism, were found to be up-regulated in the left IMM (Solomonia et al., 2011). These subunits are encoded by mitochondrial DNA (Khalimonchuk and Rodel, 2005). Further study of proteins in the mitochondrial/membrane fraction from the IMM revealed learning-related changes, restricted to the left IMM, in (1) membrane cognin; (2) a protein resembling the P32 subunit of splicing factor SF2; (3) voltage-dependent anionic channel-1; (4) dynamin-1; and (5) heterogeneous nuclear ribonucleoprotein A2/B1. There were also, in the left IMM, learning-related changes in transcription factors involved in mitochondrial biosynthesis without significant change in DNA copy number (Meparishvili et al., 2015). These changes are accompanied by increased rates of mitochondrial fission and fusion, but these processes were balanced, indicating that overall numbers of mitochondria in the IMM are stable 24 h after imprinting training. See Solomonia and McCabe (2015) for a summary of learning-related biochemical changes in the IMM following imprinting.

Regulation of protein synthesis was investigated by enquiring whether there were changes in levels of micro RNAs (miRNAs), which inhibit protein synthesis by pairing with bases in the 3′-untranslated regions of mRNA and either blocking translation into protein or destroying the RNA. A preliminary screen using the left IMMs from a small number of strongly or weakly imprinted chicks implicated a particular miRNA in imprinting on the basis of statistical significance and expression level. Levels of this miRNA (gga-miR-130b-3p) in the left IMM were negatively correlated with preference score and a range of criteria implicated the miRNA in a predisposition to learn (Margvelani et al., 2018; see Section "A Predisposition to Learn"). One of the protein products of this molecule is cytoplasmic polyadenylation binding protein 3 (CPEB-3), and this protein was significantly up-regulated in a learning-related manner in the left IMM as a result of imprinting training (Margvelani et al., 2018). This is thus an example of a miRNA in the IMM that is unaffected by imprinting but which predisposes a chick to learn, whereupon the protein whose synthesis is controlled by the same miRNA is up-regulated as a result of training and is intimately involved in the memory 24 h after training.

As well as the evidence for learning-related functional synaptic modification in the IMM, particularly on the left side of this structure, single unit recording in freely moving chicks has shown that neuronal responsiveness in the IMM to a visual imprinting stimulus increases as a result of imprinting training (Brown and Horn, 1994; Nicol et al., 1995; Horn et al., 2001; Nicol and Horn, 2009). Training was conducted in the presence of a hen's maternal call in order to render imprinting to the visual stimulus more effective (Bolhuis and Honey, 1994). Testing, however, was purely visual. As might be expected from morphological, pharmacological, and biochemical findings (Horn, 1985; McCabe, 1991, 2013), different effects of training were found in the left and right sides of the IMM (Nicol et al., 1995). When the visual and auditory components of the bisensory training stimulus were presented separately after training, it was found that neuronal responsiveness to the visual component had increased, whereas responsiveness to the maternal call

was reduced (Nicol and Horn, 2009). The enhanced neuronal responsiveness to the visual component was confirmed by Town (Town, 2011b), who found further that this enhancement was reduced if chicks, having been trained originally in isolation, were then reared with conspecific chicks for 9 h. The reduction was largely in the left IMM, and an increased response to a novel stimulus was observed in the right IMM. The social rearing also reduced chicks' behavioral preferences for the original training stimulus, as might be expected from previous studies of secondary imprinting (Salzen and Meyer, 1968; Cherfas and Scott, 1981; Bolhuis and Bateson, 1990; Devos and Vankampen, 1993). Neuronal responsiveness in the IMM thus changes in parallel with behavioral preferences when a second imprinting stimulus is introduced.

The pathway whereby visual information reaches the IMM was investigated by Nakamori et al. (2010), who identified a polysynaptic thalamofugal visual pathway reaching the IMM *via* synaptic connections in the interstitial nucleus of the hyperpallium apicale, and in the rostral, densocellular and periventricular parts of the hyperpallium dorsale (HD). Imprinting was associated with NR2B-containing NMDA receptors that contribute plasticity in this circuit (see also Nakamori et al., 2015). Cholecystokinin has been implicated in the role of the visual wulst in imprinting (Maekawa et al., 2007) and there is selective activation of presumed GABA-ergic parvalbumin-containing cells in this pathway (Nakamori et al., 2017).

The Effect of Sleep on Imprinting Memory

Experiments on human subjects have established that sleep after certain types of learning can enhance subsequent recall of the learned items (cf. Vorster and Born, 2015), but the underlying neural mechanisms are imperfectly understood. It was suggested (Horn et al., 2001) that neuronal responses in the IMM to a familiar imprinting stimulus may become stabilized by sleep, but this was not established until Jackson et al. (2008) recorded single units from the IMM during and after imprinting and found that undisturbed sleep within a particular temporal window after imprinting training was necessary for both stability of neuronal responsiveness to the imprinting stimulus and retention of the imprinted preference measured behaviorally. It was already known that if chicks were allowed to rest in darkness over a 6-h period after imprinting training for 2 h, responsiveness to the imprinting stimulus was enhanced significantly 24 h after the start of training (Horn et al., 2001). However, if continuous sleep was prevented during this period by occasional gentle disturbance (slowly rotating the running wheel in which the chicks were held in darkness once every 30 min), neuronal responses were unstable – neurons that had once been responsive ceased to respond and previously unresponsive neurons started to respond. This instability persisted at 24 h and chicks showed no evidence of being imprinted at that time. However, if the same disturbance was delayed for 6 h, by the 24-h time point, the neuronal response had stabilized at a significantly higher level and the chicks showed a behavioral preference for the imprinting stimulus. There was increased activity in the lower theta range (5–6 Hz) of the electroencephalogram during the 6-h period after training had finished, during which sleep disruption was effective in disrupting both neuronal responsiveness and retention of the preference acquired through imprinting (Jackson et al., 2008). In view of the finding of Marshall et al. (2004) and Marshall et al. (2006) that transcranial electrical stimulation of the human brain at a frequency of 0.75 Hz (corresponding to the frequency of slow-wave sleep) enhanced declarative memory recall, Nicol and McCabe (2014) electrically stimulated the brains of chicks at 5 Hz [the frequency enhanced during the post-training sleep period in the experiments of Jackson et al. (2008)] or 0.75 Hz (the main frequency of slow-wave sleep) during the 6-h period after training when sleep was necessary for stabilization of IMM neuronal responsiveness and behavioral retention. Stimulation at both these frequencies protected against loss of the preference acquired during imprinting.

O'Reilly and Johnson (1994) found that the responsiveness of units in Layers 1 and 2 of their neural network model became unstable in the early stages of imprinting as the balance between strengthening and weakening of connections was becoming established. It is therefore noteworthy that instability in neuronal responsiveness in the IMM was observed shortly after imprinting by Horn et al. (2001) and that this instability was strongly reduced by sleep in a specific 6-h period after the end of training (Jackson et al., 2008).

The models of O'Reilly and Johnson and Bateson and Horn (1994) rely on Hebbian plasticity, and therefore it is appropriate to enquire where such plasticity has been described in the IMM. Associative long-term potentiation and depression are well-established forms of synaptic plasticity based on Hebbian mechanisms, which depends on activation of NMDA receptors (see e.g., Morris, 2003). Investigation of the IMM *in vitro* has revealed plasticity resembling glutamate receptor-mediated long-term potentiation (Bradley et al., 1991, 1993, reviewed in Matsushima and Aoki, 1995; Bradley et al., 1999) and susceptibility of imprinting to local pharmacological blockade of NMDA receptors in the IMM (Mccabe et al., 1992).

The Sensitive Period for Imprinting

Mechanisms underlying the sensitive period have been studied by correlational measurements, determining which physiological changes parallel the sensitive period, in combination with physiological and pharmacological interventions to modify the sensitive period.

The start of the sensitive period for visual imprinting is clearly dependent on the ontogeny of the neural systems necessary for perception of an imprinting stimulus, together with motivational and motor systems required to express the imprinted behavior. This is not to say that experience does not influence the sensitive period, since sensory stimulation is necessary for complete development of sensory systems, which are themselves subject to sensitive periods (see e.g., Feldman, 2009). A detailed example of an effect of sensory stimulation on a sensitive period comes from experiments on auditory imprinting in ducklings, where post-hatch ability to acquire a preference for the conspecific adult maternal call requires previous experience of embryonic contact-calls in the

egg (reviewed in Gottlieb, 1987). Moreover, the ability to imprint to a non-conspecific call (the maternal call of a chicken) was not detected in ducklings reared in isolation, but only occurred when ducklings experienced tactile contact during social rearing (Gottlieb, 1993). An influence of visual experience on subsequent visual imprinting was described by Bateson and Wainwright (1972), who found that exposure of chicks hatched and reared in darkness to white light for 30 min before exposure to imprinting stimuli increased the efficacy of visual imprinting. This effect was tentatively ascribed to activation of visual pathways by the white light.

Barbiturate anesthesia has been found to extend the sensitive period (Macdonald, 1968). An anesthetic dose of a ketamine/xylazine mixture administered on the day of hatching enabled chicks to become imprinted to a jungle fowl model on day 8 post-hatch, whereas this did not occur in saline-treated controls (Parsons and Rogers, 1997). This effect was later ascribed to the inhibitory action of ketamine on the NMDA subtype of glutamate receptor, since the effect was reproduced using the specific NMDA receptor antagonist MK-801 (Parsons and Rogers, 2000). It is worth noting that the sensitive period in these experiments was defined in terms of a preference for a model of a fowl, raising the possibility that specific patterns within this naturalistic stimulus may have been especially important in these experiments.

Thyroid hormone has been strongly implicated in control of the sensitive period for imprinting in chicks. It was reported by McNabb (2006) that thyroid hormone levels peak around the time of hatching in precocial birds. Also, the gene for Type 2 iodothyronine deiodinase (Dio2), the enzyme that catalyzes conversion of thyroxine (T4) to triiodothyronine (T3), is up-regulated when chicks become imprinted (Yamaguchi et al., 2008). Yamaguchi et al. (2012) also found that T3 levels in brain peaked around hatching and increased as a result of imprinting. Dio2 inhibitors administered either systemically or into the IMM, blocked visual imprinting and T3, and administered either intravenously or into the IMM could increase the efficacy of a visual imprinting stimulus and extend the sensitive period for several days. Moreover, both imprinting and exogenous T3 facilitated imprinting on a second stimulus (Yamaguchi et al., 2012), suggesting that T3 might contribute to neural mechanisms underlying updating of the representation of an imprinting stimulus. Nucleotide diphosphate kinase 2 has been implicated in the action of T3 by the demonstration that inhibition of this enzyme in the IMM blocks the action of T3 in extending the sensitive period (Yamaguchi et al., 2016). The intermediate hyperpallium apicale (IMHA) receives output from the IMM and has been implicated in imprinting by Aoki et al. (2015). The IMHA receives a projection from the IMM and is the site of an increase in the level of Wnt-2b, a glycoprotein that regulates neuronal growth, when imprinting occurs (Yamaguchi et al., 2018). Blockade of Wnt-2b action in the IMHA prevents expansion of the sensitive period by T3, leading to the proposal that T3 causes up-regulation of Wnt-2b in the IMHA, thus playing a crucial role in the regulation of the sensitive period. The action of T3 in the IMM has been ascribed to differential effects on gamma-aminobutyric acid (GABA) receptors, namely sub-types A (ionotropic) and B (metabotropic). From the results of injecting GABA-A and GABA-B agonists and antagonists into the IMM, it was concluded that the T3-dependent sensitive period depends on a balance between the activities of these two receptor subtypes. It is suggested that GABA-B activity is necessary for imprinting and that GABA-A receptor activity suppresses imprinting (Aoki et al., 2018).

Predispositions

The predisposition of domestic chicks for face-like objects was discovered on account of its resistance to lesions of the IMM, the forebrain region thought to store information about the imprinting stimulus (Horn and McCabe, 1984). It would appear, therefore, that the predisposition is governed by one or more systems outwith the IMM. Mayer et al. (2016) measured c-fos protein expression in chicks that preferred a model of a jungle fowl to a scrambled version of the same model and in chicks having the opposite preference, that is, in chicks that respectively showed a predisposition and those that did not (cf. Johnson and Horn, 1988). No significant difference was found in the hyperpallium apicale (HA, homologous to part of the mammalian visual cortex) or in the tectum [suggested by Johnson (2005) as possibly being one of the regions controlling an analogous predisposition in human neonates]. In the IMM, c-fos protein expression was significantly greater in chicks without the predisposition. The results thus do not implicate the HA or the tectum in the predisposition but indicate that neuronal activity in the IMM is influenced by the predisposition, although it is known that the IMM is not necessary for the predisposition to be expressed. The results raise the interesting possibility that a predisposition is responsible for a net suppression of neuronal activity in the IMM.

A certain amount is known about the properties of the predisposition. It can be induced by mild, non-specific sensory stimulation such as handling, or exposure to white light or a hen's maternal call (Hampton et al., 1995). There is a sensitive period about 10–40 h after hatching during which the predisposition may be induced (Johnson et al., 1989), and this period can be delayed by general anesthesia (Bolhuis and Horn, 1997) and the noradrenaline-depleting neurotoxin DSP4 (Davies et al., 1992), but DSP4 does not abolish the predisposition (Davies et al., 1985). Preference for the jungle fowl is positively correlated with the concentration of plasma testosterone and can be enhanced further by injection of testosterone (Bolhuis et al., 1986).

The neural basis of the predisposition to follow biological motion has been investigated by c-fos protein immunocytochemistry. Exposure of a chick to a living, behaving conspecific increased expression in the septum and the amygdaloid regions nucleus tenia and arcopallium as compared with chicks that did not experience this exposure (Mayer et al., 2017b). The septum and preoptic area were differentially activated by a living, behaving conspecific in comparison with a rotating model of a conspecific, i.e., not expressing biological motion (Mayer et al., 2017a). Moreover, these two regions were selectively activated by another animacy cue, namely a spontaneous change in speed of an object, compared to constant speed (Lorenzi et al., 2017). Thyroid hormones have also been implicated in this predisposition, by experiments in which T3 was injected into chicks that were imprinted on a rotating object not exhibiting biological motion

and then tested by being given a choice between animate and inanimate motion. Exogenous T3, known to extend the sensitive period for imprinting (Yamaguchi et al., 2012), was found also to enhance the preference for biological motion, providing a physiological link between imprinting, the sensitive period for imprinting, and the predisposition to prefer biological motion (Miura et al., 2018).

A Predisposition to Learn

A technique for detecting a learning-related change has the potential also to yield evidence for the presence of processes contributing to a predisposition. Many investigations of the role of the IMM in imprinting have enquired whether there is a correlation between a measure of the strength of imprinting – a preference score derived from a choice between the familiar imprinting stimulus and a novel stimulus – and a quantitative measurement of a physiological process. Appropriate choice of training period duration can result in some chicks learning nothing despite exposure to the imprinting stimulus and other chicks becoming strongly imprinted – simply, they learn better. If chicks learning nothing show no significant change in the measurement, if the strongly imprinted chicks show a strong change in the measurement, and there is a significant correlation between the measurement and preference score, one is led to conclude that the measurement is related to learning. One would expect training to have induced a learning-related change if, in addition, residual variance from the correlation (i.e., variance about the regression line) is no lower than the variance of untrained control chicks (McCabe and Horn, 1988; Horn and Johnson, 1989; McCabe, 2013). This is because an effect of training that is related to learning would add to the variance in control chicks and would reveal itself in a significant correlation with preference score; residual variance about the regression line would have the same origin as in untrained chicks. In contrast, a variance about the regression line that is significantly lower than the control variance is evidence, not for an effect of training, but merely a resorting of the control values. For example, chicks with high levels of the physiological measurement could be predisposed to learn well and chicks with low levels of the measurement predisposed to learn poorly. Evidence of this kind for a predisposition was found by Margvelani et al. (2018) when investigating the effect of micro-RNA (miRNA) expression in the IMM. miRNA profiling identified a miRNA (gga-miR-130b-3p) whose expression was negatively correlated with preference score. In addition, the residual variance about the regression line was significantly lower than the variance of untrained control chicks. For the reasons outlined above, it was inferred that this micro-RNA was not affected by training but was present at control levels in poor learners and low levels in good learners. That is, its concentration reflects a predisposition to learn well or badly and is a predictor of how well chicks will learn when trained with an imprinting stimulus – (see Margvelani et al. (2018) for detailed). Interestingly, levels of a protein controlled by this miRNA, cytoplasmic polyadenylation element binding protein 3 (CPEB-3), was positively correlated with preference score (this direction of correlation is expected because miRNA inhibits protein translation), and the data indicate that training affect CPEB-3 level in a learning-related manner. The miRNA, as one of the factors controlling protein level, reflects a predisposition and is not affected by training.

It is not known whether the predisposition to learn referred to above is related to the other predispositions discussed in this review: there are clearly several types of predisposition and their relationships to one another remain to be elucidated. The correlational technique outlined here (see also Margvelani et al., 2018) is a powerful way of determining how inevitable differences between individual animals may predispose the animals to specific types of behavior.

Transient Preference for Novelty

There has been little investigation of the neural mechanism underlying a temporary preference for slight novelty during the early phase of imprinting. The neural network models of O'Reilly and Johnson (1994) and Bateson and Horn (1994) do not account for this phenomenon, although the latter suggest that this behavior could be simulated by adding habituation to the properties of the input to the recognition layer of the network from the analysis layer. There may be other possibilities, for example, metaplastic modification of the Hebbian synapses in the recognition layer, namely reducing the efficacy of Hebbian modification by recent activation of the synapses involved (Abraham and Richter-Levin, 2018).

Classification Together of Temporally Juxtaposed Stimuli

The demonstration by lesion studies that the IMM is necessary for both acquisition and retention of a preference acquired through imprinting also revealed a functional difference between the left and right sides of this region. A series of experiments indicated that the left IMM is responsible for long-term storage of a representation of the imprinting stimulus and that the right IMM also has a storage function, but of a different nature. If the left IMM is lesioned shortly after training and the right remains intact for approximately one more day, storage occurs in a region, identified as S′ (Cipolla-Neto et al., 1982), which must lie outside the IMM because the IMM at that point is no longer present. Conversely, if the order of lesioning is reversed, i.e., the right IMM lesioned before the left, the chicks show no memory for the imprinting stimulus: the remaining left IMM is critical for retention of the preference. Thus, S′ becomes functional under the influence of the right (reviewed in Horn, 1985; McCabe, 1991). It is therefore possible to arrange for chicks to be imprinted without S′ becoming operational, by lesioning the right IMM shortly after training. It is also possible for other chicks to possess a functional S′ with no IMM, by lesioning the IMM bilaterally after S′ has become operational. It is then possible to compare the properties of the two memory systems: S′ and the IMM. Presentation of two imprinting stimuli to chicks in close temporal juxtaposition results in behavior indicating that the two stimuli are classified together (Chantrey, 1974, 1976). This may be demonstrated by training chicks with two stimuli

presented sequentially according to a random schedule in short intervals of duration 10–30 s and inter-stimulus intervals of 5–25 s ("mixed" training). Chicks trained in this way subsequently learn to discriminate between the two stimuli in a heat-rewarded conditioning procedure, but more slowly than chicks that have been subjected to the two stimuli for the same total time, but in longer, separate intervals, each of duration 53 min ("separate" training) (Honey et al., 1995). These authors found that chicks in which S′ was intact also showed this effect. However, if the chicks became imprinted and S′ was not allowed to become operational, the inferred ability to classify stimuli together was lost (Honey et al., 1995). It was concluded that the IMM system can store information about the imprinting stimulus, but S′ is required for the flexibility of processing that permits classification together.

Recognition of Individuals

Johnson and Horn (1987) found that chicks can learn to distinguish between two different jungle fowl models after being imprinted to one of them; moreover, this ability was abolished by lesions to the IMM. Town (2011a) socially reared chicks in groups of six and then recorded the responses of IMM neurons, in these chicks, to video recordings of familiar and unfamiliar chicks in groups, in the presence of conspecific calls. Note that both testing and social rearing involved simultaneous visual and auditory stimulation. Under these conditions, neuronal responsiveness to the familiar chicks was lower than to novel chicks, this effect predominating in the right IMM. Although a group of chicks rather than an individual animal was used in this experiment, the results provide evidence of remarkable learning-dependent discrimination between naturalistic stimuli, such as may be engaged in learning the features of an individual. As noted previously, responsiveness of IMM neurons to the visual and auditory components of a familiar audio-visual imprinting stimulus are different (Nicol and Horn, 2009). Responsiveness to a bisensory stimulus may be different again and not necessarily in linear combination of the constituent modalities.

The question of the IMM's responsiveness to stimuli sharing only some of the features of an imprinting training stimulus was addressed by Town and McCabe (2011). In these experiments, chicks were trained with an artificial visual stimulus accompanied by a maternal call, followed by determination of IMM neurons' responsiveness to combinations of familiar and novel versions of the visual and auditory components of the training stimulus. As reported previously (e.g., Brown and Horn, 1994), neuronal responsiveness to the visual component of the training stimulus was increased by imprinting, whereas responsiveness to a novel visual stimulus was not. Responsiveness to unisensory auditory stimuli was equivocal: there was a significant interaction between stimulus familiarity and training condition but no clear indication of how either of these factors contributed, possibly because of the small number of animals involve. A particularly strong increase in responsiveness was observed when the familiar visual stimulus was presented with a novel maternal call, leading to the suggestion that IMM neurons may be sensitive to changes in the context of a familiar visual stimulus (Town and McCabe, 2011). It is also apparent from these results that a response to a bisensory stimulus is not necessarily the sum of responses to its unisensory components: there can be considerable interaction between the underlying processes.

Despite the obvious need for caution in comparing neuronal activity in the IMM with behavior arising from imprinting and despite the different timescales involved, there is a noteworthy parallel between increased neuronal responsiveness to a familiar visual stimulus in a novel auditory context (Town and McCabe, 2011) and the behavioral preference for slight novelty observed in the early stages of imprinting (Jackson and Bateson, 1974; Bateson and Jaeckel, 1976). Such behavior was incorporated into the model of Bateson (1973, 1974). Bateson and Horn (1994) consider such behavior when discussing their neural network model, postulating, in addition to the formal implementation of the model, attenuation of input into recognition modules as a result of continuous exposure to the same stimulus. Bateson (2000) proposed a similar addition to the model in the light of experiments investigating chicks' classification together of imprinting stimulus features (Honey and Bateson, 1996).

IMPRINTING TO SEVERAL OBJECTS AND RELATIONAL CONCEPT LEARNING

Neurobiological analysis has yet to make headway with these behavioral phenomena, important though they undoubtedly are in the life of a young animal and at least implied by existing neural network models (Martinho and Kacelnik, 2016).

CONCLUSION

By establishing a social bond between a newly hatched chick and a potentially protective conspecific adult, imprinting can substantially increase the chick's chances of survival. The contribution of imprinting to social cohesion is therefore of great biological importance. Imprinting is also experimentally tractable. Therefore, much is known about its behavioral characteristics and the underlying neural mechanisms. Modeling the behavior associated with imprinting has yielded useful insights and predictions at the behavioral level, but such models also require physiological validation, which currently is incomplete. If such validation can be accomplished, the relevant models may make an important contribution to understand social behavior at the physiological level.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Evolution of Pallial Areas and Networks Involved in Sociality: Comparison Between Mammals and Sauropsids

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Birds are extremely interesting animals for studying the neurobiological basis of cognition and its evolution. They include species that are highly social and show high cognitive capabilities. Moreover, birds rely more on visual and auditory cues than on olfaction for social behavior and cognition, just like primates. In primates, there are two major brain networks associated to sociality: (1) one related to perception and decision-making, involving the pallial amygdala (with the basolateral complex as a major component), the temporal and temporoparietal neocortex, and the orbitofrontal cortex; (2) another one related to affiliation, including the medial extended amygdala, the ventromedial prefrontal and anterior cingulate cortices, the ventromedial striatum (largely nucleus accumbens), and the ventromedial hypothalamus. In this account, we used an evolutionary developmental neurobiology approach, in combination with published comparative connectivity and functional data, to identify areas and functional networks in the sauropsidian brain comparable to those of mammals that are related to decision-making and affiliation. Both in mammals and sauropsids, there is an important interaction between these networks by way of cross projections between areas of both systems.

Keywords: medial amygdala, BST, social cognition, affiliation, dorsal ventricular ridge, six part pallial model, orbito frontal cortex, pallial amygdala

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INTRODUCTION

In primates, several studies have shown a correlation between social network size and the volumes of specific parts of the telencephalic pallium, including the orbitofrontal cortex, the cortical and basolateral amygdala, the temporo-parietal cortical junction, and the superior temporal sulcus (Lewis and Barton, 2006; Powell et al., 2010, 2012; Bickart et al., 2011; Kanai et al., 2011). In contrast, such a correlation is not observed when other neocortical areas and the hippocampal formation are considered. One important aspect to remark is that areas related to sociality are reciprocally connected and are clustered together in functional MRI (fMRI) studies (Bickart et al., 2012). In particular, fMRI in humans described two distinct brain networks positively correlated to sociality, one involved in social perception (the impressions and inferences that one individual of a group makes on another, from selective attention to inferences of intentionality) and another one involved in social affiliation (the association of

individuals within a group, including social attachment) (Byrne and Bates, 2007; Bickart et al., 2012). The network related to social perception involves the "ventrolateral amygdala" (including the pallial amygdala, encompassing the cortical and basolateral or basal amygdala complex), the fusiform gyrus (for face recognition) and other temporal neocortical areas, and the orbitofrontal cortex; the network related to social affiliation involves the medial amygdala, the ventromedial prefrontal cortex and adjacent subgenual and anterior cingulate cortical areas, the ventromedial striatum (largely including nucleus accumbens), and the ventromedial hypothalamus (Bickart et al., 2012).

While social cognition in primates relies more on visual and auditory cues, and less so on olfactory cues, the opposite is true in most non-primate mammals. Comparative studies of brain areas and networks involved in sociality are important because: (1) they can identify useful non-primate models for studying in depth the neural basis of social cognition at molecular, cellular, and circuit levels, (2) they can help to identify selective pressures and developmental mechanisms behind evolutionary change (MacLean, 2016), and (3) they can help to identify minimal neural requirements for achieving sophisticated cognitive tasks, such as predict intentionality of others and other aspects of theory of mind. In this sense, birds are extremely interesting animals because some species are highly social, show high cognitive capabilities, use vocalization in social contexts (a capacity that is particularly well developed in songbirds; Hessler and Doupe, 1999), and include at least a family (corvids) with capacity for physical reasoning, for remembering the past and plan for the future and for thinking about another's perspective (Clayton and Emery, 2015; Boucherie et al., 2019). In addition, birds – like primates – rely more on visual and auditory cues than on olfaction for social behavior and cognition (Clayton and Emery, 2015; Mayer et al., 2017). Thus, although sophisticated aspects of social cognition in corvids and primates were achieved by independent evolution, birds are good models for studies on the neural basis of sociality and its evolution. However, the pallium of birds and mammals have greatly diverged during evolution, which has made the comparison of pallial areas and interpretations quite controversial (discussed by Reiner et al., 2004). For inter-species brain comparisons to make sense, it is mandatory to first unravel the brain building plan (Bauplan or morphoplan), which is shared by different vertebrates. Developmental studies, particularly those on combinatorial expression patterns of early regulatory genes in relation to the topological framework of the neural tube, have become extremely useful to unravel the brain morphoplan, with its basic divisions comparable across vertebrates (Nieuwenhuys and Puelles, 2016). The conclusions of this type of approach support that a large lateroventral part of the avian and reptilian pallium (called the dorsal ventricular ridge) derives from pallial embryonic divisions that gives rise to the pallial amygdala and other areas of the so-called piriform lobe in mammals (Puelles et al., 2000, 2017; Medina et al., 2011, 2017a; Abellán et al., 2013; Desfilis et al., 2018), a proposal also supported by results of fate mapping (Hirata et al., 2009; Soma et al., 2009; Waclaw et al., 2010; Bupesh et al., 2011; Puelles et al., 2016a; García-Moreno et al., 2018; Rueda-Alaña et al., 2018), tract-tracing studies (Bruce and Neary, 1995; Martínez-García et al., 2007), and more recently, by single-cell transcriptome (Tosches et al., 2018). In this account, we will review the different data pointing to the presence of a pallial amygdala-like region in the sauropsidian dorsal ventricular ridge, as well as the possible existence of an area comparable to the orbitofrontal cortex. We will also review the known connections and functions of these areas and discuss their putative implication in social cognition.

THE EVOLUTIONARY DEVELOPMENTAL BIOLOGY APPROACH TO UNRAVEL THE PALLIUM MORPHOPLAN

Understanding the evolutionary origin of the neocortex has inspired a vast amount of research and still generates vehement and thrilling debates as well as new publications, often with different conclusions (for example: Karten, 1997; Puelles, 2001; Butler et al., 2011; Dugas-Ford et al., 2012; Belgard et al., 2013; Tosches et al., 2018). The neocortex derives from the telencephalic pallium, but the continuing controversy on its evolution relates to the uncertainty on how many pallial divisions there are and how they are compared across vertebrates. The traditional comparative neuroanatomy approach, excellent for analyzing the gradual evolutionary variations in relatively well conserved brain areas and fiber tracts (for example, the basal ganglia and the dopaminergic nigrostriatal projection; Reiner et al., 1998), have not provided a satisfactory answer to the question on the evolutionary origin of the neocortex, which has involved a high degree of divergence. The evolutionary developmental biology (evodevo) approach, integrated as part of the extended evolutionary synthesis, is complementary to the traditional approaches for understanding evolution and has the power of providing developmentally based explanations of the organism (or organ) form and function, as well as new venues for both exploring the mechanisms behind innovation and answering the question of how novel complex traits originate (Hall, 2003; Moczek et al., 2015). The latter often relates to developmental changes in genetic networks involved in patterning, specification, cell proliferation, and/or other aspects of development, which emerge and spread in populations under favorable conditions for ecological (natural *sensu* Darwin) or sexual selection (i.e., if the variation provides advantage for survival and/or reproduction).

The evodevo approach is successfully being applied to study brain evolution, providing highly powerful tools for: (1) understanding the brain morphoplan and comparing its basic building blocks or divisions across species (Puelles et al., 2000; Medina and Abellán, 2009; Moreno et al., 2010; González et al., 2014), (2) identifying hidden or obscure cases of deep homology, or partial homology (Shubin et al., 2009), (3) identifying homologous brain areas and networks involved in particular functions and behaviors, such as those related to sociality (Medina et al., 2011; O'Connell, 2013), (4) unraveling the genetic and developmental mechanisms involved in convergence

(Pfenning et al., 2014; Whitney et al., 2014) and in evolutionary variations (for example, eye loss in blind cavefish: McGaugh et al., 2014; variations in optic tectum size: Striedter and Charvet, 2008). Importantly, homologous brain divisions typically show similar combinatorial expression patterns of highly conserved transcription factors and other regulatory proteins during early (phylotypic) stages of development of different vertebrates (Puelles et al., 2000; Puelles and Medina, 2002; Medina, 2007; Nieuwenhuys and Puelles, 2016). Variations in expression patterns of developmental regulatory genes occur more often during late development and are behind cases of morphological divergence (Medina et al., 2013).

Puelles and colleagues were the first in using this approach to identify the same basic building divisions in the embryonic telencephalon of mouse and chicken (Puelles et al., 2000). In the pallium, they identified a novel ventral pallial division (VP) as a partition of the classical piriform lobe or lateral pallium, leading to the proposal of a tetrapartite model of pallial divisions, with medial, dorsal, lateral and ventral pallia (this proposal was recently revisited by Puelles et al., 2017). According to the initial proposal, the VP of mouse and chicken showed expression of pan-pallial transcription factor-related genes (such as Pax6 in the ventricular zone and Tbr1 in the mantle), but lacked ventricular zone expression of Emx1 (typical in the rest of the pallium) and Dlx2 (typical of the subpallium) (Puelles et al., 2000). This division was located just above the pallio-subpallial boundary and showed the olfactory tract at its surface (Puelles et al., 2000; Puelles, 2001). This initial proposal was reinforced later by the identification in mouse of transcription factors expressed in the ventral pallium but not in adjacent pallial and subpallial divisions (such as Dbx1, expressed in the VP ventricular zone, but not in the dorsal pallium nor the subpallial ganglionic eminences; and Lhx9, highly expressed in the VP mantle, but not in the subpallial striatum/pallidum and only transitorily in the dorsal pallium outer layer, in relation to Cajal-Retzius cells) (Dbx1: Yun et al., 2001; Medina et al., 2004; Lhx9: Rétaux et al., 1999; Abellán et al., 2009; Medina et al., 2011). The VP has also been identified in non-mammals on the basis of Lhx9 expression, in combination with other regulatory genes, although this division does not express Dbx1 in non-mammals (Moreno et al., 2004; Abellán et al., 2009; Medina et al., 2011; Vicario et al., 2017; Desfilis et al., 2018).

According to the tetrapartite pallial model, the VP includes the olfactory bulb at its rostral pole, followed caudally by olfactory pallial areas in all vertebrates (Puelles, 2001; revisited by Puelles et al., 2017). In mammals, this includes the olfactory cortex, and more caudally the cortical amygdalar areas, all of which receive input from the main and/or accessory olfactory bulbs (Puelles, 2001; Puelles et al., 2017). It also includes several nuclei located deep (in topological terms; i.e., considering the radial dimension) to the olfacto-recipient cortical areas, such as the endopiriform nuclei and the basal amygdalar complex (Puelles et al., 2017). In birds and reptiles, it produces a ventral part of the dorsal ventricular ridge (DVR), which in birds includes the so-called nidopallium and the arcopallium, both of which are located deep to some olfactory superficial areas (Puelles et al., 2017).

However, although the existence of a VP sector in the pallium is currently unquestionable, its rostrocaudal extension and number of derivatives are currently a matter of debate. First, the VP derivatives have been clearly traced in mice using migration assays (Soma et al., 2009; Bupesh et al., 2011) and Dbx1 cell lineage tracing in transgenic reporter mice (Hirata et al., 2009; Waclaw et al., 2010; Puelles et al., 2016a). According to these results, the VP progenitors give rise to the ventral parts of the piriform cortex and endopiriform nuclei (but not their dorsal parts) and to large part of the pallial amygdala except its caudal pole (Puelles et al., 2016a). Second, in birds and reptiles, VP derivatives would not include the caudal DVR, encompassing the avian arcopallium and the reptilian dorsolateral amygdala and nucleus sphericus, which show discrepant expression patterns with those of the nidopallium/anterior DVR during development (Abellán et al., 2014; Medina et al., 2017a; Desfilis et al., 2018). Third, based on cell lineage tracing and gene expression patterns, the VP derivatives do not include the whole olfactory bulbs, but only part of them (as discussed by Desfilis et al., 2018).

TOWARD A MODEL OF SIX PALLIAL DIVISIONS?

The controversial data exposed above on the VP extension and derivatives, together with the difficulty found when trying to compare the embryonic pallium of mammals, birds and reptiles using gene expression patterns, prompted us to propose an alternative model of six pallial divisions, which is currently under evaluation (Medina et al., 2017a; Desfilis et al., 2018). The six part pallial model takes into consideration the experimental and genetic cell lineage results on VP derivatives in mouse, together with the gene expression patterns that are discrepant with a VP profile in different vertebrates (for example, expression of Emx1 in the ventricular zone, which happens in the rostral and caudal poles) in order to better delimit the VP and its derivatives (Medina et al., 2017a; Desfilis et al., 2018). According to the six part pallial model, the lateral pallium (LP) includes the dorsal part of the piriform lobe (the latter is neatly delineated by Lmo3 expression, encompassing VP and LP; Abellán et al., 2009). In contrast to VP, LP is poor in Dbx1-lineage cells (Puelles et al., 2016a) and Lhx9 (Abellán et al., 2009) and includes the dorsal parts of the olfactory cortex and endopiriform nuclei (Abellán et al., 2009, 2014; Desfilis et al., 2018). In birds, the LP includes the so-called mesopallium, which expresses Lmo3 but not Lhx9 in the mantle (Abellán et al., 2009).

Dorsal to VP and LP, the six part pallial model proposes the existence of a different pallial sector, which is also distinct from the dorsal pallium and is called the dorsolateral pallium (DLP; Abellán et al., 2014; Medina et al., 2017a). This division is distinguished from early developmental stages in different amniotes, as a sector showing moderate to high expression of Emx1, Lhx2, and Lhx9 in the ventricular zone and/or mantle (Abellán et al., 2009, 2014; Desfilis et al., 2018), and it extends from rostral to caudal levels (Desfilis et al., 2018).

In mouse, the DLP also shows strong expression of Nr4a2/Nurr1 from early stages and includes the claustroinsular complex (Watson and Puelles, 2017; discussed by Desfilis et al., 2018). Note that this complex is attributed to the LP and compared to the avian mesopallium in the current version of the tetrapartite pallial model (Puelles et al., 2016b, 2017; Watson and Puelles, 2017; see also Smith et al., 2018, where this sector is compared between different mammals), but the problem with this proposal is that while the mesopallium is part of the piriform lobe and rich in Lmo3, the claustroinsular complex is above and poor in Lmo3 (see data in Abellán et al., 2009) (**Figure 1B**). Thus, according to the six part pallial model, the claustroinsular complex is not comparable to the mesopallium and is not part of the lateral pallium, but belongs to the DLP. The DLP also extends rostrally into the olfactory bulb (Desfilis et al., 2018) and caudally contains the lateral entorhinal cortex in mammals and comparable areas in sauropsids (Abellán et al., 2014; Medina et al., 2017b; Desfilis et al., 2018).

Caudal to VP, the six part pallial model proposes the existence of another pallial division, the ventrocaudal pallium (VCP), which is rich in expression of Lhx2, Lhx9, and Emx1 in the ventricular zone and mantle (Medina et al., 2017a; Desfilis et al., 2018). In chicken and lizard, this division is clearly distinguished from early stages and relates to a ventricular sector located caudally, which is easier to visualize in sagittal or horizontal sections (Abellán et al., 2009; Desfilis et al., 2018). In chicken, the VCP includes the arcopallium; while in the long-tailed lacertid lizard, it includes the dorsolateral amygdala and nucleus sphericus (Medina et al., 2017a; Desfilis et al., 2018). In chicken and lizards, the VCP is clearly separated from VP by a cell poor lamina, giving additional support for the distinction of VCP as a major division and not simply a subdivision of VP (discussed by Desfilis et al., 2018). Note that cell poor glial palisades as the one that separates VP and VCP are only observed in the boundary between major divisions, such as pallium versus subpallium, nidopallium versus mesopallium, mesopallium from DLP, and DLP from hyperpallium, but not between internal subdivisions within major regions as the nidopallium or mesopallium. In mouse, the VCP is also distinguished in the caudalmost pallium from early stages, characterized for its strong expression of Lhx2, Lhx9, and Emx1, and because it lacks ventricular zone expression of Dbx1 (Medina et al., 2017a). The VCP of mouse appears to give rise to the caudal pole of the pallial amygdala (Medina et al., 2017a).

As noted above, more studies are needed to evaluate the validity of this six part pallial model or morphoplan in amniotes and, if so, to investigate which of the six divisions are present in anamniotes. However, we believe that the six pallial divisions proposed in the model are truly independent entities based on their distinct embryonic genoarchitecture from early stages, unique topological position, separation by cell-poor laminae, and presence across different amniotes, including mammals, birds, and reptiles (Desfilis et al., 2018). Moreover, so far, we found that the use of this morphoplan can be highly useful for comparative purposes and will help to decipher the evolutionary origin of the pallial areas or regions found in different vertebrates. In the context of this article, it can help to investigate the presence in non-mammals of areas homologous to the pallial amygdala and the orbitofrontal cortex, which in mammals are an important part of the brain networks involved in social cognition.

THE PALLIAL AMYGDALA ACROSS VERTEBRATES

According to the six part pallial model, in mammals, the pallial amygdala derives from the VP and the VCP (Medina et al., 2017a). The VP gives rise to the nidopallium in birds and a large part of the DVR in reptiles, while the VCP produces the arcopallium in birds and the dorsolateral amygdala and nucleus sphericus in reptiles (Desfilis et al., 2018). The avian nidopallium and the reptilian DVR have often been compared to the neocortex or to specific cell subpopulations of the neocortex (Reiner, 1991; Butler, 1994a; Karten, 1997; Dugas-Ford et al., 2012; Briscoe and Ragsdale, 2018). These various proposals consider that, even though the sauropsidian DVR and the mammalian neocortex possess many non-homologous, divergent areas, they also contain a conserved set of neuron subtypes and connections, which are considered homologous (Karten, 1997; Dugas-Ford et al., 2012; Briscoe and Ragsdale, 2018). The problem with this is that the DVR and the neocortex originate in different embryonic compartments of the pallium, as noted above (see also discussions on this subject by Puelles, 2001; Aboitiz et al., 2003; Medina et al., 2013). However, a major problem is that the pallia of sauropsids and mammals have undergone greatly divergent routes, and the derivatives of the same (homologous) embryonic compartments of the pallium are highly dissimilar in sauropsids and mammals. This is in line with analysis of the transcriptome (including expression of more than 5,000 genes) of the different pallial regions, which do not support homology of adult derivatives of pallial compartments between sauropsids and mammals (with the only exception of the hippocampal formation, derived from the medial pallium) (Belgard et al., 2013). According to these data, regions that share the same embryonic origin exhibit no greater transcriptomic similarity than regions derived from different embryonic sectors, such as the chicken nidopallium and mouse neocortex (Belgard et al., 2013). However, when single-cell transcriptome (by mRNA sequencing) has been carried out, which allows selection of only glutamatergic neurons of pallial lineage (discarding interneurons of subpallial origin and glial cells), the results have been different and show unequivocal similarity between the turtle posterior DVR and the mouse pallial amygdala, with the only exception of the lateral nucleus of the amygdala, which correlates with the anterior DVR (Tosches et al., 2018). By contrast, these data did not support the cell-type homology between turtle DVR and mammalian neocortex (Tosches et al., 2018), although parts of neocortex and DVR show some spectacular cases of evolutionary convergence (such as that between the

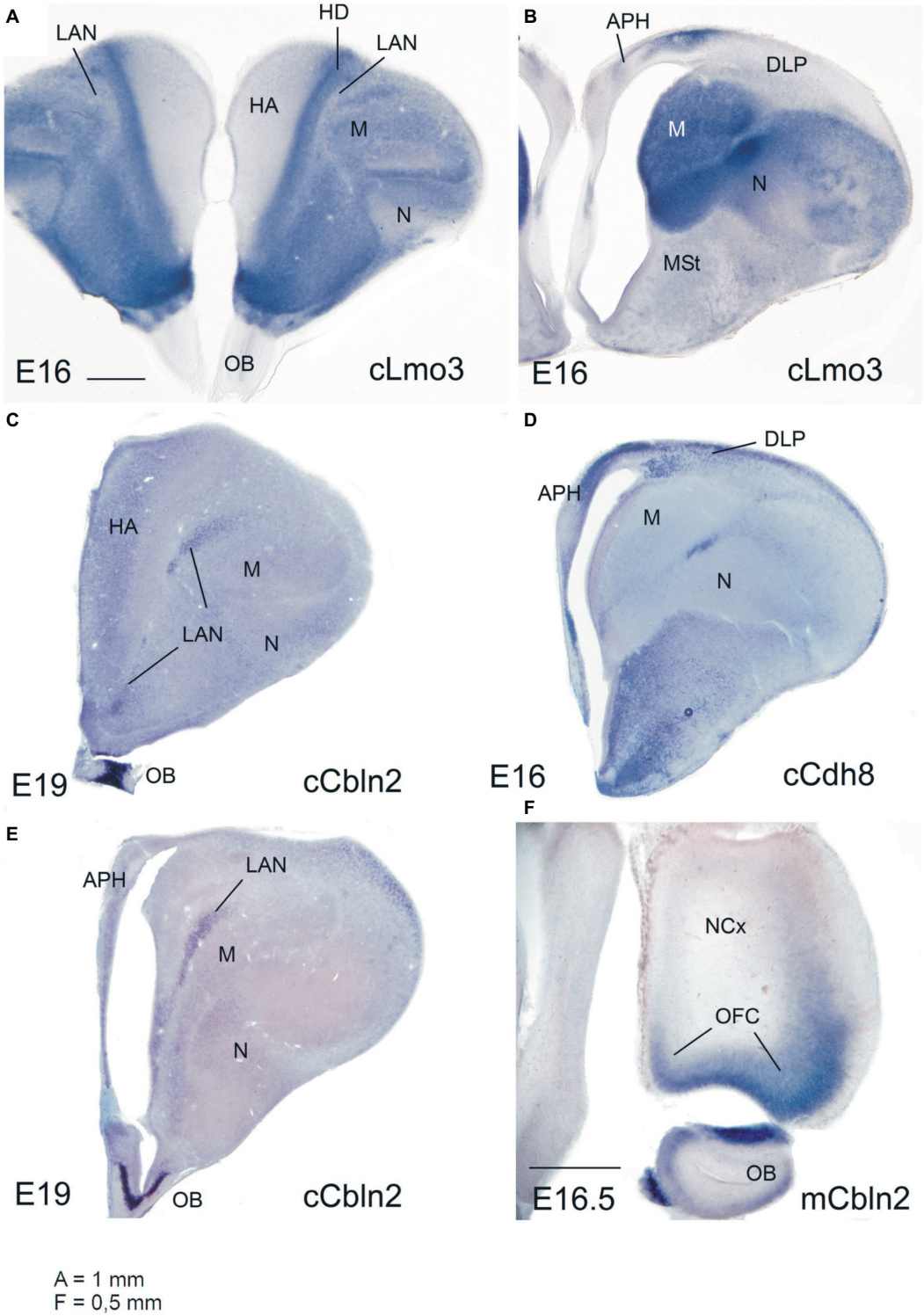

FIGURE 1 | Expression of the mRNA of selected regulatory genes in the embryonic telencephalon of chicken and mouse. **(A,B)** Lmo3 expression in chicken (E16): like in mammals, this is rich in the ventral pallium (nidopallium, N) and lateral pallium (mesopallium, M), in parts of the dorsal pallium (densocellular hyperpallium, HD) and parts of the medial pallium (parahippocampal area, APH). In contrast, it is extremely weak in the dorsolateral pallium (DLP), including its rostral extension (laminar pallial nucleus or LAN). **(C,E)** Cbln2 expression in the chicken (E19): note the distinct expression in the LAN, resembling that in the mouse orbitofrontal cortex (OFC). **(D)** Cdh8 expression in chicken (E16): note the expression in the DLP, resembling that in the mouse claustrum (which is also part of DLP). **(F)** Cbln2 expression in the OFC of E16.5 mouse (which belongs to the rostral pole of the mouse DLP). Other abbreviations: HA, apical hyperpallium; MSt, medial striatum; OB, olfactory bulb. Scale bar: A = 1 mm (applies to **A–E**). *F* = 0.5 mm.

avian caudal nidopallium and the mammalian prefrontal cortex; Güntürkün and Bugnyar, 2016; see discussion below).

Regarding the connections, both the avian/reptilian DVR and the mammalian pallial amygdala receive sensory input from comparable thalamic nuclei (Bruce and Butler, 1984; Butler, 1994a,b; Bruce and Neary, 1995; Guirado et al., 2000). Notably, these nuclei belong to the collothalamus (Butler, 1994b) and locate in the same thalamic compartment: the ventral tier for the auditory-related nuclei (comparable to the mammalian medial geniculate nucleus) and intermediate tier for the visual-related nuclei (including the avian/reptilian nucleus rotundus, which is comparable to the suprageniculate nucleus of mammals; note that the lateral posterior and pulvinar thalamic nuclei – sometimes compared to nucleus rotundus – are located in the dorsal thalamic tier, instead of the intermediate thalamic tier) (Guirado et al., 2000; Puelles, 2001; Puelles and Medina, 2002; Medina et al., 2011, 2017a). In mammals, the collothalamic sensory input targets the lateral and basomedial amygdalar nuclei, while in birds and reptiles, the same thalamic nuclei target segregated areas along the rostrocaudal dimension of the DVR (in birds, these include the entopallium for visual input and field L2 for auditory input; Bruce and Butler, 1984; Bruce and Neary, 1995; Guirado et al., 2000; discussed by Medina et al., 2017a).

In addition to the thalamic input, the pallial amygdala (in particular, the basal nuclear complex of the amygdala, Pessoa, 2008; Medina et al., 2017a) and the avian/reptilian DVR (particularly its posterior part, named posterior dorsal ventricular ridge in lizards, Lanuza et al., 1998; and caudolateral nidopallium in birds, Kröner and Güntürkün, 1999) are extensively connected with other pallial areas, including sensory association centers and are considered high integration centers (discussed by Medina et al., 2017a). Moreover, both project to subpallial areas that include the extended amygdala, which channels descending pathways to similar centers of the hypothalamus and brainstem, involved in the control of endocrine, autonomic, and motor systems (Vicario et al., 2014; Medina et al., 2017a). Notably, both the pallial amygdala (including the basal complex) and the posterior DVR are involved in stress and fear responses and social behavior (reviewed by Medina et al., 2017a). On the one hand, in both birds and mammals, the central extended amygdala areas receiving pallial amygdala-like input (including the arcopallium and parts of the caudal nidopallium and subnidopallium in birds; Atoji et al., 2006; Hanics et al., 2017) project to hypothalamic areas involved in acute and chronic stress responses (Phelps and LeDoux, 2005; Nagarajan et al., 2014; Vicario et al., 2014). Like in mammals, in birds, at least part of the central extended amygdala (lateral bed nucleus of the stria terminalis or BSTL) as well as the pallial amygdala-like areas of the DVR that project to BSTL also become active during stress and are involved in fear behavior (Saint-Dizier et al., 2009; Nagarajan et al., 2014). On the other hand, in birds and mammals, the medial extended amygdala and part of the pallial amygdala become active upon animal exposure to conspecific-related stimuli (olfactory cues in rodents, Choi et al., 2005; visual cues in chicks, Mayer et al., 2017) and are part of the social cognition network (Choi et al., 2005; Mayer et al., 2017).

In spite of these similarities, it is also clear that the pallial amygdala and the DVR also show many dissimilar features regarding cytoarchitecture, chemoarchitecture, connections, and functions (reviewed by Reiner et al., 2004 and Jarvis et al., 2005). These are due to divergent processes during evolution after separation from the last common ancestor. These differences deserve deeper evaluation and more investigation since they can help to understand not only mechanisms behind the evolutionary divergence, but also differences on how different animals process information to solve biologically-relevant problems (similar or different depending on their way of life and their specific ecological and/or social context). For example, rodents are mostly nocturnal animals (with exceptions) and depend highly on olfactory cues for feeding, detecting danger, and social communication, while birds have a relatively less developed olfactory system and rely more on visual and auditory cues (Abellán et al., 2013; Clayton and Emery, 2015). Thus, brain networks and areas dedicated to olfactory or visual processing may show a different degree of elaboration in these different animals. Similarly, brain networks involved in social cognition are expected to show a different degree of elaboration in social versus solitary species and depending on the size of the social group.

IS THERE AN ORBITOFRONTAL CORTEX IN NON-MAMMALS?

In mammals, the orbitofrontal cortex (OFC) is defined as the ventral and oldest part of the prefrontal neocortex (Kringelbach and Rolls, 2004). In human studies, it is often included as part of the medial prefrontal cortex. However, it is located at the frontier between the neocortex and allocortex and shows some peculiarities in cytoarchitecture (with agranular or dysgranular areas, as typical of transition areas) and connections that make it different from the rest of the prefrontal cortex (in primates, this is particularly so for its posterior or limbic part, Barbas, 2007). Regarding the connections, the posterior OFC of primates and comparable areas of rodents (including the infralimbic cortex, among other areas) are reciprocally connected with primary olfactory structures, including the anterior olfactory area and the piriform cortex (Barbas, 1993, 2007; Illig, 2005). The posterior OFC also projects to the main olfactory bulb (Illig, 2005), and there is a report of a very weak direct projection from the main olfactory bulb to a posteromedial part of OFC (the infralimbic cortex) in rats (Neafsey et al., 1986), although the latter has not been confirmed in other studies with rodents (for example, Hintiryan et al., 2012, for the mouse connectome project) or primates (Barbas, 1993; Carmichael et al., 1994). In addition, the posterior OFC is reciprocally connected with the agranular insular cortex (Illig, 2005) and with the basal complex of the pallial amygdala (Ghashghaei et al., 2007; Reppucci and Petrovich, 2016). In contrast, it only shows scarce connections with other parts of the prefrontal cortex (Illig, 2005; Barbas, 2007), such as the anterior and dorsal prefrontal areas showing eulaminar (granular) structure in primates, which display a typical neocortical lamination (Barbas, 2007). The posterior OFC and the basal

complex of the pallial amygdala (with which the OFC is reciprocally connected) receive input from visual and auditory association cortices (Barbas, 2007), and both show overlapping projections to the accumbens and adjacent parts of the striatal "emotion processing network" (Heilbronner et al., 2016). The OFC is thus an integration center that is involved in evaluation of the emotional significance of stimuli, in decision-making based on likely-reward, and in mediation of reward- and fear-guided behavior (Elliott et al., 2000). Together with the amygdala, it plays an important role in predicting the reward value of odors (Schoenbaum et al., 1999; Illig, 2005) and other sensory stimuli (Barbas, 2007). In addition, it participates with the amygdala in a network involved in social perception (Bickart et al., 2011, 2012; see also Adolphs, 2003).

The prefrontal cortex – derived from the dorsal pallium – is generally assumed to be an innovation of mammals, although an analogous area (i.e. functionally similar but not homologous) has been described in the caudolateral nidopallium of birds (Güntürkün, 2005). However, the dorsal pallium is quite small in reptiles (Desfilis et al., 2018; Tosches et al., 2018), making unlikely the existence of a homologue of the prefrontal cortex in non-mammals. Interestingly, the OFC was recently proposed to be part not of the neocortex (i.e., dorsal pallium), but of a newly defined pallial sector named DLP (Desfilis et al., 2018). In contrast to the dorsal pallium, the DLP is well developed in birds and reptiles (Abellán et al., 2014; Medina et al., 2017b; Desfilis et al., 2018). This proposal would be partially in line with that posed in the structural model by García-Cabezas et al. (2019), in which they suggest that agranular and dysgranular cortical areas such as those of the posterior orbitofrontal and other limbic cortices (like the agranular and dysgranular insular cortex) are phylogenetically more ancient than eulaminar neocortical areas. In our model, the agranular/dysgranular areas of OFC and insula would be part of DLP, while the eulaminar cortices would derive from the dorsal pallium. In agreement with the structural model, the DLP stands at the base of the dorsal pallium from rostral to caudal levels.

During embryonic development, the mouse DLP is enriched in expression of Nr4a2/Nurr1 and Cadherin 8 (mainly in relation to the claustrum; Medina et al., 2004; Watson and Puelles, 2017) and Cerebellin 2 (Cbln2, preferentially expressed in the insular cortex and OFC) (Allen Developing Mouse Brain Atlas; for OFC see also **Figure 1F**). The chicken DLP also shows expression of these genes (**Figures 1C–E**; Reiner et al., 2011; Puelles et al., 2016b), with Nr4a2/Nurr1 and Cbln2 partly segregated to different subdivisions. Notably, in the chicken DLP, expression of Cbln2 extends rostrally into a pallial subdomain intercalated between the hyperpallium and the mesopallium (**Figures 1C,E**; Reiner et al., 2011). This same subdivision is also poor in Lmo3 (**Figure 1A**) and was previously called "laminar pallial nucleus" (LAN) due to its relation to the lamina frontalis superior (Suárez et al., 2006). This subdivision is reciprocally connected with the olfactory bulb (Atoji and Wild, 2014), and we suggested that it may represent the avian OFC-homologue (Desfilis et al., 2018). In lizards, the rostral pole of DLP also appears to include an olfactory area (Desfilis et al., 2018) previously identified as part of the anterior olfactory area (Martínez-García et al., 1991). Thus, like in mammals, the OFC-like area of birds and lizards is reciprocally connected with olfactory structures. Moreover, at least in birds, the OFC-like area (the LAN, sometimes identified as the rostral pole of the densocellular hyperpallium) is also reciprocally connected with caudal DVR areas, such as the caudal nidopallium and arcopallium (Kröner and Güntürkün, 1999; Atoji et al., 2006), which are considered homologous to at least part of the pallial amygdala, as explained above (Bruce and Neary, 1995; Puelles, 2001; Martínez-García et al., 2002, 2007; Medina and Abellán, 2009; Medina et al., 2017a; Desfilis et al., 2018; Tosches et al., 2018).

BRAIN NETWORKS INVOLVED IN SOCIALITY IN MAMMALS: FACTS AND MODEL-BASED PREDICTIONS

As mentioned above, in humans and other mammals, there are at least two distinct brain networks involved in sociality, whose strength is positively correlated to the size and complexity of the social group: (1) a network related to social perception, involving the pallial amygdala, several neocortical areas (including visual and auditory association areas), as well as the orbitofrontal cortex, and (2) a network related to social affiliation, involving the medial amygdala, some neocortical areas (including parts of the anterior cingulate and prefrontal cortices), the ventromedial striatum (largely including the nucleus accumbens), and the ventromedial hypothalamus (Bickart et al., 2012; see also Adolphs, 2003). Some of these connections are represented in **Figure 2**. In addition, there is another network involving the central extended amygdala, which is related to fear and aversion, that participates in social aversion (Bickart et al., 2012; see also Adolphs, 2003; and Phelps and LeDoux, 2005).

Regarding the first network that involves ample pallio-pallial connections, the structural model of cortical organization proposed by García-Cabezas et al. (2019) predicts the laminar pattern and strength of the connections of different types of cortices. According to this model, cortical areas with the same lamination type display stronger connections between them than with cortical areas showing a different lamination type. The predictions are confirmed for the case of the OFC, since its agranular/dysgranular parts are reciprocally and strongly connected (with the connections originating and ending in all layers) with similar parts of the insular, perirhinal, and entorhinal cortices (Barbas, 2007; García-Cabezas et al., 2019). This would also agree with predictions of our evodevo-based model, which suggests preferential or stronger connections between areas or cells with the same developmental origin and molecular profile (Medina et al., 2017a). Notably, the agranular/dysgranular areas that are strongly and reciprocally connected locate along the rostrocaudal dimension of the same pallial division, the DLP, according to our model (Desfilis et al., 2018).

The structural model also predicts that connections between cortices of different lamination types would be weaker

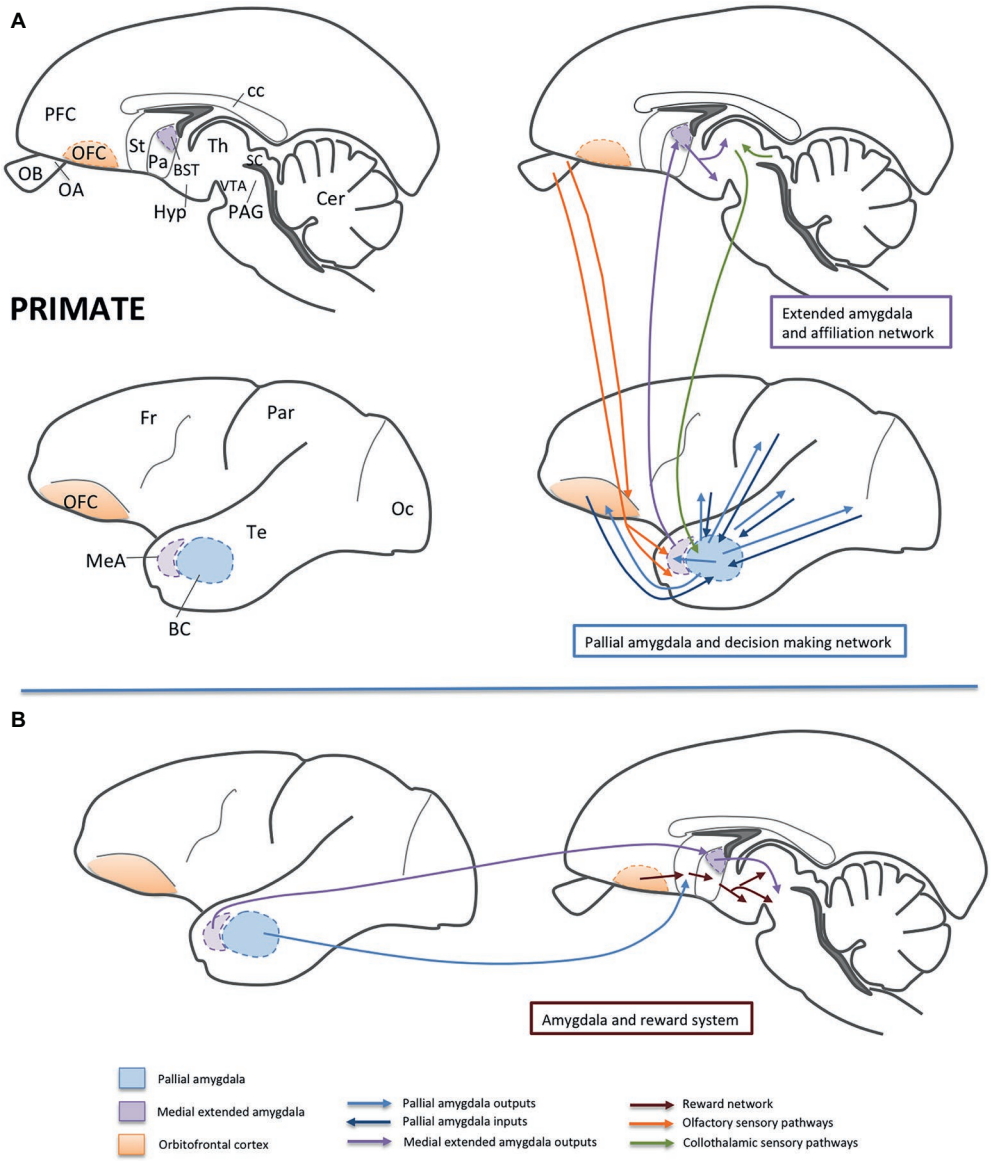

FIGURE 2 | Schemes of parasagittal sections of the primate brain (at different mediolateral levels), representing some of the areas and functional networks related to sociality. For simplicity, only part of the connections is shown. Panel **(A)** shows the networks related to affiliation (involving the medial extended amygdala; i.e., the medial amygdala and BSTM) and to decision-making (involving the basal amygdalar complex of the pallial amygdala and the orbitofrontal cortex). Olfactory and collothalamic sensory inputs are also shown in the schemes. Panel **(B)** shows the influence of the amygdala on the reward system, mainly by way of projections from the pallial amygdala to the ventral striatum and from the BSTM to the ventral tegmental area. Abbreviations: BC, basal amygdalar complex; BST, bed nucleus of the stria terminalis; cc, corpus callosum; Cer, cerebellum; Fr, frontal lobe of NCx; Hyp, hypothalamus; MeA, medial amygdala; NCx, neocortex; OA, anterior olfactory area; OB, olfactory bulb; Oc, occipital lobe of NCx; OFC, orbitofrontal cortex; Pa, pallidum; PAG, periaqueductal gray; Par, parietal lobe of NCx; PFC, prefrontal cortex; SC, superior colliculus; St, striatum; Te, temporal lobe of NCx; Th, thalamus; VTA, ventral tegmental area.

and involve only part of the layers, and this would apply for the connections between OFC with the visual and auditory association neocortical areas: the projections originate in deep layers of agranular/dysgranular OFC and terminate in superficial layers (specially layer I) of eulaminar cortices, and the projections from eulaminar cortices to agranular/dysgranular OFC originate in superficial layers and terminate in middle layers (García-Cabezas et al., 2019). The structural model does not extend predictions to the connections between cortical areas and the pallial amygdala, since the latter does not show a laminar organization. New experimental data in rats have shown that in the basolateral amygdala, different neurons project to either the prefrontal cortex (including the infralimbic cortex) or the lateral hypothalamus (Reppucci and Petrovich, 2016). Developmental studies in mouse showed that the basolateral amygdala includes neurons of at least two different lineages, Emx1 and non-Emx1 (the latter are the typical ventral pallial cells that belong to Dbx1 and/or Lhx9 lineages, as reviewed

by Medina et al., 2017a). Our evodevo-based model of six pallial divisions would predict that cells of the basolateral pallial amygdala that project to the prefrontal cortex would preferentially belong to Emx1 lineage (and would target a region rich in Emx1-lineage cells), a proposal that requires investigation. Thus, our evodevo-based model of six pallial divisions represents a more ample framework for understanding the pallium and for predicting connections between its subdivisions, which can extrapolate to the pallium of non-mammals to evaluate the degree of conservation or variation, offering new venues for a better comprehension of the organization and evolution of functional networks involving the pallium.

BRAIN NETWORKS INVOLVED IN SOCIALITY IN NON-MAMMALS

Are there networks in non-mammals similar to those of mammals involved in sociality? Although more neuroethological studies are needed, the answer is yes (**Figure 3**). However, there are differences in these networks between mammals and non-mammals. Regarding the network involving the pallial amygdala, while in mammals, the predominant reciprocal connections are with the neocortex (dorsal pallium) and OFC (noted above to be part of DLP), in sauropsids, the pallial areas primarily interconnected with the amygdala are not only neocortical-like (dorsal pallium; Wulst or hyperpallium in birds) and OFC-like (the LAN in rostral DLP), but also include parts of other pallial sectors such as the LP (mesopallium). However, in birds and, especially, in reptiles, most of the sensory association information (visual, somatosensory, and auditory) is processed and transmitted within the ventral pallium, from rostral/intermediate to caudal parts (Lanuza et al., 1998; Kröner and Güntürkün, 1999; Güntürkün, 2005). In particular, the anterior DVR of birds and reptiles include different nuclei that receive sensory input directly from the collothalamus or the brainstem (Butler, 1994a,b). The DVR thalamorecipient nuclei have been compared to the thalamorecipient lateral nucleus and/or the basomedial nucleus of the mammalian basal amygdalar complex (Bruce and Neary, 1995; Medina et al., 2017a; and Desfilis et al., 2018), a conclusion also partially supported by data from single cell transcriptome (which shows high similarity between glutamatergic cells of anterior DVR and lateral amygdalar nucleus; Tosches et al., 2018). These sensory nuclei of the DVR project to adjacent association areas, which in turn project to the caudal or posterior DVR (Lanuza et al., 1998; Kröner and Güntürkün, 1999). This is where the most important high integration centers are located in sauropsids: the caudal nidopallium and arcopallium in birds and the equivalent parts in the posterior DVR of reptiles (Lanuza et al., 1998; Kröner and Güntürkün, 1999; Güntürkün, 2005). Based on similarity of connections and important role in cognition (including decision-making and working memory), the caudolateral nidopallium of birds have often been compared to the prefrontal cortex of mammals (Kröner and Güntürkün, 1999; Güntürkün, 2005; Güntürkün and Bugnyar, 2016), but if we consider the topological location, embryonic origin and gene expression profile, together with some of the connections, it becomes clear that the avian caudal nidopallium (and posterior DVR of lizards) is part of the ventral pallium and at least partially homologous to the basal complex of the amygdala of mammals (Lanuza et al., 1998; Puelles, 2001; Medina et al., 2017a). However, the pallial amygdala is also part of the same functional network that, together with the prefrontal cortex, plays a critical role in cognition, including decision-making. Therefore, another way of viewing the avian caudolateral nidopallium (and the corresponding high integration area of the lizard posterior DVR; Lanuza et al., 1998) is as a distinct pallial amygdala-like caudal area that work together with a prefrontal-like rostral area as part of a functional network involved in cognition (as discussed by Medina et al., 2017a).

Nevertheless, the homology of DVR with basal complex of the amygdala is only partial, since the degree of enlargement and elaboration of the DVR is very high in extant reptiles, and especially in birds, and includes multiple subdivisions and complex connections between them. In addition, the basal complex of the amygdala is also very large in some mammals, such as primates, but this enlargement has been accompanied by a great expansion and elaboration of areas of the neocortex (dorsal pallium) with which the mammalian pallial amygdala is reciprocally connected. As a consequence, some of the connections that reach the basal complex of the amygdala in mammals and the caudal/posterior DVR in sauropsids likely evolved independently. This is particularly so for part of the auditory input, which directly reaches a specific area of the DVR in sauropsids (field L2 in the caudomedial nidopallium of birds) and the lateral/basomedial nuclei of the basal amygdalar complex of mammals, originating from a homologous medial geniculate-like nucleus of the collothalamus (reviewed by Medina et al., 2017a). In addition, in mammals, high order auditory information also reaches the basal complex of the amygdala from an association area of the temporal neocortex, but this second link is apparently missing in sauropsids. In birds, the auditory information is processed in secondary association areas of the nidopallium adjacent to field L2, and from here it is transmitted to the caudolateral nidopallium and arcopallium (in the posterior DVR), where it is integrated with information of other modalities coming from different pallial divisions (Kröner and Güntürkün, 1999).

As noted above, both the caudal nidopallium and the arcopallium (including the so-called posterior pallial amygdala according to Reiner et al., 2004; and Atoji et al., 2006) are reciprocally connected with a thin area intercalated between the hyperpallium and the mesopallium (Kröner and Güntürkün, 1999; this is included as part of the densocellular hyperpallium by Atoji et al., 2006), an area proposed by us to be comparable to the mammalian OFC (Desfilis et al., 2018). The mammalian OFC plays an important role in decision-making, including choices in social contexts (Campbell-Meiklejohn et al., 2012; Watson and Platt, 2012; Xia et al., 2015). In mammals, it appears that the brain makes simple choices by assigning value to the options under consideration, and the pallial amygdala-to-OFC projection is essential for this and more important

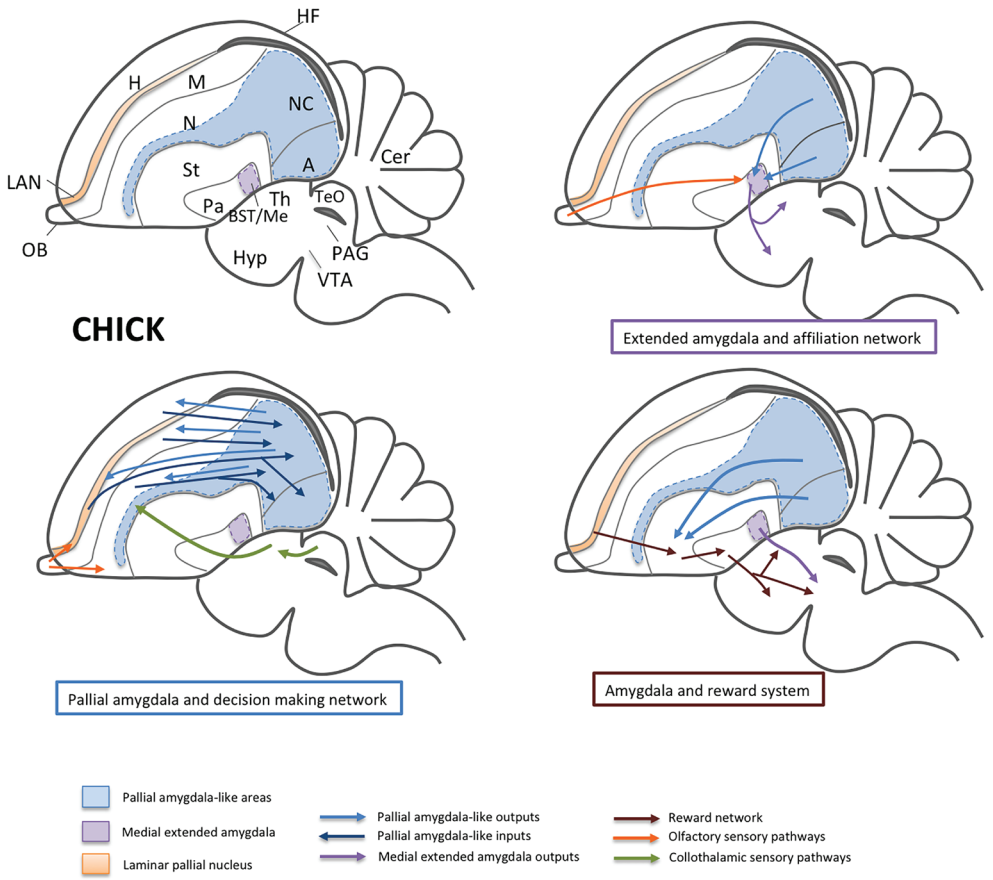

FIGURE 3 | Schemes of a lateral view of the chick brain, representing some of the areas and functional networks related to sociality. For simplicity, only part of the connections is shown. Separate schemes show the networks related to affiliation (involving the medial extended amygdala; i.e., the medial amygdala and BSTM) and to decision-making (involving the nidopallial and arcopallial areas comparable to the pallial amygdala, and the LAN, comparable to the orbitofrontal cortex). Olfactory and collothalamic sensory inputs are also shown in the schemes. In a separate scheme, the influence of the amygdala on the reward system is represented, mainly by way of projections from the pallial amygdala to the ventral striatum and from the BSTM to the ventral tegmental area. Abbreviations: A, arcopallium (ventrocaudal pallium); APH, parahippocampal area; BST, bed nucleus of the stria terminalis; Cer, cerebellum; DLP, dorsolateral pallium; H, hyperpallium (dorsal pallium); Hyp, hypothalamus; LAN, laminar pallial nucleus (rostral part of DLP comparable to the orbitofrontal cortex); M, mesopallium (lateral pallium); Me, medial amygdala; N, nidopallium (ventral pallium); NC, caudal nidopallium; OB, olfactory bulb; Pa, pallidum; PAG, periaqueductal gray; St, striatum; TeO, optic tectum of the midbrain (comparable to the superior colliculus); Th, thalamus; VTA, ventral tegmental area.

than the reciprocal OFC-to-amygdala projection (Jenison, 2014). In birds, neurons of the caudolateral nidopallium display value-related activity (Dykes et al., 2018). In addition, the caudal nidopallium plays a role in decision-making, and at least in crows is involved in complex tasks using flexible working memory (Güntürkün, 2005; Güntürkün and Bugnyar, 2016; Hartmann et al., 2018). Regarding social contexts, mate choice decisions are extremely important because they affect the genetic constitution of offspring and, in some species, the quality/quantity of parental care (Sockman, 2007). Songbird females make mate choices based on song features (Sockman, 2007). In European starlings, females prefer long songs, and these induce higher ZENK activation in the caudomedial mesopallium and in the auditory-related caudomedial nidopallium (Sockman, 2007). Moreover, enriched social contexts induce an increment in neurogenesis in the caudal nidopallium (Lipkind et al., 2002). However, it is unclear whether the projection from the caudal nidopallium to the OFC-like area plays a role in making choices in social contexts. Regarding other areas of the network, the arcopallium does not appear to be involved in food selection-related choices (Aoki et al., 2006b), but the lateral arcopallium has been shown to contribute to social facilitation of foraging efforts in domestic chicks (Xin et al., 2017). More studies are needed to evaluate the implication of the arcopallium in social choices.

The caudal nidopallium, subnidopallium, and arcopallium (and the equivalent areas in reptiles) project to the extended amygdala and striatum (Lanuza et al., 1998; Kröner and Güntürkün, 1999; Atoji et al., 2006; Hanics et al., 2017). In particular, the arcopallial descending projections target the medial striatum (comparable to the nucleus accumbens and related to reward) (Aoki et al., 2006a; Hanics et al., 2017). Like in mammals, the projection through the medial striatum may modulate reward, including social reward (O'Connell and

Hofmann, 2011; Frith and Frith, 2012; Dölen et al., 2013). In songbirds, nidopallium and arcopallium are part of sophisticated brain networks involved in song learning and production, which are fundamental for social interactions in these species (reviewed by Jarvis et al., 2013; Pfenning et al., 2014). Male vocalizations, both sexually motivated (to attract females) and "undirected," require the involvement of the reward system, although different patterns of dopamine activity and opioid release are involved (Riters, 2012; Riters et al., 2017).

The pallial amygdala descending projection through the extended amygdala relates to other aspects of social behaviors and to emotional responses (reviewed by Martínez-García et al., 2007; Medina et al., 2017a). In the extended amygdala of birds and reptiles, it is possible to distinguish between the central extended amygdala and the medial extended amygdala (Abellán and Medina, 2009; Vicario et al., 2014, 2015, 2017; Medina et al., 2017a). Like in mammals, the central extended amygdala (including the lateral bed nucleus of the stria terminalis or BSTL) has been associated to fear responses, stress, and aversion (Saint-Dizier et al., 2009; Nagarajan et al., 2014), while the medial extended amygdala has been related to different aspects of social behavior, including affiliation, sexual, and agonistic behaviors (reviewed by Martínez-García et al., 2007; Medina et al., 2017a). Notably, in chicken, the arcopallium includes areas projecting to the medial extended amygdala, which become active following visual exposition of naive animals to conspecifics (Mayer et al., 2017). In agreement with this observation, the arcopallium contains many cells that respond to certain color and shape stimuli (Scarf et al., 2016). The visual information seems to reach the arcopallium indirectly by way of the collothalamic pathway; the information first reaches the entopallium and is then transmitted to the arcopallium by intratelencephalic projections (as discussed by Mayer et al., 2017). The arcopallium also projects to the tectum, closing a tecto-tectal loop, which like in humans may be involved in early social orienting responses (Mayer et al., 2017).

Like in mammals, in birds and reptiles the medial extended amygdala (including the medial bed nucleus of the stria terminalis or BSTM) controls social behavior by way of projections to the preoptic area and hypothalamus (reviewed by Martínez-García et al., 2007; Medina et al., 2017a). In mammals, this pathway is part of the network involved in affiliation (Bickart et al., 2012), which is modulated by vasopressin, oxytocin, and their receptors (Hammock and Young, 2006; Donaldson and Young, 2008; Goodson, 2013). The affiliative behavior is mediated by the central projections of vasopressin and oxytocin containing cells, located mainly in the supraoptic (SO) and paraventricular (PVN) hypothalamic nuclei, as well as in the medial extended amygdala (primarily the BSTM) (De Vries and Miller, 1998). These cells project to the amygdala (including the basolateral complex, central and medial nuclei), but have also projections to brain areas of the reward network (including the nucleus accumbens, the ventral tegmental area, and the prefrontal cortex), the hippocampal formation (related to memory formation), the septum, and the anterior olfactory area (Walum and Young, 2018). Vasopressin (vasotocin, VT) and/or oxytocin (OT) receptors are found in all these areas, but there are significant variations of their expression in species exhibiting differences in pair-bonding, such as the monogamous prairie voles versus the non-monogamous meadow and montane voles (Hammock and Young, 2006; Donaldson and Young, 2008). The nucleus accumbens, the ventral pallidum, the prefrontal cortex, and the amygdala are among the areas showing higher receptor density in monogamous compared to non-monogamous voles (Insel et al., 1994; Young and Wang, 2004). Intracerebroventricular infusion of OT facilitates partner preference formation in both sexes, and this seems to be mediated through the reward system (Walum and Young, 2018). It appears that social interactions facilitate dopamine release from axon terminals in the nucleus accumbens and prefrontal cortex (the dopaminergic input comes from the ventral tegmental area), as well as OT release in multiple areas including those of the reward system (reviewed by Walum and Young, 2018). Blocking OT receptors in the nucleus accumbens or the prefrontal cortex (both part of the reward network) prevents the formation of mating-induced partner preference (Walum and Young, 2018). However, striking individual variations are also found within monogamous prairie voles regarding the density of oxytocin receptors in nucleus accumbens (Walum and Young, 2018). It appears that a high density of oxytocin receptors in this nucleus confers resilience to neglect in a paradigm involving neonatal social isolation and relates to the ability to form partner preferences later in life (Walum and Young, 2018). Regarding vasopressin, it plays a role in social recognition, territorial scent marking, and aggressive behaviors (Walum and Young, 2018). In prairie vole males, it facilitates pair-bonding, which is mediated by VT1a receptors. These show higher density in the ventral pallidum of the monogamous species compared to the non-monogamous ones, and blocking VT1a receptors in the ventral pallidum of the monogamous prairie voles inhibits formation of partner preference (Walum and Young, 2018). VT1a signaling in the lateral septum is also important for some aspects of pair-bonding and is possibly related to social recognition. However, VT1a receptors show higher expression in the septum of non-monogamous species of voles than in the monogamous one (Walum and Young, 2018).

While only 9% of mammals are socially monogamous, most birds (90%) are monogamous (although this does not imply sexual exclusivity, for example Dolan et al., 2007; Lukas and Clutton-Brock, 2013). In birds, the SO, PVN, and BSTM also include populations of vasopressin (vasotocin) and oxytocin (mesotocin) cells (Aste et al., 1998; Panzica et al., 1999; De Vries and Panzica, 2006; Vicario et al., 2017). Like in rodents, in songbirds, there are striking interspecific and intraspecific variations in the expression of vasopressin and oxytocin receptors (Leung et al., 2011). In zebra finch, different VT-like and OT-like receptors are found in parts of the nidopallium and arcopallium, including auditory and vocal control related areas (Leung et al., 2011). In addition, in zebra finch and white-throated sparrow, VT-like receptors (including VT1a-like or VT4) are found in the septum, parts of the reward system (medial striatum, ventral pallidum, ventral tegmental area), and part of the medial extended amygdala (BSTM) (Leung et al., 2011). Moreover, OT-like receptors (VT3) are found in

the hippocampus in sparrows, although not in that of finches (Leung et al., 2011). Activation of oxytocin and vasopressin 1a like receptors increases partner preference and/or gregariousness in zebra finches (Goodson, 2013). Vasopressin also increases aggressive competition for mates in finches (Goodson, 2013). It is likely that the action of these peptides is mediated in part through the reward system, although it may involve different receptors (through cross-binding of oxytocin and vasopressin to several of them). In songbirds, many pallial areas of the anterior and posterior circuits controlling song learning and production express VT and OT receptors, and it is likely that these peptides modulate social interactions through these neural systems as well.

In conclusion, birds and reptiles share with mammals some networks of those described in mammals for social perception, salience, and decision-making (the network including the pallial amygdala) and for social affiliation and recognition (the network including the medial amygdala). At least in birds, these networks play roles resembling some of those described in mammals, such as decision-making (including social contexts) and affiliation. However, while some of the connections of the networks likely derived from those present in the common ancestor, others appear to have evolved independently. More studies are needed to better understand the role of these networks in social cognition and behavior in birds (and reptiles), as well as the similarities and differences of distinct subcomponents with those found in different mammals. One interesting similarity between birds and primates relates to the fact that the networks in birds preferentially involve visual and auditory cues, and in songbirds include different areas of the auditory and song control systems. Finally, it is important to remark that in mammals, birds, and reptiles, there is an important interaction between the different networks by way of cross projections between areas of both systems (for example, see O'Connell and Hofmann, 2011). Future functional studies need to address the consequences of the anatomical cross talks observed between these networks.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to the research and ideas that led to preparation of this article. LM prepared the first version of the manuscript. ED and AA discussed and revised it. AA prepared the *in situ* hybridization material shown in **Figure 1**. LM and ED prepared the schemes shown in **Figures 2, 3**.

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Valproate Exposure *in ovo* Attenuates the Acquisition of Social Preferences of Young Post-hatch Domestic Chicks

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Embryonic exposure to valproic acid (VPA) is known to produce sociability deficits, resembling human autistic phenotypes, in several vertebrate species. Animals living in groups prefer the proximity of peers and have the ability to perceive and to respond to social signals for modifying behavior. Chicks of Galliform birds, known to display early preference behaviors, have been used extensively for adaptive learning studies. Young precocial birds seem to be useful models also for studying the effect of embryonic VPA treatment. Here, domestic chicken eggs were injected with sodium valproate (200 µl of 35 µmol/L solution) or with vehicle (distilled water) on the 14th day of incubation. After hatching, the chicks were tested for one-trial passive avoidance learning at day 1, vocalization due to isolation as a measure of stress level (day 2), approach preference to large versus small groups of age-matched conspecifics (day 5), and to those with normal versus blurred head features (day 7). In addition, we tested the preference of birds to conspecifics reared in group versus those reared in isolation (day 9), as well as the preference of chicks to familiar versus non-familiar conspecifics (day 21). Our findings confirm previous reports concerning an adverse effect of VPA on embryonic development, including a tendency for aborted or delayed hatching and, occasionally, for locomotor disorders in a small percentage of birds (eliminated from later studies). Otherwise, VPA treatment did not impair motor activity or distress level. Memory formation for the aversive stimulus and discrimination of colors were not impaired by VPA treatment either. Innate social predispositions manifested in approach preferences for the larger target group or for the birds with natural facial features remained unaffected by VPA exposure. The most prominent finding was attenuation of social exploration in VPA-exposed birds (expressed as the frequency of positional switches between two stimulus chicks after the first choice), followed by a deficit in the recognition of familiar conspecifics, unfolding at the end of the third week. Social exploration and recognition of familiar individuals are the key elements impaired at this stage. The results underline the importance of early social exploration in ASD.

Keywords: autism spectrum disorder, avian, social cohesion, embryonic development, developmental disorder, social brain network

INTRODUCTION

Autism spectrum disorder (ASD) is currently envisaged as a neurodevelopmental disorder associated with altered social behavior. In addition to a multitude of human studies, it was necessary to find reliable animal models for investigation into various aspects of ASD. One such model for laboratory rodents is prenatal exposure to valproic acid (VPA), a known antiepileptic substance and mood stabilizing agent (Rodier et al., 1996; Schneider and Przewlocki, 2005; Wagner et al., 2006; for recent reviews, see Roullet et al., 2013; Nicolini and Fahnestock, 2018). Social impairment accompanies all forms of ASD. *In utero* VPA causes sociability deficits postnatally in the adult animals (Roullet et al., 2010; Kim et al., 2011; Moldrich et al., 2013), and VPA-treated animals are also known to exhibit anxiety, depression-like behavior, and abnormal nociception thresholds (Wu et al., 2017). Embryonic exposure to VPA was found to produce an autistic-like phenotype even in fish (Baronio et al., 2017; Chen et al., 2018). The mechanism of action of VPA is rather complex but the key factor eliciting developmental and behavioral alterations seems to be that VPA is an inhibitor of histone deacetylase (Moldrich et al., 2013), potentially affecting gene expression and transcription engaging the Wnt1 signaling pathway, a robust "caudalizing" factor in rostrocaudal patterning of the developing CNS (Wiltse, 2005; Jang and Jeong, 2018).

Social behavior has been a widely studied phenomenon in life sciences. Most of the studies investigating the neurobiological bases of sociability exploit the fact that animals need to form bonds, at least temporarily, in order to reproduce. These bonds may represent strong contacts between sexual partners during or even beyond the time of mating, or between parents and offspring. The basis for such bonding may be hardwired innate preferences and affections and/or specific early learning mechanisms such as filial (Lorenz, 1937; Cherkin, 1969; Di Giorgio et al., 2016) and sexual imprinting (Irwin and Price, 1999). It is well known that many animals living in groups are often linked stronger to group members than to other conspecifics, they can even form stronger coalitions (often based on genetic relatedness) within such groups (Henzi and Barrett, 1999; Bergman, 2010). Effective cooperation within a group requires the preference for proximity of group members, suppression of aggression toward conspecifics, ability to perceive and respond to social signals and to change (often synchronize) behavior accordingly (Anacker and Beery, 2013). Group cohesion and evaluation of socially relevant information have been implicated as essential factors in various animal models of autism (Seebacher and Krause, 2017).

Earlier studies on pair bonding and, less extensively, on parent-offspring relations, have identified a network of brain regions associated with social behavior (Goodson, 2005). Present in a wide variety of vertebrate animals, this network seems to regulate most of the social interactions in these relations (O'Connell and Hofmann, 2011). Less investigated, the neural correlates of group forming (Goodson et al., 2009; Goodson and Kingsbury, 2011), group cohesion, and responsiveness to group signals in more general terms (not only in those of reproductive significance) might well engage the same brain regions and follow the same organizational principles (Wilson et al., 2016). In the present study, we focus our attention on the latter category.

The choice of the domestic chick as a model species for the current investigation was based on the assumption that certain key phenomenological elements of sociability, essential for survival, are associated with distinct neural systems that are common to vertebrate animals. Based upon previous findings in mammals, we chose to investigate another, phylogenetically distant species, the domestic chicken. Newly hatched chicks of Galliform birds are known to display various types of preference behavior, e.g., genetically determined preference for color of Japanese quail (Kovach, 1980; Csillag et al., 1995), or predispositions toward social stimuli of domestic chicks (Zachar et al., 2008, 2016; Di Giorgio et al., 2016). A precocial nidifugous species with a remarkably mature brain structure at hatching, the domestic chicken has long been used for investigation of early adaptive learning (imprinting or passive avoidance training, for an overview, see Rose, 2000), and it also appears to be a useful model for studying the effect of embryonic VPA treatment on acquired and innate behaviors. Traditionally, domestic chicken eggs have long been used for developmental studies and *in ovo* manipulations, and this propensity could be exploited here by injecting the eggs with VPA or vehicle at the critical time of development.

VPA has been examined in birds as a teratogenic agent (Whitsel et al., 2002; Tureci et al., 2011; Hsieh et al., 2013; Akhtar et al., 2015). Concerning ASD, administration of VPA into the egg caused an impairment of social behavior (but not of imprinting) in chicks (Nishigori et al., 2013). In a recent study (Sgadò et al., 2018) on visually naïve young post-hatch chicks (1–3 days of age), VPA treatment *in ovo* was found to attenuate the manifestation of the innate predisposition for an inanimate chicken (stuffed hen) over an object with scrambled features of chicken. This effect on (socially related) visual predisposition was not accompanied by a similar impairment of filial imprinting, in agreement with the Nishigori study (Nishigori et al., 2013). Recently, Lorenzi et al. (2019) showed that innate preference for an object autonomously changing speed over one moving with a constant speed was affected in chicks by embryonic VPA treatment. While it is increasingly evident that domestic chicks might offer a useful and inexpensive alternative model for studies of autism-related social deficits, the aptitude of the model could be further corroborated by a refined analysis of the affected elements behaviors, also extended to a later period of postembryonic maturation. In the previous studies little attempt has been made to test whether the impairment of the social behavior is a part of a more general attenuation of locomotor activity or cognitive deficit. Also, it was described that VPA negatively influences the group-forming and group-cohesion of young chicks (Nishigori et al., 2013), however, it is unknown whether the recognition of the siblings as social stimuli was attenuated, or else social exploration and social learning as such were impaired. In the present study, we tested the role of innate preferences, as well as the learned component of social recognition in chicks treated *in ovo* with VPA from hatching to 3 weeks of age. Moreover, we intended

to exclude the role of aspecific effects of VPA by using behavioral tests for early learning and non-social stimulation, such as the presence of a predator, to corroborate the validity of the VPA model in chicks. By so doing, we are focusing primarily on the early development of social recognition and the dichotomy between innate and acquired preferences.

MATERIALS AND METHODS

Animals

Eggs of Hunnia Broiler hybrid chicks were purchased from a local distributor (Bábolna Bio kft.) and placed into an incubator kept at 37.8°C with 55% relative humidity, reduced to 37.5°C after the first week and, finally, to 37.3°C 3 days before hatching (during this last period the relative humidity was 70%). The hatching temperature was continuously monitored by a cell phone based remote surveillance application. Eggs containing live embryos were selected by candling on days 7 and 13, discarding the eggs that showed no signs of development. On the 14th day of the incubation, 200 µl of 35 µmol/L sodium valproate (dissolved in sterile, pyrogen-free distilled water) was injected into the air sac of 32 eggs. The rest of the eggs (42) were injected with 200 µl of the above vehicle only, and served as controls. Injections were performed similarly to the methods described in previous studies (Nishigori et al., 2013; Sgadò et al., 2018). Briefly, the eggs were placed on a supporting cup, with the air sac (previously located and marked) facing upwards. The egg shell was wiped with a disinfectant solution, allowed to dry and perforated with a sterile hypodermic needle. Then, the solutions were injected manually, using a small gauge hypodermic needle and syringe, slowly enough to avoid backflow and steadily enough not to penetrate the inner shell membrane. After retraction of the hypodermic, the burr hole was sealed with candle wax. All animals were kept and treated according to the regulations of the ethical committee of the Semmelweis University, and all experiments were approved by the Ethical Committee on Animal Experimentation, and permitted by the Food Chain Safety and Animal Health Directorate of the Government Office for Pest County (Permit Number: XIV-I-001/2269-4/2012). Procedures were in harmony with the EU Council directives on laboratory animals (86/609/EEC).

Behavioral Tests

We performed six different behavioral tests on 26 of the control and 18 of the VPA treated chicks. The rest of the controls were used as stimulus chicks in the choice between isolation-reared and group-reared individuals. Of these, nine were kept isolated and 6 as a group. Ambient temperature in the experimental room was 30°C.

Taste Aversion Learning and Positive Reinforcement (Day 1)

Chicks remained in the dark incubator after hatching for cca. 24 h without access to food or water. Afterwards, they were put in 25 cm × 25 cm boxes with one wall made of a mirror to reduce isolation stress. After 45 min of habituation a colored bead glued to the end of a 3 mm diameter rigid plastic tube was introduced to the chicks from above by the experimenter for 20 s. The tube was connected to a plastic syringe filled with tap water. When the chicks pecked at the bead, water droplets were administered through the bead as a reward. The presentation of the water reinforced bead was repeated two more times with 5-min intertrial intervals. For the fourth trial a bead with a different color was introduced, which had previously been dipped into methyl anthranilate (MeA), a bitter tasting substance, as negative reinforcement. All chicks that pecked on the MeA-covered bead showed a clear disgust response (beak opening, retreating, head shaking). Chicks that did not peck on the water beads at least two times out of the three trials, or failed to peck at the MeA bead, were excluded from the analysis. The color of the two types of stimuli alternated among individuals to avoid any effect of innate color preference (Zachar et al., 2008). Four hours later, the chicks were tested on dry beads of the same colors: MeA bead first, then the water bead with a 5-min intertrial interval. Number of pecks were recorded at every trial.

From day 2 onwards, the chicks were kept in their home cages heated by infrared light bulbs, in groups of 6 animals under a 12/12-h dark/light cycle with water and food available *ad libitum*.

Isolation Test (Day 2)

The chick was placed into a 42 cm × 34 cm × 44 cm cardboard box for five and a half minutes. The box then was covered with a Samsung Syncmaster 710v LCD monitor which also served as illumination. The behavior and the vocalization of the animal were recorded by a digital video camera with a wide angle lens through a small hole at one upper corner of the box. Through a 20 cm × 25 cm window on one side of the box another LCD monitor was visible. 150 s after the start of the session a 6 cm wide silhouette of a flying common buzzard (*Buteo buteo*) appeared on the overhead screen moving across for 4 s. After another 120 s a real size video recording of 5 age matched chicks was started to be played at the side monitor for 60 s. The experimental video recordings were analyzed by using Solomon coder (András Péter, http://solomoncoder.com). The recorded variables were: the latency of the first move and first distress call, the time spent moving, number and time of escape jumps and defecations. The precise time, number and amplitude of vocalizations were identified by a detailed analysis of the sound files extracted from videos (sampling frequency: 48,000 samples/s, quality: 16 bits).

For the sound analysis, we used the Seewave package (Sueur et al., 2008) in R (R Core Team, 2018). First, the spectrogram was generated (FFT length: 512, overlap: 50%) and all chicken calls were searched in the recordings. We characterized the calls by the amplitude of the strongest frequency based on the generated spectrogram, and the time position at that point. Only calls more intense than −45 dB were taken into account, where 0 dB corresponds to the amplitude of a 1 kHz reference signal (generated with maximum amplitude). The −45 dB threshold was chosen because the sound pressure of the noise

generated by the movement of the chickens was less than this value. We managed to automatize the process using the R script. All automatic measurements were checked by visual inspection to exclude the potential errors of the automatic process. The −45 dB threshold served as a minimum reference level, and, in the following calculations, the intensity of calls were compared to this level resulting in sound pressure levels potentially ranging between 0 and 45 dB. The amplitude of vocalizations had been multiplied by the number of vocalizations in 10-s periods to obtain a variable characteristic to both the number and the loudness of the vocalizations in every 10 s of the experiment.

Social Preference Tests

Larger Versus Smaller Groups (Day 5)

On post-hatch day 5, chicks were placed into a 90 cm × 40 cm runway apparatus (**Figure 1A**) facing one of the longer walls. Video recordings of three and eight chicks were played on Samsung Syncmaster 710v monitors at the opposite ends of the runway. The sides of the small group and large group video were alternating between subsequent individuals to control for any side preference. The runway was divided into three equal compartments (large group compartment, central compartment, and small group compartment). The test was recorded by an overhead camera and the videos were analyzed using the Solomon coder. Time spent in each part, as well as the first choice (touching one of the monitors), the latency to reach one end of the runway and the switches between the two stimulus chicks after the first choice were recorded. The test lasted for 4 min. Chicks that did not leave the central compartment during the test were excluded from the analysis.

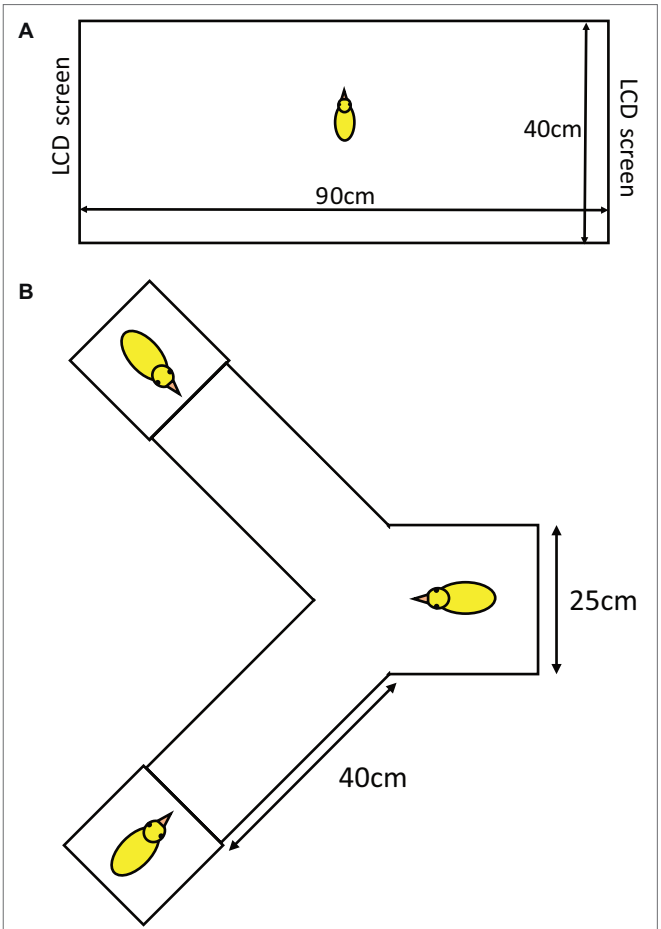

FIGURE 1 | Experimental setup for the runway tests **(A)** and the Y-maze test **(B)**.

Normal Versus Distorted Video Stimuli (Day 7)

On post-hatch day 7, chicks were placed into the same runway apparatus (**Figure 1A**) that had been used on day 5, facing one of the longer walls. Real size motion picture images of five chicks were played on monitors fixed at opposite ends of the runway. The two recordings were exactly the same except that, on one side, the facial characters (eyes and beak) were blurred on all chicks (using a digital video editing software). The sides on which the blurred and normal movies were played alternated between subsequent individuals to control for any side preference. For technical reasons, no video recordings were available for this test. The first choice of the chicks (touching one of the monitors) was recorded by the experimenter. The test lasted for 4 min. Chicks that did not leave the central compartment during the test were excluded from the analysis.

Choice Between Isolation-Reared and Group-Reared Individuals (Day 9)

On post-hatch day 9, the chicks were placed into a Y-maze (**Figure 1B**) to choose between two 9-day-old unfamiliar chicks. These were placed into 25 cm × 25 cm goal boxes at the end of the two arms of the maze. The boxes were separated from the maze by transparent glass walls. One of the stimulus chicks had been kept alone, isolated from the other conspecifics since hatching, meanwhile the other chick was kept in a group of six before the experiments. The experimental chick was placed at the starting box which lay 40 cm apart from each goal box. The test was recorded by an overhead camera and the videos were analyzed using the Solomon coder. Latency of first touch of the glass wall, the first choice, and the switches between the two stimulus chicks after the first choice were recorded. The test lasted for 4 min. Chicks that did not leave the central compartment during the test were excluded from the analysis.

Choice Between Familiar and Unfamiliar Individuals (Day 21)

On post-hatch day 21, the chicks were placed into a 45 cm × 160 cm runway facing one of the longer walls. In a goal box at one end of the runway, three familiar individuals (chicks that had been kept in the same home cage with the experimental chick from post-hatch day 2) were placed. In the goal box at the other end of the runway 3, unfamiliar individuals had been placed behind a wire mesh. The experimental chicks

were allowed to spend 4 min in the runway. The target animals were swapped around between the two goal boxes in subsequent tests to control for any lateral preferences, and to keep the birds more alert. VPA-treated subject chicks were always tested with VPA-treated target (reference) chicks, while control subjects with control targets in both goal boxes; therefore, the only difference between the two sides was the factor of familiarity. After being used as target chicks, the animals were returned to their home cage for at least 90 min before they were used again as subjects for testing.

Statistical Analysis

The sample size of each experimental group varied among the different experiments, since chicks that were passive in the preference tests or in the taste aversion learning test were omitted from the analysis. There was no significant difference between the two groups (VPA-treated and control) in the number of passive individuals in any of the tests, and there were no consistently passive individuals across the tests. The two experimental groups were compared using Student's *t*-tests, when the variances were equal and Welch's *t*-test when they were not. The effect of predatory stimulation on the distress call intensity in the open field test was assessed by repeated measures ANOVA with the treatment as independent factor. In the social preference tests, χ^2 tests of homogeneity were used, for establishing whether the number of individuals choosing between the targets was different from random choice.

RESULTS

General Observations

Success rate of hatching was 41/42 eggs in the control group, while the VPA-treated eggs hatched at a poorer rate of 23/32. Five out of the 23 VPA-treated chicks showed visible motor disorders (also noted by Nishigori et al., 2013), including serious muscle tone impairment: some of these chicks held their legs in an unnatural position, and were not able to move properly. In all impaired chicks, muscle weakness also appeared: some chicks were able to stand only for a few seconds, then they always sat down. All of the five chicks affected by motor deficits were excluded from the experiments. There was no apparent difference between the remaining VPA chicks and control chicks in their motor behavior; all birds showed intact righting reflex: they stood up within 2 s after being laid flat on their backs.

Taste Aversion Learning and Positive Reinforcement (Day 1)

VPA-treated chicks pecked more on water-reinforced beads than control chicks (Student's $t > 2.59$, d.f. = 26, $p < 0.05$, **Figure 2**) during the first three training trials. Both groups stopped pecking at the MeA-covered bead after tasting the bitter substance (**Figure 2**). Chicks that failed to peck in at least two out of the three positive training trials, or in the MeA trial, were excluded from further analysis. When tested 4 h later, both groups avoided the dry bead with the same color as the MeA-covered bead and readily pecked at the other dry bead with the color of the water-reinforced stimulus (**Figure 2**). However, control chicks tended to peck more frequently on the positively reinforced bead than did the VPA chicks ($t = 2.25$, d.f. = 17, $p = 0.038$, **Figure 2**). Both VPA and control chicks were able to discriminate between the positively and negatively reinforced stimuli after 4 h: every chick pecked more on the water-reinforced bead than on the MeA-reinforced one (VPA: $t = 5.7$, d.f. = 9, $p < 0.001$; control: $t = 7.1$, d.f. = 8, $p < 0.001$, **Figure 2**).

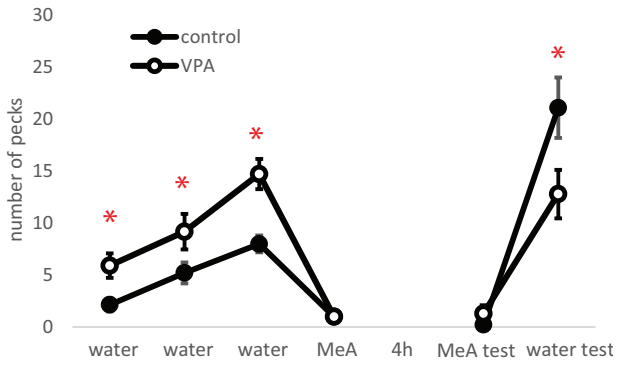

FIGURE 2 | Number of pecks on target beads in taste aversion learning (mean ± s.e.m.). Asterisks denote significant differences between the VPA and control groups.

Isolation Test (Day 2)

There was no difference between the VPA and control chicks in the motor activity (time spent moving: Student's $t < 0.46$, d.f. = 36, $p > 0.648$) or in the latency of first move either after the start of the isolation (Student's $t = 0.86$, d.f. = 36, $p = 0.395$) or after the onset of the predatory stimulus (Student's $t = 0.33$, d.f. = 36, $p = 0.74$). Both groups showed an almost equal amount of escape behavior (jumping: Student's $t = 0.4$, d.f. = 36, $p = 0.675$) and defecation (Student's $t = 1.71$, d.f. = 36, $p = 0.095$) during the isolation, suggesting a similar distress level.

VPA chicks tended to emit fewer and/or less intense vocal calls than control chicks, however, these differences were significant only in two 10-s periods (T5, T22, **Figure 3A**) over the whole duration of the isolation experiment (Student's $t > 2.3$, d.f. = 36, $p < 0.05$). The p here is statistically uncorrected for the large number of comparisons. Both VPA and control chicks reacted to the predator with a reduced vocalization in the subsequent 10 s (repeated measure ANOVA: $F = 8.77$, d.f. = 36, $p = 0.005$, **Figure 3**) and their reaction did not differ ($F = 0.46$, d.f. = 36; $p = 0.832$). However, if we selectively calculated the frequency of high-intensity (20 dB<) vocalization activity (representing distress calls) within the two stages of the experiment (before and after the presentation of predator), there was significant difference between the two groups in the latter stage ($t = 1.72$, d.f. = 35, $p = 0.094$; $t = 2.20$, d.f. = 35, $p = 0.037$, respectively, **Figure 3B**).

Social Preference Tests

Larger Versus Smaller Groups (Day 5)

The VPA treatment did not affect the preference toward a larger group of conspecifics over a smaller one as measured when the chicks were 5 days old. Approximately 80% of both VPA and control chicks chose the larger group as a first choice and the two groups did not differ in their choice (χ^2 = 0.18, n = 18, p = 0.671, **Figure 4A**). The latency of the first choice also did not differ between the two groups (t = 0.78, d.f. = 16, p = 0.939, **Figure 4B**), suggesting similar levels of social motivation in the two groups. VPA chicks spent more time at the proximity of the larger group than of the smaller group (paired t-test: t = 4.76, d.f. = 11, p < 0.001, **Figure 4C**), whereas, in the controls, the difference between the time spent with the larger and smaller groups failed to reach statistical significance (paired t-test: t = 1.24, d.f. = 10, p = 0.243, **Figure 4C**). Notably, the latter is likely due to a tendency for control chicks to explore both ends of the runway more intensely through frequent switches of position between the target screens after their primary choice. Such difference in switching frequency becomes significant in later tests with older animals (see below).

Preference for Normal Over Distorted Social Stimuli

One-week-old chicks were tested whether they prefer video movies of normal conspecifics over the same movie without visible facial features (blurred head). Both groups were more inclined to choose the normal social stimulus (control: χ^2 = 4.57, n = 14, p = 0.033; VPA: χ^2 = 5.3, n = 12, p = 0.021, **Figure 5**).

Preference Toward Socialized Chicks Over Isolated Ones

To test whether VPA affects the detection of subtle details in the behavior of conspecifics at the age of 9 days, chicks were tested whether they prefer normally socialized conspecifics over those reared in isolation. Neither group showed significant preference toward any of the stimulus types as a first choice (control: χ^2 = 1.64, n = 22, p = 0.201; VPA: χ^2 = 2.88, n = 17, p = 0.09, **Figure 6A**). There was no difference between the two groups in the latency of the first choice either (t = 0.6, d.f. = 36, p = 0.554, **Figure 6B**). Both groups tended to spend more time at the proximity of the isolated chicks (VPA: 57.9 ± 9.9%, control: 55.4 ± 7.1%) than of the group-reared ones (VPA: 27.2 ± 9.7%, control: 31.1 ± 7.0%) but the difference was subsignificant. The two groups did not differ in their time spent at any of the stimulus chicks (t = 0.2, d.f. = 37, p = 0.841). However, chicks in the control group often abandoned their first choice and explored the other stimulus individual as well, while the VPA-treated chicks tended to stick to their first choice. The number of positional switches between the two stimulus individuals was significantly greater in the control group (Welch's t = 2.67, d.f. = 22.45, p = 0.014, **Figure 6C**).

Recognition of and Preference for Familiar Individuals Over "Strangers"

Three-week-old chicks preferred their sympatric, familiar conspecifics: they chose them primarily (χ^2 = 4.84, n = 25, p = 0.028, **Figure 7A**) and spent more time in their proximity

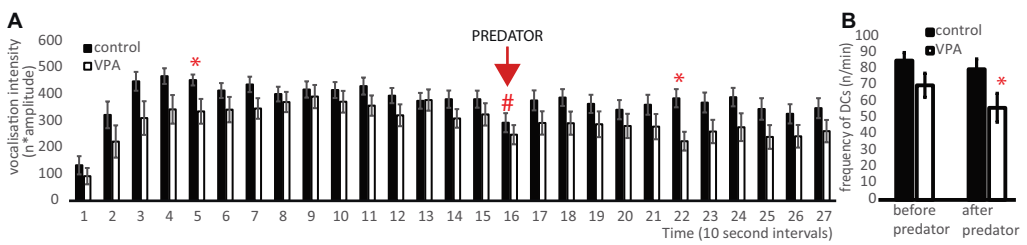

FIGURE 3 | (A) Vocalization intensity during the isolation test in 10-s intervals (mean ± s.e.m.). Asterisks represent significant difference between VPA and control groups. Hash mark denotes significant difference compared to the previous 10-s intervals in both experimental groups. **(B)** Frequency of distress calls (DCs) during the isolation test (mean ± s.e.m.). Asterisks represent significant difference between VPA and control groups.

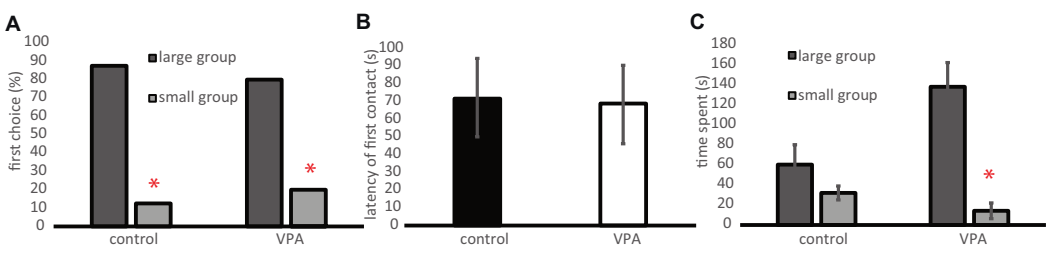

FIGURE 4 | (A) Percentage of chicks approaching one end of the runway as their first choice between video recordings of a large group vs. a small group of chicks. Asterisks denote a choice significantly different from random choice. **(B)** Latency of the first choice (physical contact with the screen, mean ± s.e.m). **(C)** Time spent at each end of the runway (mean ± s.e.m). Asterisk denotes significant difference between the times spent in the proximity of the two screens.

(t = 2.41, d.f. = 25, p = 0.024, **Figure 7B**). VPA-treated chicks failed to develop such a preference at the age of 3 weeks; they chose randomly between familiar and unfamiliar individuals (χ^2 = 0.91, n = 12, p = 0.768, **Figure 7A**) and spent an equal amount of time in their proximity (t = 0.31, d.f. = 11, p = 0.76, **Figure 7B**). Similar to the previous tests, control chicks tended to explore the goal boxes at both ends of the runway: they switched sides 3–4 times more often after the first choice than did VPA-treated chicks (t = 2.1, d.f. = 28, p = 0.046, **Figure 7C**).

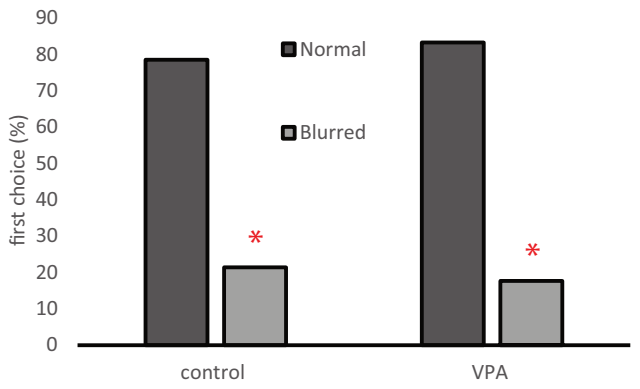

FIGURE 5 | Percentage of chicks approaching one end of the runway as their first choice between video recordings of normal individuals vs. digitally distorted ones (head blurred). Asterisks denote a choice significantly different from random choice.

DISCUSSION

Newly hatched domestic chicks have often been used as models in studies of behavioral neuroscience (Bolhuis and Honey, 1998; Rose, 2000; Zachar et al., 2008), because, despite their young age, they are capable of displaying complex behaviors, while, at the same time, an extensive previous experience does not interfere with their behavior (Rose, 2000). Chicks prefer the close proximity of conspecifics. One widely used behavioral paradigm is filial imprinting (Horn, 1998; Matsushima et al., 2003), which is not entirely devoid of social context, since it is the trigger of social bond forming between parent and offspring. Chicks also react to social isolation by displaying behaviors aimed at reuniting with conspecifics (Gallup and Suarez, 1980), and they prefer larger groups of siblings over smaller ones (Zachar et al., 2016). The drive to reinstatement can be evaluated by measurement of distress vocalization (Takeuchi et al., 1996; Yazaki et al., 1999). Such innate gregariousness likely relies on the social brain network, since recognition of and exposure to just one same-age conspecific partner activates brain regions of the social brain network in naïve domestic chicks (Mayer et al., 2017). This suggests that, even if lacking time to form social bonds, affiliation to siblings is likely processed similarly to other social behaviors.

The present findings seem to confirm previous reports concerning an adverse effect of VPA on embryonic development, including a tendency for aborted or delayed hatching and, occasionally, for locomotor disorders. However, those VPA chicks that have overcome these initial deficits will perform

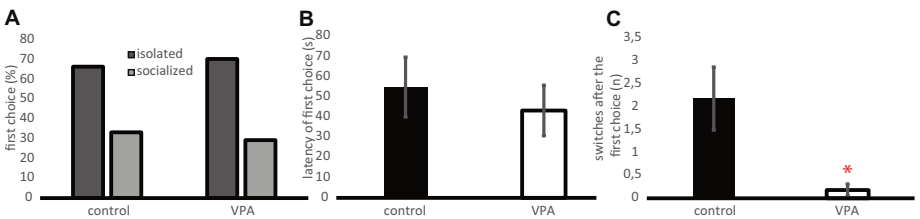

FIGURE 6 | (A) Percentage of chicks approaching one end of the Y-maze as their first choice between a socially reared (socialized) chick and another one reared in isolation (isolated). The first choice of the VPA and control chicks did not differ significantly. **(B)** Latency of the first choice (physical contact with goal box, mean ± s.e.m.). **(C)** Number of positional switches performed by the experimental chicks between the two goal boxes after the first choice (mean ± s.e.m.). Asterisk denotes significant difference between VPA and control chicks.

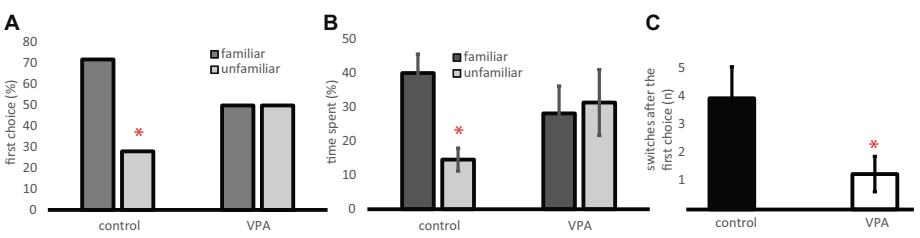

FIGURE 7 | (A) Percentage of chicks approaching one end of the runway as their first choice between a familiar and an unfamiliar chick. Asterisk denotes a choice significantly different from random choice. **(B)** Time spent at each end of the runway (mean ± s.e.m.). Asterisk denotes significant difference between the two goal boxes. **(C)** Number of positional switches between the two goal boxes performed by the experimental chicks after their first choice (mean ± s.e.m.). Asterisk denotes significant difference between VPA and control chicks.

normally in locomotor tests and remain in good physical condition. Judged by visual observation (not quantified, off-record) over the entire period of experiments, VPA chicks tend to display normal social behaviors not overtly dissimilar from control peers (aggregation, synchronized activities such as joint sleeping and waking cycles, joint pecking at objects). Also, their distress level appears to be similar to that of controls, apart from minor differences at selected periods of monitoring. In agreement with the results on filial imprinting in previous reports (Nishigori et al., 2013; Sgadò et al., 2018), appetitive (water reward) and taste aversion (MeA) learning, as well as discriminative learning (recognizing the color bead) of chicks are unaffected by embryonic VPA treatment. Whereas cognition remains unimpaired, the observed elevation of pecking frequency during positive water reinforcement training in VPA chicks may be due to an increased tendency of stereotypic pecking. The latter is known to be related to dopaminergic overstimulation, also in birds (Kabai et al., 1999; Zsedényi et al., 2014). Repetitive and stereotypic behaviors are among the standard symptoms of human ASD (Bangerter et al., 2017). On the other hand, this does not explain the observed elevation of pecking of control birds during the test phase. Another viable hypothesis to explain the increased pecking on water reinforcement is in line with the observation of excessive water consumption in autistic humans (Mills and Wingy, 2015).

The existing reports on the early behavioral effects of embryonic VPA treatment of domestic chicks in relation to ASD (Nishigori et al., 2013; Sgadò et al., 2018; Lorenzi et al., 2019) seem to agree upon the point that VPA does not impair filial imprinting. In line with this is our observation that another form of early adaptive learning that is devoid of social context (passive avoidance learning) is not attenuated by VPA either (present study). However, at variance with the reports by Sgadò et al. (2018) and Lorenzi et al. (2019), we did not find significant differences between VPA-treated and control birds in other behavioral tasks relevant to early social preferences: distress calls evoked by isolation (suspended by predator sighting), and approach preference for larger over smaller group of conspecifics (with natural or blurred facial features), at least, in the period between 1 and 9 days of age.

It has to be noted that, in the study by Nishigori et al. (2013), vocalization of the VPA exposed birds was found to be reduced. In our case, such an overall reduction was suggestive as a trend but not significant, except for high-intensity (distress) calls in selected periods of observation. However, the response to predator sighting (abrupt silencing of distress calls) was uniformly present both in the experimental and in the control group, indicating that the predisposition of chicks to avoid danger from overhead attacks was unaffected by VPA exposure.

The apparent contradiction between the report of Sgadò et al. (2018) and our current findings concerning susceptibility of innate preferences (predispositions) to embryonic VPA challenge can likely be ascribed to meaningful differences in the experimental conditions. In the cited study, social predisposition of naïve, dark-reared chicks was assessed by a choice between two stimuli of "face configuration" (a stuffed hen or a scrambled version of it), on post-hatch day 2, whereas imprinting to similar objects was carried out no later than day 3. In our study, the earliest measure of innate behavior was distress vocalization evoked by social isolation on post-hatch day 2, followed by an approach preference for larger groups of conspecifics on day 5. Thus, in our case, the modality and salience of the stimuli triggering the behavior clearly differed from those applied in the study by Sgadò et al. (2018).

While the current findings do not suggest an impairment of innate preferences by VPA exposure in the categories studied (however, they do not preclude the existence of such effect in other categories), our results point to an important novel aspect of VPA-dependent alterations of sociability, developing during the first 3 weeks of life. The first subtle sign of disturbance emerged at day 9, when VPA-exposed chicks showed reduced exploration of conspecifics (while they still did not differ significantly from controls in their choice between socially reared and isolation-reared partners). The intensity of social exploration (number of times the birds switch proximity position between group-reared and isolation-reared partners) proved to be a useful and important parameter, potentially indicating behavioral plasticity and flexibility in control chicks, as opposed to rigidity and perseverance in VPA-exposed chicks. Notably, 3-week-old chicks showed a similar difference between the numbers of positional switches in the familiarity tests (again, control birds outperforming VPA birds). Presumably, the reduced number of side switches in VPA chicks cannot be ascribed to impaired locomotor activity, since it has been found to be unaffected in running wheel test (Nishigori et al., 2013) or by measuring open field activity (in the present study). Still, it cannot be excluded that the observed deficit affected general, rather than just socially driven, exploration.

The results of the present study answer an important, hitherto unadressed, question raised by the work of Nishigori et al. (2013) by suggesting that the observed deficits in group-forming and group-cohesion were due to attenuated motivation for exploring social stimuli, rather than to impaired stimulus recognition. It is expected that, without proper exploration, the individual recognition required for the complex social life of adult domestic chicks would fail to develop by the third week.

At 3 weeks of age, by which time the control birds have acquired the capability of distinguishing familiar sympatric birds from non-familiar partners (Koshiba et al., 2013), the VPA-exposed chicks perform markedly poorer in this task. It has to be noted that, in our study, we did not attempt to determine the precise nature of the "familiarity stimulus." According to the study by Koshiba et al. (2013), familiar recognition in domestic chicks of 15 days of age is based primarily on acoustic (rather than visual or olfactory) cues. According to the above-cited authors, it is unknown if chicks are capable of recognizing familiar peer calls based on individual chick recognition. Nevertheless, visual cues underlying the recognition of familiar conspecifics of chicks have been reported to appear well before that period (Vallortigara, 1992; Vallortigara and Andrew, 1994), and, on the whole, conspecific recognition can likely be ascribed to a complex multimodal input from primary and reinforcing stimuli (Bolhuis, 1991), and a reciprocal communication between subject and target.

In summary, VPA administration on the 14th day of incubation *in ovo* impaired certain acquired (primarily exploratory) social behaviors and social memory of young post-hatch chicks, but it failed to cause robust defects in their hardwired predispositions. The most prominent findings included an attenuation of social exploration, followed by a remarkable deficit in the recognition of familiar conspecifics, unfolding at the end of the third week after hatching. These novel results underline the importance of further investigation into the differences in early social exploration, potentially contributing to the early diagnosis of ASD.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

All animals were kept and treated according to the regulations of the ethical committee of the Semmelweis University, and all experiments were approved by the Ethical Committee on Animal Experimentation, and permitted by the Food Chain Safety and Animal Health Directorate of the Government Office for Pest County (Permit Number: XIV-I-001/2269-4/2012). Procedures were in harmony with the EU Council directives on laboratory animals (86/609/EEC).

AUTHOR CONTRIBUTIONS

GZ, AT, and AC designed the experiments. AT was mostly involved in the technical execution of behavioral tests. GZ, AT, and SZ analyzed the video recordings. AT, LG, and ÁÁ carried out the embryonic manipulations. The manuscript was written and finalized by GZ and AC.

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