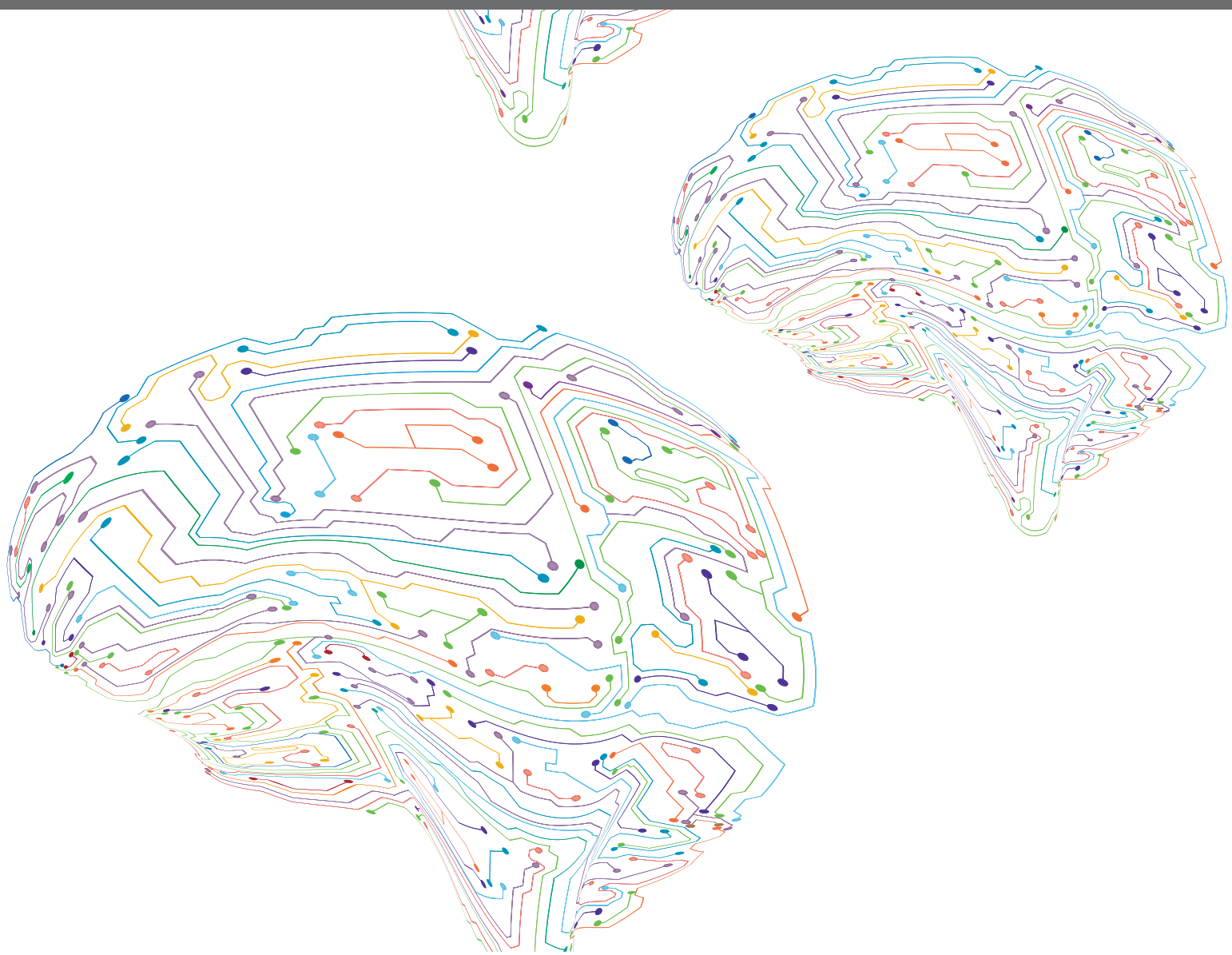
THE NEUROETHOLOGY OF SOCIAL BEHAVIOR

EDITED BY: Joel D. Levine, Ana Silva and Gervasio Batista
PUBLISHED IN: Frontiers in Neural Circuits and
Frontiers in Behavioral Neuroscience



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ISSN 1664-8714
ISBN 978-2-88976-264-4
DOI 10.3389/978-2-88976-264-4

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THE NEUROETHOLOGY OF SOCIAL BEHAVIOR

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Citation: Levine, J. D., Silva, A., Batista, G., eds. (2022). The Neuroethology of Social Behavior. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-264-4

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frontiers | Frontiers in Neural Circuits

EDITORIAL
published: 09 May 2022
doi: 10.3389/fncir.2022.897273

Editorial: The Neuroethology of Social Behavior

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Keywords: neuroethology, social behavior, neuroscience, evolution, neural mechanism

Editorial on the Research Topic

The Neuroethology of Social Behavior

One of the great challenges in modern biology is to understand how social behaviors arise through a combination of genetic and environmental factors, and how they are implemented by brain circuits. Increasingly, studies in the field and laboratory have shown that group dynamics are critical to the life of individuals and that wherever groups are observed, behavior is modulated by individual members as well as by the presence of others. We pursued this Frontiers Research Topic to seek Neuroethological perspectives on principles of neural circuits and social behavior across organisms and fields.

Neuroethology takes advantage of species diversity to study the neural underpinnings of natural behaviors. Each organism provides unique answers to diverse long-standing problems that are fundamental to understanding brain function and social processes. Invertebrates, for instance, have been pivotal in tracking the genetic basis of social cohesion. Newborn birds have increasingly become ideal systems to untangle *nature vs. nurture* questions, given their innate responses to social stimuli and precocious learning capacities. The well-understood circuits controlling vocal and electric communication in fish shed light on the architecture of social signals. Field studies in frogs have forged new evidence on the molecular basis of social behavior and the origins of its natural variability. The present collection of articles represents the diversity of theoretical and experimental approaches necessary to establish a compelling view of social behaviors. From flies to humans, authors stage the richness of studying social phenomena at multiple levels, from groups of individuals to genes, and confirm the importance of picking the right organisms to address specific questions.

Early life experience determines brain maturational trajectories that affect a number of social and non-social behaviors. Similarly, parental care has long-lasting effects on progeny as Zeng et al. exemplify. Autry and O'Connell discuss and compare behavior and brain approaches to tackle generalizable and distinctive principles in parenting. A major point raised by the authors is that parental care strategies across organisms need to be understood in terms of their ecological challenges. In turn, field studies become an important aspect of information in understanding parenting across species. In laboratory settings, the continuously growing toolbox for neuronal manipulation allows the resolution of specific circuits driving parental care. As argued by Autry and O'Connell it is at the crossroads of ethological and neurogenetic inroads that future avenues will open.

Whether brains are innately tuned to social stimuli has been elusive to experimental inquiry. Newborn chicks, extensively used in filial imprinting studies, have been previously shown to be attracted to face-like visual stimuli and patterns of biological motion. Lorenzi et al. report that the

Edited and reviewed by:
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Received: *15 March 2022*
Accepted: *04 April 2022*
Published: *09 May 2022*

Citation:
Batista G, Levine JD and Silva A (2022) Editorial: The Neuroethology of Social Behavior. Front. Neural Circuits 16:897273. doi: 10.3389/fncir.2022.897273

chicks' preference toward animated objects is controlled by thyroid hormones. In addition, Adiletta et al. demonstrated that embryonic exposure to Valproic Acid (an agent used to model autistic-like behaviors in preclinical studies) disrupts the innate orienting behaviors toward face-like stimuli. Thus, Adiletta et al. argue that chicks might be useful to model face processing deficits in ASD.

Two teleost model systems, weakly electric fish and vocal fish, stand out for their contributions to the understanding of the neuroendocrine basis of social communication. These distantly related teleost groups that produce either vocalizations or electric organ discharges have been traditionally compared. Dunlap et al. discuss an updated view on the behavioral neuroendocrinology of vocal and electric fish and offer complementary insights into social communication biology. Dunlap et al. revise recent findings in both teleost systems and make direct comparisons to highlight how these analogous communication systems have evolved similar and different mechanisms.

Weakly electric fish have also recently emerged as advantageous model systems for the study of complex social behaviors, in which electric signaling is part of their displays. The extensive knowledge of electrocommunication set the stage for more complex evolutionary comparisons based on social behavioral strategies. Both song sparrows and banded knifefish display territorial aggression uncoupled from reproduction. Quintana et al. summarize recent findings on the neuromodulation of non-breeding aggression in fish and birds to establish general principles in the regulation of social behavior. Notably, neurosteroids (steroids synthesized locally in the brain) and neuropeptide Y play important roles in non-breeding aggression across organisms.

The advanced genetics of *Drosophila melanogaster* and broadly available tools to dissect specific circuits governing behaviors puts this organism at a privileged site to investigate the basis of sociability. However, flies have been considered solitary for decades. The recent use of social network analysis has revealed that groups of flies present a structure rooted in their genes. Jezovit et al. review several studies to highlight methodological differences and common findings to inspire new ideas in the field of fly social networks.

While human social interactions are difficult to translate into animal models, complex social traits have been successfully replicated in preclinical studies. Leong et al. describe how social learning and its underlying inter-brain synchrony can be modeled in mice. In such settings, the authors argue that optogenetic manipulation of social dyads might open novel avenues for future studies. On the other hand, the authors discuss major caveats to this approach to promote discussion in the field. Li et al. offer their view on how animal models can be useful in discovering novel therapeutic strategies for social deficit disorders such as autism and schizophrenia.

In an era where cutting-edge experimental tools are available across taxa, the scientific community needs to embrace comparative biology as a keystone of future studies of social behaviors. As we understand the peculiarities of species-specific social behaviors, an evolutionary perspective will grant us new experimental paradigms and theoretical concepts to extract common principles linking brain function and social performance. From an evolutionary perspective sexual reproduction, communications, group structure, and problem-solving are important problems that need to be understood and explained. We believe that the papers in this Frontiers Research Topic will drive more conversations about this important issue and invite researchers from around the world to adopt the neuroethological approach to the study of social behavior.

AUTHOR CONTRIBUTIONS

GB, JL, and AS wrote and edited the article. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We thank authors who contributed their articles to this Research Topic. We also thank the reviewers who kindly accepted to review the manuscripts submitted to this Research Topic and gave important comments to improve the manuscripts.

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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 07 April 2021
doi: 10.3389/fnbeh.2021.637678

Attachment Insecurity in Rats Subjected to Maternal Separation and Early Weaning: Sex Differences

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Attachment insecurity in the forms of attachment anxiety and avoidance is associated with mental disorders in humans. In this research field, rodents, especially mice and rats, are commonly used to study social behaviors and underlying biological mechanisms due to their pronounced sociability. However, quantitative assessment of attachment security/insecurity in rodents has been a major challenge. The present study identified attachment insecurity behaviors in rats subjected to maternal separation (MS) during postnatal days (PD) 2–16 and early weaning (EW) during PD 17–21. This MSEW procedure has been used to mimic early life neglect in humans. After MSEW, rats continued to survive until early adulthood when they were subjected to open-field, social interaction, and elevated-plus maze tests. Compared to CNT rats in either gender, MSEW rats moved longer distances at higher velocities in the open-field. The MSEW rats also showed lower ratios of travel distance at central zone over that on whole arena of the open-field compared to CNT rats. In social interaction test, male CNT rats preferred to investigate an empty cage than females; whereas female CNT rats spent more time with a partner-containing cage as compared to males. This gender-specific difference was reversed in MSEW rats. On elevated-plus maze female CNT rats exhibited more risk-taking behaviors as compared to male counterparts. Moreover, female MSEW rats experienced a greater difficulty in making a decision on whether approaching to or averting from which arms of elevated-plus maze. Taken together, male MSEW rats behaved like attachment anxiety while females' phenotype is alike to attachment avoidance described in humans. These results shall prompt further application of MSEW rat in abnormal psychology and biological psychiatry research.

Keywords: attachment anxiety, attachment avoidance, maternal separation, early weaning, rat, sex differences

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *04 December 2020*
Accepted: *16 March 2021*
Published: *07 April 2021*

Citation:
Zeng H, Yu Z, Huang Q and Xu H (2021) Attachment Insecurity in Rats Subjected to Maternal Separation and Early Weaning: Sex Differences. Front. Behav. Neurosci. 15:637678. doi: 10.3389/fnbeh.2021.637678

INTRODUCTION

Attachment refers to a selective and enduring bond between individuals including romantic attachment between adults and infant–caregiver attachment. In the latter scenario, attachment describes a complex and highly specific bond established between an infant and his/her caregiver (Bowlby, 1982). There is increasing evidence that quality of care affects emotionality and emotion regulation throughout the life course (Waters et al., 2000). It was reported that individuals reared in institutional settings exhibited deficits in emotion regulation, attachment to primary caregivers,

and cognitive development (O'Connor et al., 2003; Kreppner et al., 2007; Zeanah et al., 2009; Tottenham et al., 2010). A stable sense of attachment security results from interactions with attachment figures who are available in times of need, sensitive and responsive to bids for proximity and support (Bowlby, 1973). With a secure attachment, a person tends to have a high level of self-esteem, self-stability and satisfaction as it facilitates emotion regulation and enhances affiliative behaviors between peers (Canterberry and Gillath, 2013). In contrast, insecure attachment is likely due to having an unresponsive, rejecting, inconsistent, or insensitive caretaker (Ainsworth and Bell, 1970). Clinical studies have shown that attachment insecurity is associated with some of mental health problems including depression (Catanzaro and Wei, 2020), anxiety (Bosmans et al., 2020), obsessive-compulsive disorder (Doron et al., 2012), post-traumatic stress disorder (Ein-Dor et al., 2010), suicidal tendencies (Gormley and McNiel, 2010), and eating disorders (Illing et al., 2010).

A person's sense of attachment security is reflected by his/her location in the two-dimensional conceptual space defined by attachment anxiety and avoidance (Mikulincer and Shaver, 2007). People with low scores on these two dimensions generally feel secure and tend to employ constructive and effective affect-regulation strategies; whereas those with high score on either the attachment anxiety or avoidance dimension (or both) often have a sense of insecurity and tend to rely on secondary attachment strategies (either deactivating or hyperactivating their attachment system) to cope with threats (Cassidy and Kobak, 1988). In clinical and research practice, adult attachment style can be assessed using several self-report instruments, such as the Experiences in Close Relationships (Brennan et al., 1998), the Attachment Style Questionnaire (Hazan and Shaver, 1987), and the Relationship Questionnaire (Bartholomew and Horowitz, 1991).

Most psychological scholars concede that the core human psyche is a product of biological evolution resulting from natural selection (Panksepp, 2006). In line with this consensus, it is believed that many other animals also have emotional feelings, including anger, fear, maternal care, separation distress, social bonding, and playfulness (Panksepp, 1998, 2005). Indeed, animal studies including those on imprinting in birds (Bateson, 1966), early olfactory learning in rabbits (Hudson, 1993), and the development of affectional bonds in nonhuman primates (Harlow and Suomi, 1970) have significantly facilitated the development of attachment theory. And animal models of disrupted infant–caregiver relationship have been used to investigate the neurobiology of infant attachment and fear, as well as the maturation of emotion circuits (Callaghan et al., 2014). Particularly, adolescent and adult rats that had received less maternal care or unpredictable shock during infancy expressed anxiety-like behaviors and heightened stress responses (Macrì et al., 2008; O'Mahony et al., 2009; Sarro et al., 2014). Moreover, parental separation was shown to enhance active avoidance learning in juvenile rodents (Abraham and Gruss, 2010) while early life handling enhanced contextual conditioning in P18 rats (Beane et al., 2002). These previous findings support the view that translational models of disrupted infant–caregiver relationship are critical in understanding mental health trajectories in humans.

Different from human studies that assess human attachment style using several self-report instruments as reviewed above (Hazan and Shaver, 1987; Bartholomew and Horowitz, 1991; Brennan et al., 1998), quantitative assessment of attachment security/insecurity in animals has been a major challenge. In trying to circumvent this challenge, this animal study employed the laboratory Sprague-Dawley (S-D) rat, an ideal subject for studies of maternal care (Numan, 1994), and adapted a paradigm of maternal separation and early weaning (MSEW), which was initially designed by George et al. (2010) for mice. This paradigm has been used to mimic early life neglect in humans and is believed to influence brain development and consequently bring forth a predisposition toward mental and behavioral disorders (Carlyle et al., 2012; Strüber et al., 2014). After MSEW, rats continued to survive into early adulthood and then subjected to open-field, social interaction, and elevated-plus maze tests. The three behavioral tests have been used to estimate the explorative activity and anxiety level (Hiroi and Neumaier, 2009), social behavior (Smolensky et al., 2019), and risk-taking/anxiety-like behavior (Tillmann and Wegener, 2019) in rats, respectively. Compared to controls, MSEW rats showed higher anxiety level and social behavior deficiency in open-field and social interaction tests, as well as a risk-taking behavior on the elevated-plus maze. These behavioral abnormalities were not reported in previous studies that either applied maternal separation (MS) (Park et al., 2018; Isobe and Kawaguchi, 2019; Ströher et al., 2019) or early weaning (EW) to rats (Kanari et al., 2005; Ito et al., 2006; Shimozuru et al., 2007). In the previous studies that applied MSEW paradigm to mice (George et al., 2010; Carlyle et al., 2012), different behavioral tests were employed thus did not result in the same results as what reported in this study. Moreover, female rats were included in this study given that females were frequently overlooked in previous preclinical research due to the concern that female reproductive cycle would lead to behavioral variance in subjects. This addition allowed us to compare behavioral abnormalities in male and female MSEW rats. Interestingly, male MSEW rats behaved like attachment anxiety while females' phenotype is alike to attachment avoidance described in humans.

MATERIALS AND METHODS

Animals

Female S-D rats at gestational week 2 were purchased from the animal center of the Southern Medical University (Guangzhou, China) and housed in an air-conditioned room at the vivarium of Shantou University Medical College. The animals had free accesses to food and water in the room with controlled temperature in the range of 23 ± 1°C and a 12:12 h light cycle. The delivery day was defined as PD 0. An even number (with equal number in male and females) up to ten pups of each litter and their dam were culled for the next MSEW procedure or being used as controls. All animal handling and use were carried out in accordance with the guidelines set up by the Animal Care and Use

Committee of Shantou University Medical College and approved by the committee.

MSEW Procedure

The maternal separation (MS) started on PD 2, by removing a pup from his/her dam and placing the pup in a small carton (10 × 9 × 9 cm) for 4 h per day during PDs 2–5, and 6 h per day during PDs 6–16. The MS duration increased with age because the younger the pups, the more susceptible to starvation as demonstrated in our primary experiment, in which MS for 6 h per day during PDs 2–5 led 50% of pups to die. During the separation period, which started at the same time (8:00 am) every day, pups in cartons (one pup per carton) were kept at an infant incubator (YP-100; Ningbo David Medical Device Co., Ltd., Ningbo, China) which was kept well ventilated at a controlled temperature (34°C during PDs 2–5, 32°C during PDs 6–9, 30°C during PDs 10–14, and 28°C during PDs 15–16) and a constant humidity (60%) under the light condition of 20 lux at a room of 3.5 × 4.5 m. Before and after MS, all pups in the MSEW groups (n = 20/group in either sex) were brought back to the cage where their dam was living, but the maternal behaviors were not monitored during the reunion period. Early weaning (EW) occurred on PD 17 when a home-made soft diet (powdered rodent chow in tap water) was provided to the pups kept at cartons (one pup per carton). Starting at PD 22, the MSEW rats of a same litter were housed in group (5 pups/cage, 485 × 350 × 200 mm) by sex. The pups in Control groups (n = 20/group in either sex male) were raised by their dams under the standard laboratory condition as described above and weaning started at PD 22. The body weight of all pups was weighed at PD 7, 14, 21, and 30, respectively. The schematic diagram of above procedures was shown in **Figure 1**. Nothing was done to control the estrous circle of females as the MSEW procedure was applied to immature rats in this study (rats take about 3 weeks to mature and begin fending for themselves). And meta-analyses have shown that naturally cycling female mice and rats present no more variance in broadly categorized behavioral measures than males (Prendergast et al., 2014; Becker et al., 2016; Beery, 2018).

Behavioral Tests

The behavioral tests carried out in this study include open-field test, social interaction test, and elevated-plus maze test. They were administered during PDs 60–62, once a day in the order of increasing aversiveness to minimize the impact of immediate behavioral testing on subsequent tests. Before the commencement of behavioral tests, rats were transported to the testing room (about 10 square meter size) and stayed there overnight for adaptation.

Open-Field Test

The wooden open field box (100 × 100 × 60 cm) was painted in black and sheltered by a blue drape in the behavioral test room, which was lighted with three white fluorescents (in a total of 15 lux) placed 160 cm above the arena. Each individual rat was placed in the center of the open-field box and allowed to move freely for 12 min. The first 2 min were defined as the adaptation period and the data from this period was not included for analysis. A video tracking system (EthoVision XT 9.0; Noldus Information Technology, Wageningen, Netherlands) was used to monitor the tested rat. For each tested rat, the moving distances on the whole arena (TD) and its central zone (CD, the central part of 50 × 50 cm), and time spent on the central zone (CT) were recorded. The ratio of CD/TD was calculated. The TD was considered an index of locomotor activity and CD/TD index of anxiety level. In addition, the moving velocity (MV) of rats in the open-field was also calculated. The floor and inner walls of the box were cleaned with 70% ethanol after each test.

Social Interaction Test

This test was carried out in the same open-field box lighted by the same white fluorescents as in the open-field test. It consists of two sessions and an interval between sessions. Each session persisted for 150 second (S) while the interval persisted for 1 min thus the whole test persisted for 6 min as described previously (Challis et al., 2013). The procedure was also successfully employed in the other animal studies that measured social behaviors of rodents (Krishnan et al., 2007; Browne et al., 2018; Zhang et al., 2018). Before the test, all rats were housed in group (5 rats/cage) as mentioned above. During the first session, an empty (E session) wire mesh cage (12 × 12 × 18 cm) was placed at one end of the open-field arena (100 × 100 cm) where a tested rat was allowed to move freely. During the second session, the conditions were identical except that an unfamiliar conspecific partner (C session) had been introduced into the cage before a tested rat was placed in the open-field box. The partner was matched with the tested rat

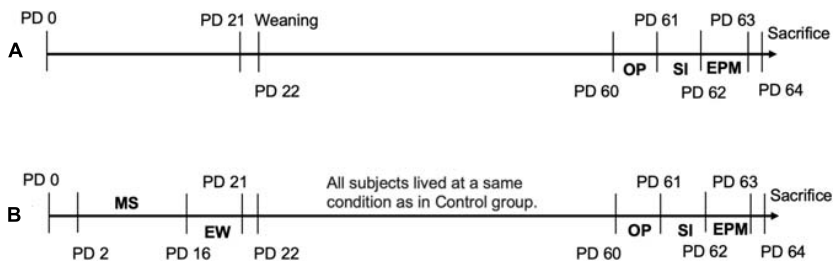

FIGURE 1 | Experimental schedule and MSEW paradigm. **(A)** Shows the experimental schedule set up for rats in Control group. **(B)** Shows the MSEW paradigm and experimental schedule for rats in MSEW group. EPM, elevated plus maze test; EW, early weaning; MS, maternal separation; OP, open field test; PD, postnatal day; SI, social interaction test.

in gender, age, and body weight, but they were neither littermates nor cage mates. Between the two test sessions, the tested rat was removed from the box and placed back into his/her home cage for 60 S. The video tracking system was used to monitor the tested rat. The time spent by the tested rat at the interaction zone (a 16-cm-wide corridor around the cage) was recorded.

Elevated-Plus Maze Test

The elevated-plus maze consists of four radial arms (two closed, 50 × 10 × 40 cm; two open, 50 × 10 × 2 cm) elevated 60 cm above the floor. Under the same lighting condition as that in the open-field test, rat was placed at the central junction, facing a closed arm, and the activity of the rat on the elevated-plus maze was recorded during the subsequent 10 min. The first 2 min were defined as the adaptation period and the performance of the rat in the remaining 8 min was analyzed. The time spent by a tested rat on the central junction (Tcj), open (To) and closed arms (Tc), and the number of entries to these locations (Ncj, No, and Nc) were recorded. The ratio of To/Tc was calculated and considered an index of anxiety level. In the preliminary experiment, MSEW rats spent much more time on open arms of the elevated-plus maze compared to CNT rats. We speculated that this abnormal behavior in MSEW rats was indicative of a risk-taking behavior instead of an anxiolytic effect induced by the paradigm. In order to confirm and further interpret this abnormal behavior, we elongated the test time from the standardized 5 to 8 min and included Tcj and Ncj for data analysis.

Statistical Analysis

SPSS17.0 (IBM Corp., Armonk, NY, United States) was used to analyze all the data which were expressed as mean ± SD. The Shapiro–Wilk test was used to test the data for normality. For social interaction data, independent paired *t* tests were done to compare mean values from E and C sessions of a same group (CNT or MSEW), and from CNT and MSEW groups in a same E or C session. For the other data, two-way ANOVA was done before post-hoc comparisons (*F*-test). The significant threshold was set at 0.05.

RESULTS

The Weight Gain of Rats and Effect of MSEW

Infant rats rely on attaching to his/her dam for care and nourishment. MSEW may exert significant impacts on rat pups in respect of physiological and psychological parameters. We wanted to establish a reliable MSEW paradigm that has no or a minimum effect on physiological parameters of subjects. In preliminary experiments, MS lasted for 6 h/day during PD 2–5 and 8 h/day during PD 6–16. This protocol led to a high fatality (about 50%) in MSEW rats during the MS period. As such, the procedure was modified as reported here, i.e., 4 h/day during PD 2–5 and 6 h/day during PD 6–16. This modified procedure caused no rat death. The data of body weight measured at PD 7, 14, 21, and 30 were analyzed by two-way ANOVA. For male rats, two-way ANOVA showed (1) no significant interaction between treatment and time ($F_{(3,159)}$ = 0.558, p = 0.644), (2) a significant effect of measuring time on body weight of rat pups ($F_{(3,159)}$ = 2,813.101, p = 0.000), i.e., the body weight of rat pups increased with age, (3) MSEW showed no effect on weight gain of rat pups ($F_{(1,159)}$ = 0.046, p = 0.831) (**Figure 2A**). Similar results were found in female rats, i.e., there was no significant interaction between time and treatment ($F_{(3,159)}$ = 0.939, p = 0.423), the body weight of female pups increased with age ($F_{(3,159)}$ = 2,261.789, p = 0.000), but MSEW had no effect on weight gain ($F_{(1,159)}$ = 0.483, p = 0.488) (**Figure 2B**).

In addition, another two-way ANOVA was carried out with gender and measuring time as two main factors. The results showed significant interactions between gender and measuring time in both CNT ($F_{(3,159)}$ = 3.588, p = 0.015) and MSEW ($F_{(3,159)}$ = 7.025, $p < 0.001$) rats. Both gender ($F_{(1,159)}$ = 8.083, p = 0.005) and measuring time ($F_{(3,159)}$ = 1,746.947, $p < 0.000$) had significant effects on body weight of rat pups in CNT and MSEW groups. Post-hoc comparisons showed that male CNT rats were heavier than females at PD 30 (**Figure 2C**). As for MSEW rats, females had lower body weight than males at PD 14 and thereafter (**Figure 2D**).

Effects of MSEW on the Performance of Rats in Open-Field Test

In the open-field test, we analyzed the parameters TD, CD, CD/TD, CT, and MV as shown in **Table 1**. Both males and females in either CNT or MSEW rats showed comparable performances in terms of the parameters mentioned above. But differences were obvious between CNT and MSEW groups in either males or females. Specifically, two-way ANOVA revealed that there was no interaction ($F_{(1,59)}$ = 0.113, p = 0.738) between gender and treatment in regard of TD, but each of the main factors had a significant effect (treatment, $F_{(1,59)}$ = 44.539, p = 0.000; gender, $F_{(1,59)}$ = 5.141, p = 0.027) on this parameter. Post-hoc comparisons indicated that male and female MSEW rats moved longer TDs compared to CNT groups (**Figure 3A**), but no difference between males and females in both CNT and MSEW rats. As for CD, there was no interaction ($F_{(1,59)}$ = 0.557, p = 0.458) between the two main factors. Treatment ($F_{(1,59)}$ = 10.199, p = 0.002), but not gender ($F_{(1,59)}$ = 0.338, p = 0.563), exerted a significant effect on this parameter. Post-hoc comparisons indicated that male MSEW rats had a shorter CD compared to male CNT group (**Figure 3B**). In regard of CD/TD, there was no interaction ($F_{(1,59)}$ = 0.272, p = 0.604) between the two main factors. Treatment ($F_{(1,59)}$ = 57.377, p = 0.000), but not gender ($F_{(1,59)}$ = 3.277, p = 0.076), had a significant effect on this parameter. Post-hoc comparisons indicated that both male and female MSEW rats had lower values of CD/TD compared to CNT groups (**Figure 3C**). For CT, there was no interaction ($F_{(1,59)}$ = 0.286, p = 0.595) between the two main factors. Treatment ($F_{(1,59)}$ = 12.147, p = 0.001), but not gender ($F_{(1,59)}$ = 1.162, p = 0.286), had a significant effect. Post-hoc comparisons indicated that both male and female MSEW rats spent less time at the central zone compared to CNT groups (**Figure 3D**). In regard of MV, there was

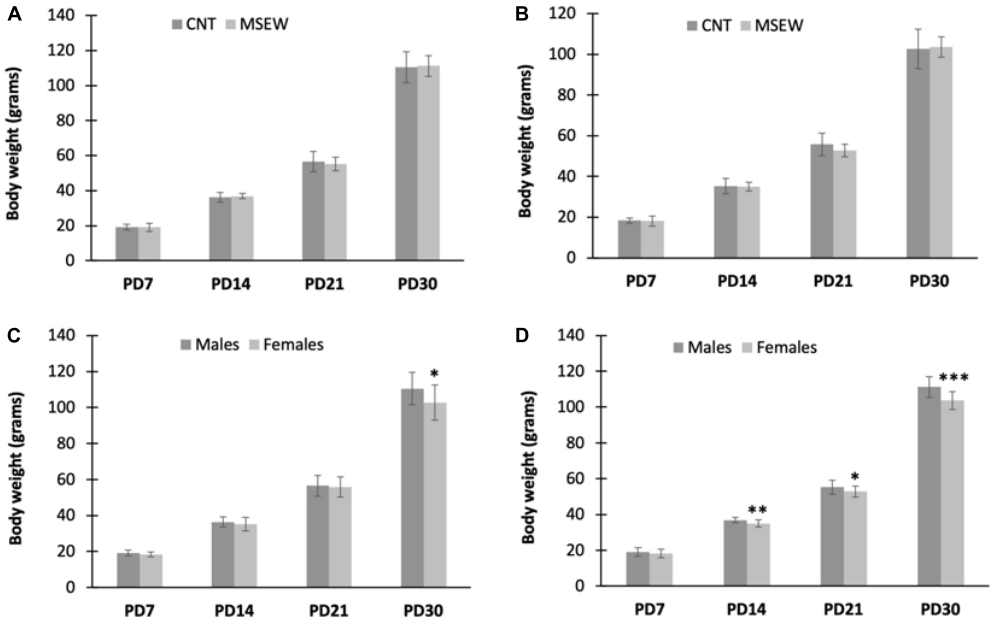

FIGURE 2 | Body weight of rats measured at four postnatal time points. **(A)** Body weight of male rats in CNT and MSEW groups. **(B)** Body weight of female rats in CNT and MSEW groups. **(C)** Body weight of male and female CNT rats. **(D)** Body weight of male and female MSEW rats. Data were expressed as mean ± SD. *n* = 20/group.

TABLE 1 | Performance of adult rats in open-field test.

	CNT		MSEW	
Measurements	**Males**	**Females**	**Males**	**Females**
TD (cm)	5,331.589 (186.64)	4,958.63 (178.45)	6,685.50 (225.70)***	6,182.62 (177.74)***
CD (cm)	462.46 (16.54)	459.03 (16.94)	380.61 (24.29)**	408.20 (23.99)
CD/TD (%)	8.83 (1.79)	9.32 (1.21)	5.73 (1.42)***	6.62 (1.46)***
CT (S)	27.21 (1.02)	28.11 (1.36)	21.37 (1.73)***	23.75 (1.69)**
MV (cm/S)	9.04 (1.17)	8.67 (1.89)	11.24 (1.41)***	10.75 (1.29)***

Data were expressed as means (SD) (n = 15).
CNT, rats in this group were raised under normal condition with no experience of MSEW; MSEW, rats in this group were subjected to MSEW procedure; CD, distance traveled at central zone of open-field; TD, distance traveled on the whole arena of open-field; CT, time spent on the central zone; MV, moving velocity of rats in the open-field.
***p < 0.01, ***p < 0.001, MSEW rats vs CNT rats in the same gender.*

no interaction ($F_{(1,59)}$ = 0.025, p = 0.874) between the two main factors. Treatment ($F_{(1,59)}$ = 32.269, p = 0.000), but not gender ($F_{(1,59)}$ = 1.295, p = 0.260), had a significant effect. Post-hoc comparisons indicated that both male and female MSEW rats moved faster in the open-field compared to CNT groups (**Figure 3E**). In summary, MSEW increased anxiety levels in either male or female rats, there was no sex difference in this regard.

Gender-Specific Performance of Rats in Social Interaction Test: Effects of MSEW

We focused on the time spent by rats at the social interaction zone around a wire mesh cage without or with an unfamiliar conspecific in the social interaction test. All data are shown in **Table 2**. First, all male and female rats in both CNT and MSEW groups spent much more time at the social interaction zone during the C session relative to E session (**Figure 4A**), confirming the presence of social preference of CNT rats, i.e., preference to investigate a novel conspecific over a novel object. This social play function keeps working in MSEW rats. Second, male CNT rats spent more time around an empty cage relative to females, suggesting that males preferred to investigate a novel object than females. In contrast, female CNT rats spent more time at the interaction zone in the presence of an unfamiliar conspecific in the cage compared to males, suggesting that females preferred to investigate a novel conspecific. These sex differences, however, were not seen between male and female MSEW rats (**Figure 4B**), suggesting that MSEW exerted different effects on the social behaviors of male and female rats. Third, male MSEW rats played for longer durations at the social interaction zone during E and C sessions as compared to controls, while female MSEW rats spent a longer duration at the social interaction zone during E session but not C session as compared to female CNT rats (**Figure 4C**). These results suggest that MSEW increased the social preference of male rats, but made female rats prefer to investigate a novel object (the empty cage), which may be indicative of an attachment avoidance behavior.

Gender-Specific Performance of Rats in Elevated-Plus Maze Test: Effects of MSEW

All data regarding the performance of rats on the elevated-plus maze are shown in **Table 3**. First, female (CNT, MSEW)

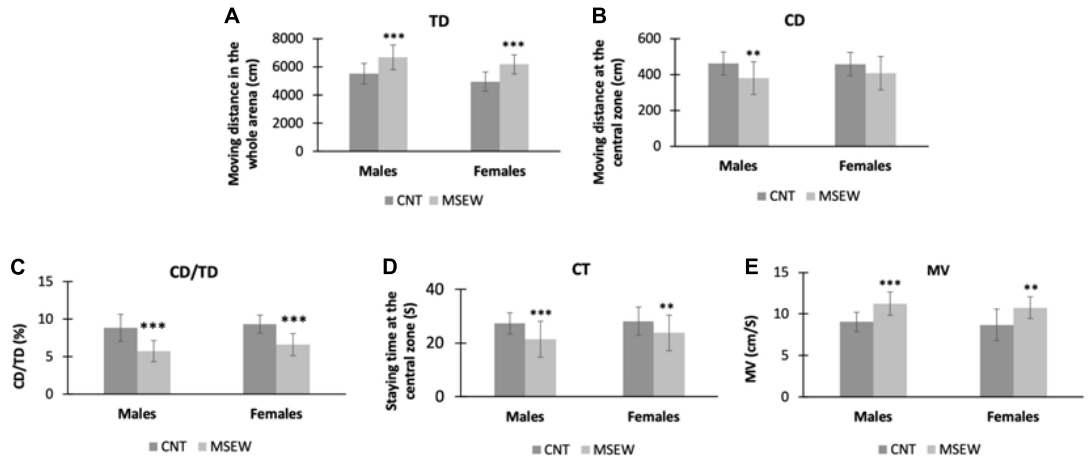

FIGURE 3 | Performance of rats in the open field test. **(A)** Moving distances of CNT and MSEW rats on the whole arena of open field. **(B)** Moving distances of CNT and MSEW rats at the central zone of open field. **(C)** The values of CD/TD of CNT and MSEW rats. **(D)** The staying time of rats at the central zone of open field. **(E)** The moving velocities of rats in the open field. Data were expressed as mean ± SD. n = 15/group. $^{**}p < 0.01$, $^{***}p < 0.001$, MSEW vs Control.

TABLE 2 | Performance of adult rats in social interaction test.

Staying time around	CNT		MSEW	
	Males	Females	Males	Females
E cage (S)	58.60 (9.55)	47.85 (15.15)*	71.59 (10.97)	71.84 (23.80)
C cage (S)	85.08 (10.72)$^{\#\#}$	112.44 (15.93)$^{**,\#\#\#}$	112.88 (8.38)$^{\#\#}$	112.20 (24.20)$^{\#\#}$

Data were expressed as means (SD) (n = 15).
CNT, rats in this group were raised under normal condition with no experience of MSEW; MSEW, rats in this group were subjected to MSEW procedure; E cage, an empty cage; C cage, a cage containing an unfamiliar conspecific.
$^{*}p < 0.05$, $^{**}p < 0.01$, males vs females in either CNT or MSEW groups.
$^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.0001$. E (cage) session vs C (cage) session.

rats entered open arms, closed arms, and central junction more frequently than males (**Figure 5A**). Second, MSEW (male, female) rats spent much more time on open arms and central junction, but less time in closed arms, as compared to CNT rats (**Figure 5B**). Third, two-way ANOVA showed a significant interaction between treatment and gender ($F_{(1,59)}$ = 4.248, p = 0.044) on values of To/Tc (%); both the treatment ($F_{(1,59)}$ = 53.932, p = 0.000) and gender ($F_{(1,59)}$ = 4.831, p = 0.032) exerted significant effects. Post-hoc comparisons showed that MSEW rats had greater values of To/Tc than CNT rats in either males or females (**Figure 5C**), implying that MSEW might have an anxiolytic effect on the rats. This interpretation seems to be contrary to the conclusion from open-field test, i.e., MSEW increased anxiety levels in either male or female rats.

To dissolve this conflict, we calculated values of Tcj/Ncj and To/No of all animal groups. These parameters reflect the staying time per visiting and are of help in confirming the so-called anxiolytic effect of MSEW on rats. We found that values of these two parameters in rats were not changed by MSEW, i.e., CNT and MSEW groups were comparable in terms of Tcj/Ncj and To/No (not shown). The results do not support the anxiolytic effect of MSEW.

DISCUSSION

This study is the first one reporting attachment-related behaviors in rats subjected to MSEW procedure during the first 3 weeks after birth. The main findings include (1) male and female MSEW rats moved longer distances on whole arena of the open-field at higher velocities and showed lower values of CD/TD compared to respective controls; (2) in the social interaction test, male CNT rats preferred to investigate a novel object than females. In contrast, female CNT rats preferred to investigate a novel conspecific compared to males. This gender-specific difference was not seen in MSEW rats. Moreover, MSEW increased the social preference of male rats, but made female rats prefer to investigate a novel object (the empty cage), which may be indicative of a social avoidance behavior (Scholl et al., 2019); (3) on elevated-plus maze, females (CNT, MSEW) rats entered open arms, closed arms, and central junction more frequently than males irrespective of MSEW experience, MSEW (males, females) rats spent much more time on open arms and central junction, but less time in closed arms, as compared to CNT rats irrespective of gender, implying an anxiolytic effect of MSEW. But values of Tcj/Ncj and To/No were comparable across all animal groups, which do not support the anxiolytic effect of this paradigm.

The present study is the first one applied the MSEW paradigm to rats while the others applied MS (Park et al., 2018; Isobe and Kawaguchi, 2019; Ströher et al., 2019) or EW (Kanari et al., 2005; Ito et al., 2006; Shimozuru et al., 2007) to rats. And a few previous studies applied MSEW procedure to mice (Carlyle et al., 2012; George et al., 2010). Long-term MS was shown to induce compensatory maternal care as seen in rat dams (Macrì et al., 2008). EW decreased play-fighting behaviors during the postweaning developmental period in Wistar rats, and increased anxiety levels during early adulthood (Shimozuru et al., 2007). In another study, EW rats showed increased locomotion and greater rearing activity in the open field but did not show anxiety increase in the open-field and elevated-plus maze tests

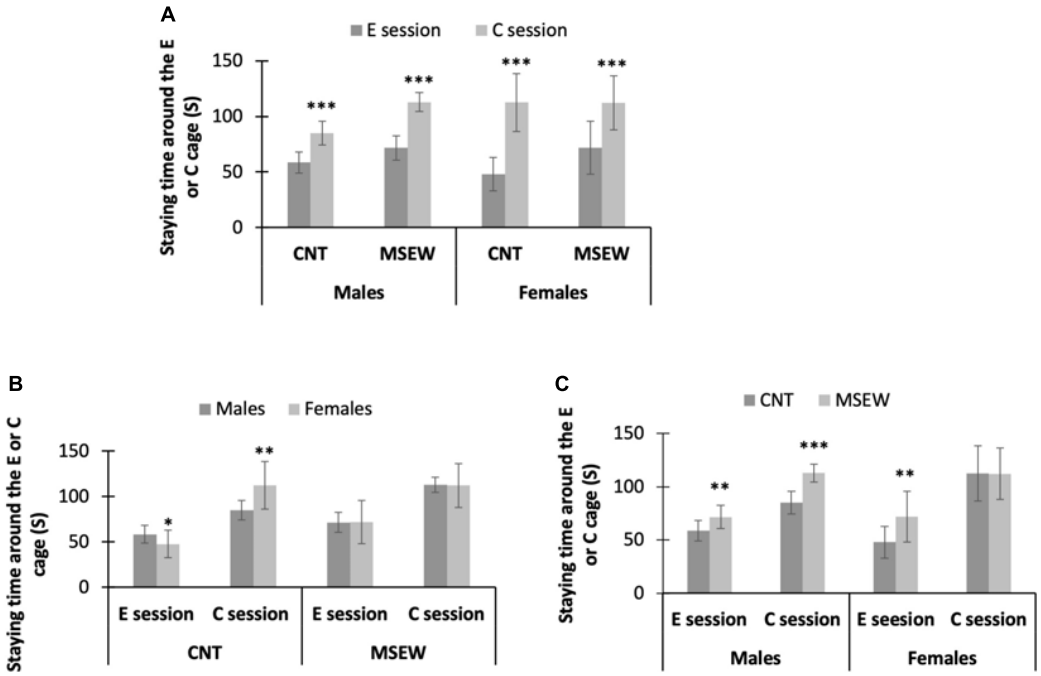

FIGURE 4 | Performance of rats in social interaction test. **(A)** The comparisons between the E session and C session, in terms of the time spent on interaction zone. **(B)** The comparisons between males and females in either CNT or MSEW rats, in terms of the time spent on interaction zone. **(C)** The comparisons between CNT and MSEW rats in either gender, in terms of the time spent on interaction zone. Data were expressed as mean ± SD. n = 15/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

TABLE 3 | Performance of adult rats on the elevated-plus maze test.

	CNT		MSEW	
Measurements	**Males**	**Females**	**Males**	**Females**
Tcj (S)	179.39 (50.33)	121.74 (21.48)$^{***,\#}$	210.44 (30.97)	151.23 (22.73)$^{***,\#\#}$
Tc (S)	254.54 (47.57)	285.78 (40.22)	211.41 (40.05)$^{\#}$	218.51 (22.15)$^{\#\#}$
To (S)	56.77 (18.28)	63.30 (16.34)	77.38 (23.51)$^{\#}$	110.57 (25.14)$^{***,\#\#}$
To/Tc	0.22 (0.07)	0.23 (0.07)	0.39 (0.02)$^{\#\#\#}$	0.52 (0.02)$^{*,\#\#\#}$
Ncj (N)	24.49 (7.00)	51.50 (21.21)***	29.60 (4.76)	57.53 (21.28)***
Nc (N)	21.13 (6.30)	45.22 (17.50)***	22.09 (4.55)	54.68 (22.38)***
No (N)	5.91 (2.18)	15.88 (7.89)***	8.74 (3.75)	25.15 (10.09)***

Data were expressed as means (SD) (n = 15).
CNT, rats in this group were raised under normal condition with no experience of MSEW; MSEW, rats in this group were subjected to MSEW procedure; Tcj, staying time (in seconds) at the central junction; Tc, staying time in closed arms; To, staying time on open arms. Ncj, number of entries into the central junction; Nc, number of entries into closed arms; No, number of entries into open arms.
$^{*}p < 0.05$, $^{***}p < 0.0001$, males vs females in either CNT or MSEW rats.
$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.0001$, CNT vs MSEW rats in either males or females.

(Ishikawa et al., 2014). MSEW mice spent less time on central part of the open-field and moved significantly faster than controls during the first 5 min of test (George et al., 2010; Carlyle et al., 2012). In line with these previous studies, MSEW rats in this study presented higher levels of anxiety demonstrated by shorter moving distance at central zone of the open-field and less time spent at the zone relative to controls. Moreover, MSEW rat moved a greater amount of distance with a faster speed on whole arena of the open-field as compared to CNT rats, indicating a higher level of locomotor activity induced by MSEW. Taken together, MSEW exerted same anxiogenic effects on male and female rats in open-field test.

In the social interaction test, both MSEW and CNT rats were able to tell an empty cage from a partner-containing cage as evidenced by spending more time at the social interaction zone in the presence of a partner-containing cage compared to the scenario of the empty cage, confirming the social preference of the rats, i.e., preference to investigate a novel conspecific over a novel object. Further analysis revealed different performance of male and female CNT rats in the social interaction test, i.e., male CNT rats spent more time with the empty cage relative to females whereas female CNT rats spent much more time with the partner-containing cage than male CNT rats did. These results suggest that male rats prefer to investigate a novel object (the empty cage) whereas females are featured with the social preference. Intriguingly, these sex-specific social behaviors are in contrast to the observation of a recent animal study in which female rats spent a greater amount of time with the novel object (empty cage) as compared to males (Scholl et al., 2019). In seeking the impact factors that may account for the contrast social behavior patterns between the rats across the two studies, we noticed a major difference between the social interaction test procedures applied in the two studies. In brief, each session of the two test sessions lasted for 5 min in the study by Scholl et al. (2019) whereas it was 2.5 min long in the present study. During a longer duration of testing, a tested

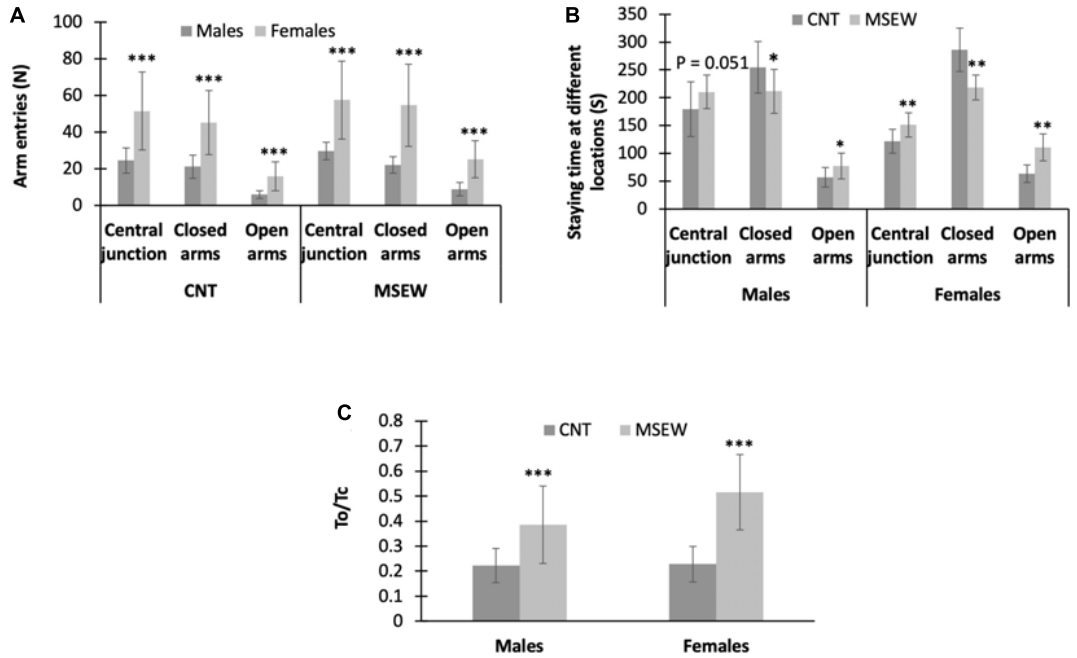

FIGURE 5 | Performance of rats on elevated-plus maze. **(A)** The comparisons between males and females in either CNT or MSEW rats, in terms of the number of entries into different parts of the elevated-plus maze. **(B)** The comparisons between CNT and MSEW rats in either gender, in terms of the time spent at different parts of the elevated-plus maze. **(C)** The comparisons between CNT and MSEW rats in either gender, in terms of To/Tc ratio. Data were expressed as mean ± SD. n = 15/group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

rat is more likely to adapt to an environment (empty cage or the same cage with an unfamiliar conspecific). With the only two studies compared, it is hard to know which test duration is more appropriate.

More importantly, these sex-specific patterns in social behavior were not seen in MSEW rats, indicating that MSEW differently impacted the performance of male and female rats in social interaction test. Specifically, MSEW made female rats spent more time with the empty cage relative to CNT rats, that is, it reversed the social behavior pattern in CNT rats in whom male (CNT) rats spent more time with the empty cage relative to females. In either case, a preference for a novel object is indicative of a social avoidance behavior (Scholl et al., 2019). Relevantly, a previous animal study reported that parental separation enhanced active avoidance learning in juvenile rodents (Abraham and Gruss, 2010). These social avoidance behaviors in animals are reminiscent of the attachment avoidance seen in humans (Mikulincer and Shaver, 2007; Mikulincer and Shaver, 2019). People with avoidant attachment rely on deactivating strategies, i.e., do not seek proximity, deny attachment needs, and avoid closeness and interdependence in relationships (Mikulincer and Shaver, 2007).

Another significant effect of MSEW on social behaviors of rats manifested as more time spent during E and C sessions by male MSEW rats as compared to CNT rats, suggesting that MSEW increased the social preference of male rats. Along with increased anxiety level of MSEW rats as shown in open-field test, the performance of male MSEW rats in social interaction test may be interpreted as a phenotype of attachment anxiety, another type of attachment insecurity seen in humans (Mikulincer and Shaver, 2007). People with attachment anxiety rely on hyperactivating strategies demonstrated by energetic attempts to achieve proximity, support, and love as they have no confidence that these resources will be provided (Cassidy and Kobak, 1988).

On elevated-plus maze, female (CNT, MSEW) rats entered open arms, closed arms, and central junction more frequently than males, MSEW (male, female) rats spent much more time on open arms and central junction, but less time in closed arms, as compared to CNT rats. These results are in line with a recent study reporting that female rats spent more time on open arms and more frequently entered open arms as compared to males. Females also traveled a greater distance than males regardless of estrus cycle stage (Scholl et al., 2019). Moreover, this less anxiety-like behavior on the elevated-plus maze has been observed in many of previous studies of female vs. male rats (Diaz-Veliz et al., 1997; Frye et al., 2000; Aguilar et al., 2003; Lopez-Aumatell et al., 2008, 2011). It was speculated that the sex differences in rodent tests of anxiety relate to sex-differences in stress-coping as evidenced by the observation that female rats showed enhanced reactive or compensatory coping strategies to stressors as compared to males (Lopez-Aumatell et al., 2008). Moreover, females have been shown to be more vulnerable to mild stress than males exposed to the same stressors as evidenced by biological measures such as altered serotonergic activity and increased corticosterone (Dalla et al., 2005, 2011).

The aforementioned data of previous studies and this one suggest that the seemingly less anxiety-like behavior of female rats may be viewed as a different form of anxiety-like behavior that are not well captured by traditional testing. Indeed, the elevated-plus maze test was used to assess risk-taking behavior of rats (Tillmann and Wegener, 2019). From this point of view, that MSEW rats spent more time on open arms and central junction but less time in closed arms as compared to CNT rats may be interpreted as a higher level of risk-taking behavior due to an anxiogenic instead of an anxiolytic effect of this paradigm. This interpretation is in line with the inference from the open-field test data, i.e., MSEW increased anxiety levels in either male or female rats. But the sex-specific effects of MSEW on behaviors of rats on the elevated-plus maze suggest that females are more vulnerable to MSEW compared to males. Following this notion, that MSEW rats spent more time on the central junction of elevated-plus maze indicates that they experienced a greater difficulty in making a decision on which arms to approach, i.e., they could not correctly cope with the threats of staying on the elevated-plus maze. Then that MSEW rats spent more time on open arms indicates an incorrect coping strategy of them in face of these danger parts of the apparatus. Taken together, the data from elevated-plus maze test provide further evidence for MSEW-induced attachment insecurity in rats.

Supporting evidence for the adverse effects of MSEW also came from the weight gain data of rats, including (1) body weights of CNT and MSEW rats were comparable at each timepoint, (2) male CNT rats were heavier than females at PD 30, and (3) female MSEW rats had lower body weight than male MSEW rats at PD 14 and thereafter. The first finding suggests that MSEW did not result in any nutritional deficits or did not induce significant changes in feeding behavior of rats during the MSEW period. This is in accordance with the previous study by George et al. (2010), in which the MSEW protocol showed no effect on weight gain of mice during PD 10–83. The second finding is fully consistent with the weight gain chart of S-D rats, in which males and females began to differ immediately after postnatal week 4. Interestingly, the gender-specific difference in rat weight gain appeared at PD 14 and continued thereafter in MSEW rats, indicating that female rats are more sensitive to MSEW while males are more tolerable to MSEW. This interpretation is in line with the behavioral data presented above indicating higher level of risk-taking behavior and attachment avoidance phenotype in female MSEW rats as compared to male counterparts featured with attachment anxiety. More importantly, the early onset of lower weight in female MSEW rats relative to males implies that attachment avoidance hurt the female subjects more than attachment anxiety did. This inference has specific relevance to extant clinical observations pointing to a higher prevalence of affective disorders such as anxiety and depression in women (Kessler et al., 1994, 2012; Seeman, 1997; Holden, 2005; Altemus et al., 2014).

In conclusion, MSEW induced emotional dysregulation in early adult rats with behavioral phenotype alike to attachment insecurity seen in humans as a consequence of early life adversity. Specifically, the behavioral phenotype of male MSEW rats is alike to attachment anxiety as evidenced by higher anxiety level detected in open-field test and much more social interaction time in both E and C sessions in the social interaction test. The phenotype of female MSEW rats is like attachment avoidance demonstrated by higher anxiety level measured in open-field test, risk-taking behaviors on the elevated -plus maze, and preference to investigate a novel object (an empty cage) in social interaction test as compared to female CNT rats. The attachment insecurity in MSEW rats made it difficult for them to make a decision on whether approaching to or averting from which arms of the elevated-plus maze. Last but not least, the delayed weight gain in female MSEW rats relative to males implies that attachment avoidance hurt the female subjects more than attachment anxiety did. This inference has relevance to the clinical observations pointing to higher prevalence of affective disorders such as anxiety and depression in women.

We are aware of a couple of limitations of this study. For instance, the maternal care behaviors of dams following the separation period were not monitored. Previous studies have shown that neonatal social isolation alters both maternal and pup behaviors in rats (Zimmerberg et al., 2003; Starr-Phillips and Beery, 2014). Technically, further social tests would be required to provide adequate proof for the conclusions from this study. These could include mating behavior, response to socially relevant cues, i.e., USV (ultrasonic vocalizations) playback paradigms or social odor tests. In a recent study, social and non-social behaviors together with concomitant emission of 50-kHz USV were measured in rats (Redecker et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of Shantou University Medical College.

AUTHOR CONTRIBUTIONS

HZ, QH, and HX designed the study. HZ and ZY conducted the experiments and collected the data. HX interpreted the results and drafted the manuscript. All authors have read and approved the final version of the submitted manuscript.

FUNDING

This work was supported by grants from the Li Ka Shing Foundation (43209502) and the Science and Technology Bureau of Guangdong Province, China (2016A030313067).

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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.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 19 April 2021
doi: 10.3389/fnbeh.2021.675994

Resurgence of an Inborn Attraction for Animate Objects via Thyroid Hormone T_3

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For inexperienced brains, some stimuli are more attractive than others. Human neonates and newly hatched chicks preferentially orient towards face-like stimuli, biological motion, and objects changing speed. In chicks, this enhances exposure to social partners, and subsequent attachment trough filial imprinting. Early preferences are not steady. For instance, preference for stimuli changing speed fades away after 2 days in chicks. To understand the physiological mechanisms underlying these transient responses, we tested whether early preferences for objects changing speed can be promoted by thyroid hormone 3,5,3′-triiodothyronine (T_3). This hormone determines the start of imprinting's sensitive period. We found that the preference for objects changing speed can be re-established in female chicks treated with T_3. Moreover, day-1 chicks treated with an inhibitor of endogenous T_3 did not show any preference. These results suggest that the time windows of early predispositions and of sensitive period for imprinting are controlled by the same molecular mechanisms.

Keywords: animacy, thyroid hormone, avian, sensitive period, plasticity, T_3

OPEN ACCESS

Edited by:
Gervasio Batista, Harvard University, United States

Reviewed by:
Brian McCabe, University of Cambridge, United Kingdom
Koichi J. Homma, Teikyo University, Japan

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Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *04 March 2021*
Accepted: *24 March 2021*
Published: *19 April 2021*

Citation:
Lorenzi E, Lemaire BS, Versace E, Matsushima T and Vallortigara G (2021) Resurgence of an Inborn Attraction for Animate Objects via Thyroid Hormone T_3. Front. Behav. Neurosci. 15:675994. doi: 10.3389/fnbeh.2021.675994

INTRODUCTION

Early experience plays a crucial role in shaping neural and behavioural development. However, early experience effects are stronger during certain periods of development (Hensch, 2005). An example is provided by filial imprinting (Spalding, 1873; Hess, 1959; Bateson, 1979; Horn, 2004; McCabe, 2013; Vallortigara and Versace, 2018). Shortly after birth or hatching, the young of some animals, usually of precocial species, learn to recognise their social partners (e.g., the mother and siblings) by simply being exposed to them. In the young domestic fowl (*Gallus gallus*), for instance, imprinting usually occurs within 24–48 h from hatching. Lorenz (1935) used the term "critical period" to refer to the fact that rather than being available throughout the lifespan, filial imprinting is shown only during a limited period of life. This time window can be extended for a few more days when a proper stimulus is not immediately available (Bolhuis, 1991). Being a flexible period with the characteristics of a self-terminating process (Bolhuis, 1991), imprinting is now better known as a "sensitive period." Sensitive periods are windows of plasticity during brain development, in which experience has a powerful effect (Hensch, 2005; Dehorter and Del Pino, 2020).

The thyroid hormone 3,5,3′-triiodothyronine (T_3) is implicated in the timing of the sensitive period for imprinting (Yamaguchi et al., 2012). In domestic chicks, imprinting causes a rapid inflow

of T_3 in the brain, particularly in the intermediate medial mesopallium (IMM), an associative telencephalic region involved in learning the features of the imprinting object (Horn, 2004; Yamaguchi et al., 2012). Via Wnt-signalling pathway, the enzyme Dio_2 (type 2 iodothyronine deiodinase), localised in vascular endothelial cells of the brain, converts the inactive form thyroxine (T_4) into the active form T_3 (Yamaguchi et al., 2012, 2018). Endogenous T_3 level in the brain peaks around the peri-hatch period, and decays within a few days if not boosted by imprinting. Injection of iopanoic acid (IOP), a potent inhibitor of Dio_2, impairs visual imprinting during the sensitive period (Yamaguchi et al., 2012). After the sensitive period, administration of exogenous T_3 allows non-imprinted chicks to imprint, re-opening the sensitive period for memory formation (Yamaguchi et al., 2012).

Re-opening of sensitive periods has been obtained also by other pharmacological agents (Batista et al., 2016, 2018; Yamaguchi et al., 2016; Aoki et al., 2018), and it has been discovered for phenomena other than filial imprinting, such as ocular dominance in non-human mammals (Hensch and Quinlan, 2018) and absolute pitch in humans (Gervain et al., 2013).

Although imprinting can occur with either naturalistic stimuli (resembling a conspecific) or artificial objects, a large amount of evidence shows the existence of spontaneous unlearned preferences for animate features (Rosa-Salva et al., 2021). These preferences act as a sort of canalisation mechanism to direct the newborns' attention, favouring exposure to stimuli that are more likely to be social partners (Di Giorgio et al., 2017; Versace et al., 2018; Rosa-Salva et al., 2021). Preferences for animacy cues, that set apart animate from non-animate objects, have been described in newly hatched chicks, comprising, e.g., preferences for face-like stimuli (Rosa-Salva et al., 2010), biological motion stimuli (Vallortigara et al., 2005; Miura and Matsushima, 2016; Miura et al., 2020) and self-propelled objects that move with variable speed (Rosa-Salva et al., 2016; for review see: Di Giorgio et al., 2017; Lorenzi and Vallortigara, 2021; Vallortigara, 2021). The same animacy cues operate on human newborns and other species, in particular for the preference for speed changes we are dealing with here (see in the human newborns Di Giorgio et al., 2021 and see for reviews: Di Giorgio et al., 2017; Lorenzi and Vallortigara, 2021; Rosa-Salva et al., 2021; Vallortigara, 2021).

These biological priors, whose main function seems to speed up learning by canalising imprinting, also operate only during transient windows of sensitivity in development. Visually naïve chicks show a spontaneous preference for the head (face-like) region of a stuffed hen during the first 2 days post-hatching, which then fades away on day 3 (Johnson et al., 1989). The spontaneous preference for objects moving with visible speed changes (Rosa-Salva et al., 2016) shows a window of sensitivity in three genetically selected and isolated breeds of chicks for only the first day of life, then disappearing on day 3 (Versace et al., 2019). Similarly, the biological motion preference occurs only within the first few days of life (Miura and Matsushima, 2012). Importantly, appearing slightly later on day 2 of life, biological motion preference exhibits also sex differences, with females being choosier than males when approaching a biological motion stimulus (Miura and Matsushima, 2012).

From the second day of life, precocious sexually dimorphic behaviours start to emerge in chicks (Andrew, 1966). Due to different levels of social motivation and aggression, males and females exhibit different attitudes towards familiar and unfamiliar individuals (Cailotto et al., 1989; Vallortigara et al., 1990; Vallortigara, 1992; Versace et al., 2017; Lemaire et al., 2020; Santolin et al., 2020). Progressively, females develop strong social cohesive behaviours with familiar subjects, while males engage more in aggressive and explorative ones (McBride and Foenander, 1962; McBride et al., 1969). At the neurobiological level, imprinting-related gene expression in the brain shows remarkable differences between sexes (Yamaguchi et al., 2012); among others, an upregulation of Dio_2 gene enabling imprinting, which appears to be quantitatively different between males and females (Yamaguchi et al., 2012).

Here we report that the thyroid hormone T_3 can modulate the timing of one window of early preferences for animacy cues, i.e., change of speed. Different from the sensitive period for imprinting, this preference does not depend on a particular experience with stimuli but rather canalises the animal's attention towards particular stimuli, working as a spontaneous, unlearned biological prior.

We devised three different experimental conditions. The first was aimed to confirm that the same window of sensitivity for the spontaneous animacy preference conveyed by visible speed changes shown in genetically selected strains (Versace et al., 2019) also exists in the strain of broiler chicks we were using in the lab. We confirmed that the preference is there on post-hatching day 1, but fades away on day 2. To check whether T_3 influences the duration of this window of sensitivity, we performed two experiments. First, in order to show that inhibition of endogenous T_3 action can abolish the animacy preference, we injected the inhibitor IOP on post-hatching day 1, and compared IOP-injected chicks with vehicle-injected peers. Second, in order to show that the animacy preference can be re-established by exogenous T_3 administration, we injected chicks with T_3 on post-hatching day 3 and compared T_3-injected chicks with vehicle-injected peers.

RESULTS

The results for the not-injected condition are shown in **Figure 1**. The permutation test revealed a significant main effect of testing Day ($F_{(1,67)} = 5.40$, $p < 0.05$), but did not reveal any effect of Sex ($F_{(1,67)} = 1.17$, $p = 0.28$) nor interaction ($F_{(1,67)} = 0.05$, $p = 0.83$; see **Supplementary Table 1** for the number of subjects tested in each condition for each sex, and for results split by sex). Chicks tested on day 1 showed a significant preference for animacy ($V_{(35)} = 458$, $p < 0.05$, $d = 0.44$), whereas chicks tested on day 3 did not show any preference ($V_{(36)} = 310$, $p = 0.72$, $d = 0.08$).

The results for chicks treated on day 1 with the IOP (Dio_2-inhibitor) are shown in **Figure 2**. The permutation test revealed a significant main effect of Treatment ($F_{(1,54)} = 5.74$, $p < 0.05$), but did not reveal any effect of Sex ($F_{(1,54)} = 1.10$, $p = 0.30$)

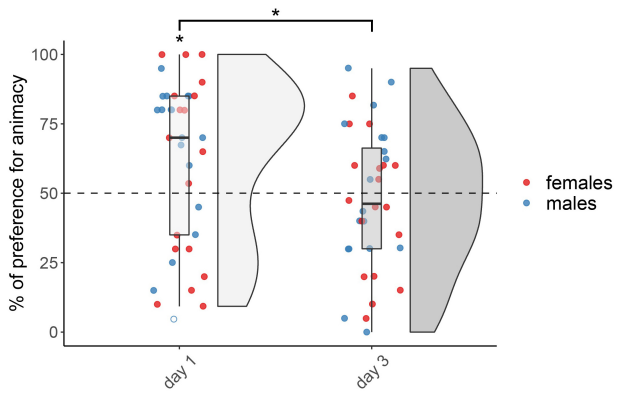

FIGURE 1 | Preference for animacy for the not-injected chicks tested 1 or 3 days after hatching. To best represent the data, we used raincloud plots. Asterisks indicate significant differences from chance (black dotted line). Black asterisk between the two groups indicates a significant difference between testing Days. * indicates $p < 0.05$. Filled red dots represent female and blue male subjects, empty dots represent outliers, which were removed from the analyses.

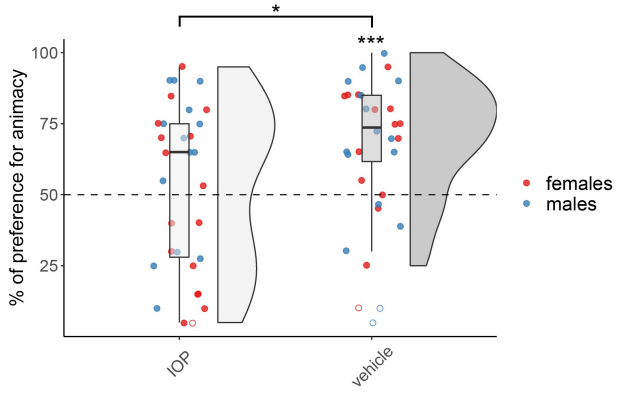

FIGURE 2 | Preference for animacy for the chicks tested 1 day after hatching injected with IOP or with vehicle. To best represent the data, we used raincloud plots. Asterisks indicate significant differences from chance (black dotted line). Black asterisk between the two groups indicates a significant difference between IOP- and vehicle-injected chicks. *** indicates $p < 0.001$, * indicates $p < 0.05$. Filled red dots represent female and blue male subjects, empty dots represent outliers, which were removed from the analyses.

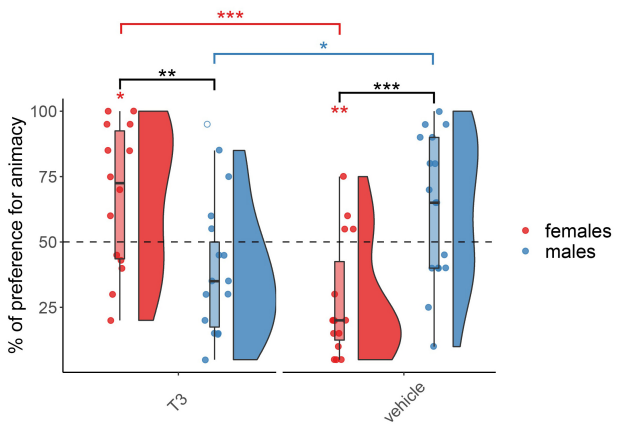

FIGURE 3 | Preference for animacy for the chicks tested 3 days after hatching injected with T_3 or vehicle. To best represent the data, we used raincloud plots. Black asterisks indicate a significant difference between sexes (females red and males blue) within each injection group. Red asterisks above a red horizontal line indicate a significant difference between females of each injection group. Blue asterisks above a blue horizontal line indicate a significant difference between males of each injection group. Red asterisks indicate a significant difference from chance in females. *** indicates $p < 0.001$, ** indicates $p < 0.01$, * indicates $p < 0.05$. Filled red dots represent female and blue male subjects, empty dots represent outliers, which were removed from the analyses.

nor interaction ($F_{(1,54)} = 0.66$, $p = 0.42$; see **Supplementary Table 1**). The vehicle-injected group showed a significant preference for animacy ($V_{(28)} = 347$, $p < 0.001$, $d = 1.01$), whereas the IOP-injected group did not show any preference ($V_{(30)} = 268.5$, $p = 0.46$, $d = 0.14$). As expected, at this age no sex differences were apparent.

The results for the chicks injected with T_3 on day 3 are shown in **Figure 3**. As expected, the permutation test revealed at this age a significant interaction between Treatment and Sex ($F_{(1,57)} = 25.02$, $p < 0.001$) but did not reveal any main effect of Treatment ($F_{(1,57)} = 1.27$, $p = 0.26$) or Sex ($F_{(1,57)} = 0.20$, $p = 0.65$). Females and males showed a different pattern within each group, T_3-injected ($W_{(29)} = 166.5$, $p < 0.01$, $d = 1.17$) and vehicle-injected ($W_{(32)} = 39.5$, $p < 0.001$, $d = 1.40$). T_3- and vehicle-injected females showed a significant difference in their preferences ($W_{(29)} = 183.5$, $p < 0.001$, $d = 1.59$). T_3-injected females showed a significant preference for animacy ($V_{(14)} = 85$, $p < 0.05$, $d = 0.63$), whereas vehicle-injected females showed a significant preference for the non-animacy stimulus ($V_{(15)} = 11$, $p < 0.01$, $d = 1.0$). T_3- and vehicle-injected males also showed a marginally significant difference in their preferences ($W_{(32)} = 61$, $p < 0.05$, $d = 0.98$). However, in spite of a trend for an inverted pattern with respect to females, T_3-injected males did not show any significant preference for the stimuli ($V_{(15)} = 27$, $p > 0.05$, $d = 0.53$) nor did vehicle-injected males ($V_{(17)} = 116$, $p > 0.05$, $d = 0.47$).

DISCUSSION

Untreated chicks showed a clear spontaneous preference for visible speed changes, a self-propelled cue to animacy (Rosa-Salva et al., 2016), on post-hatching day 1 that disappeared on day 3. Restricted sensitivity windows to animacy motion cues seem thus to exist in chicks similar to those for the head region of the mother hen and for biological motion (Johnson et al., 1989; Miura and Matsushima, 2012). Similar sensitivity windows have been described in humans as well. During the first month of life infants preferentially look at schematic face-like configurations over identical stimuli in a non-face configuration, whereas the same is not observed

at 3 and 5 months of age (Johnson et al., 1991). Also, the intensity of selective EEG responses to face-like patterns tends to decrease over time after the first hours of life (Buiatti et al., 2019).

The thyroid hormone T_3 appears to play a crucial role in the sensitivity window for spontaneous motion animacy preference. Animacy preference disappeared on day 1 in chicks injected with IOP, an inhibitor of Dio_2 the enzyme converting the inactive form T_4 into the active T_3. While vehicle-injected day 1 chicks had a significant preference for animacy, consistently with previous findings (Rosa-Salva et al., 2016).

On day 3, administration of T_3 seems to restore animacy preference, at least in females. Sex differences, which are well-known to be associated with critical period regulation by thyroid hormones (see Miura and Matsushima, 2012; Yamaguchi et al., 2012; Batista and Hensch, 2019), seem to be complicated at this age also by the effect of the injecting needle in itself. T_3-injected females preferred the animacy stimulus, while vehicle-injected females preferred the non-animacy one. Given that we do not expect any physiological effect of vehicle (as indeed this is the case for day-1 treated chicks), it seems that in older chicks the mere, though mild, pain experience of injecting the needle interacts with the expression of animacy preferences at least in females (males showed a somewhat similar effect but with inverted direction).

Precocious sex differences in social motivation and aggression are commonly observed in chicks (Andrew, 1966). Functionally, it has been argued that they may arise from different levels of social motivation (Cailotto et al., 1989; Vallortigara et al., 1990) and attitudes towards novelty (Vallortigara, 1992) in the two sexes. Females tend to engage more than males in social reinstatement and usually prefer to approach familiar individuals (Cailotto et al., 1989; Vallortigara et al., 1990; Vallortigara, 1992), whereas males tend to approach unfamiliar ones (Vallortigara, 1992; Versace et al., 2017; Santolin et al., 2020). In natural populations fowls exhibit territorial behaviour wherein single dominant cocks maintain and patrol a large territory within which several highly social-aggregated females live (McBride and Foenander, 1962; McBride et al., 1969). This social organisation may favour the prevalence of gregarious and affiliative behaviours in females and aggressive and exploratory behaviours in males.

Sex differences have also been described for biological motion preferences, whose window of sensitivity occurs exactly around day 3 (Miura and Matsushima, 2012). Therefore, it could be that the injection of T_3 affected both sexes similarly, but that they exhibited different behavioural responses coherent with their natural preferences that become apparent from day 3. Females would thus preferentially approach the animacy stimulus, engaging in social reinstatement with the most familiar, as predisposed, object. Whereas males, less socially motivated, would engage in explorative responses towards the unfamiliar, non-predisposed, object.

It could also be that the efficiency of T_3 transport from muscle through blood vessels and finally to the brain is different between males and females. This may point towards a possible limitation of the method adopted here and suggests different T_3 administration strategies.

From a neurobiological perspective, sex differences related to imprinting have been found in the brain. Several genes upregulate during imprinting, but only some do it in a sex dimorphic way. The Dio_2 gene expresses more in males than in females during imprinting, while Tubby-like protein 1 gene, producing an upstream cell signalling protein, expresses more in females than males (Yamaguchi et al., 2012). Such differences might underly the behavioural differences between the two sexes observed in the present study in older chicks.

Note that vehicle-injected females on day 3 preferred the non-animacy stimulus, whereas males had a trend to prefer the animacy one. From day 3, chicks start to be more fearful due to the rising of hormonal levels (Schaller and Emlen, 1962; Rogers, 1995). Avoidance behaviours increase progressively from day 3 on, but with different timings in the two sexes. Males show a weaker avoidance response than females until post-hatching day 4 (Schaller and Emlen, 1962). Sex differences observed in the vehicle group on day 3 might arise from the different reactions to the needle's stressful event. The fear caused by the injection in females might have evoked an avoidance response towards the most animate object, the animacy stimulus, making them walk to the opposite end of the apparatus. In males, less avoidant and more prone to aggression at this age, the same event could have evoked a tendency to approach the most animate stimulus.

It is also worth noting that early during development the levels of different thyroid hormones (T_3 and T_4, thyroxine) in the brain show sex-dependent differences. The onset of the surge of T_4 in male zebra finches (*Taeniopygia guttata*) precedes that of females, while the onset of T_3 in females precedes that of males (Yamaguchi et al., 2017). Strengthening the hypothesis of an interaction between T_3 and other sexually dimorphic hormones, response to animacy in visually naïve chicks involves brain regions rich in sex steroid hormone receptors (Mayer et al., 2016, 2017a,b; Lorenzi et al., 2017), part of the so-called *Social Behavior Network*, which appears to be highly conserved in vertebrates (Newman, 1999; O'Connell and Hofmann, 2011; Goodson and Kingsbury, 2013; Lorenzi et al., 2017).

Thyroid hormones are key regulators of vertebrates' brain development (Van Herck et al., 2013). Among others, T_3 directly influences the expression of genes by binding to its nuclear receptors, which act as transcription factors (Harvey and Williams, 2002). Therefore, abnormalities in the level of T_3 during development may result in permanent impairments. In the present study, inhibiting T_3 function gave rise to a lack of the spontaneous approach behaviour towards animacy stimuli. Abnormalities in spontaneous preferences for animacy at birth have been linked to autistic spectrum disorder (Sgadò et al., 2018; Lorenzi et al., 2019). Interestingly, human neonates at high familial risk for autism exhibit anomalous preferential looking patterns to animacy cues provided by schematic face-like and biological motion stimuli (Di Giorgio et al., 2016).

CONCLUSION

In conclusion, we showed that providing exogenous T_3 restores the predisposition to orient towards motion-animacy cue on day 3 post-hatch, whereas inhibiting the endogenous T_3 conversion prevents the predisposition to appear on day 1. The similarity of thyroid hormone effects on imprinting and on early predispositions is unlikely to be coincidental. Furthermore, re-opening the neural plasticity to the effects of environmental stimuli without restoring at the same time the biological priors for proper environmental stimulation could appear worthless. The two processes should be functionally and temporally linked, and probably for this reason they share the same molecular ground.

MATERIALS AND METHODS

Animals

All experiments were carried out in compliance with the applicable European Union and Italian laws, and guidelines for animals' care and use. All the experimental procedures were approved by the Ethical Committee of the University of Trento OPBA and by the Italian Health Ministry (permit number 1139/2015).

Fertilised eggs of the Aviagen Ross 308 strain were provided by a commercial hatchery (Azienda Agricola Crescenti, Brescia, Italy). Upon arrival, eggs were incubated under controlled temperature (37.7°C until post-hatching day 1, 33°C until post-hatching day 3) and humidity (60%) within incubators (FIEM MG140/200 Rural LCD EVO). Incubators were kept in darkness, preventing the chicks from any visual experience during incubation and hatching prior to testing. We employed a total of 190 domestic chicks (*G. gallus*; 95 females; the exact number of chicks in each condition and sex is shown in **Supplementary Table 1**). Sex was determined by feather dimorphism.

Apparatus and Stimuli

We used the same apparatus and stimuli as in previous studies investigating motion-animacy preference in newly hatched chicks (Rosa-Salva et al., 2016, 2018; Lorenzi et al., 2017, 2019). A detailed description of the apparatus, stimuli and procedure used can be found in Rosa-Salva et al. (2016) (Exp. 2). Briefly, the apparatus consisted of a white corridor (85 × 30 × 30 cm, see **Figure 4**) with two opposite high-frequency monitor screens (ASUS MG248Q, 24″, 120 Hz). Three areas subdivided the corridor: a central one (starting area: 45 cm long) and two lateral ones (choice areas: 20 cm long each). A small step (1.5 cm high) on each side delimited the boundaries between starting and choice areas. A video-camera, centrally located above the apparatus, recorded animals' behaviour. The two stimuli were displayed on the screens and represented a red circle (diameter 3 cm) moving horizontally. One stimulus was moving at a constant speed (≈4.64 cm/s on our monitors) while the other one was visibly changing speed (the slower speed being ≈3.37 cm/s and the faster one being ≈19.64 cm/s), a reliable cue to animacy (Rosa-Salva et al., 2016). We counterbalanced the position of the stimuli on the screens between subjects.

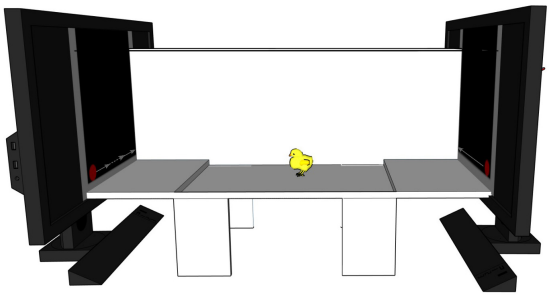

FIGURE 4 | Schematic representation of the experimental apparatus. A white corridor with two video-screens at the two opposite ends playing the stimuli. On the left monitor is depicted the stimulus changing speed (dotted line represents faster speed), on the right monitor is represented the constant moving stimulus. The chick is represented in the starting area at the centre. Two little steps divide it from the two choice areas. For representative purposes one of the two longitudinal walls is represented translucent.

Intramuscular Injections

Iopanoic acid (IOP 10 mM, TCI I0300, Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in 0.05M NaOH solution at 1 mM and rebuffered to pH = 8.5 by 6M HCl. 3,3′,5-Triiodo-L-thyronine (T_3, 100 μM, Sigma-Aldrich, T-2877) was dissolved in 0.002M NaOH and 0.9% NaCl. Vehicle solutions were also prepared to control for any effect of the injections. Respectively, the vehicle for IOP was a 0.05M NaOH solution buffered to pH = 8.5 by 6M HCl, while the vehicle for T_3 was a 0.9% NaCl and 0.002M NaOH solution (vehicle-injected groups). One hour before testing, each subject was carefully taken from the incubator in complete darkness. A black hood on the head prevented any source of visual stimulation during the intramuscular injection to thigh. Control chicks underwent the same procedure without receiving any injection (not-injected groups). Immediately after, each chick was placed back to the dark incubator. To distinguish single individuals in the darkness while keeping the same auditory environment experienced before injection, we placed the chicks in individual compartments within the same incubator.

Testing

One hour after injection (according to the different groups assigned), each chick was individually tested for the spontaneous preference for animacy for 10 min. After placing each subject in the starting area, the time spent in each sector of the corridor was recorded. In order to measure the animal preference for motion-animacy, we considered the ratio (%) of time spent near the animacy stimulus over the total choice time using the formula:

$$\text{Preference for animacy} = \frac{\text{time spent close to animacy}}{\text{time spent close to both stimuli}} \times 100.$$

The preference score could range from 0% (full preference for the non-animacy stimulus) to 100% (full preference for animacy),

while 50% represented the absence of preference. Permanence in the starting area for the total duration of the test was considered as no choice and led to the exclusion from further analyses (this occurred in about 47% of the cases). Sexing of the subjects occurred at the end of the procedure of testing.

Statistical Analysis

The statistical analyses were performed using RStudio v1.1 and the following packages: *goftest* (Faraway et al., 2019), *nlme* (Pinheiro et al., 2020), *tidyr* (Wickham et al., 2020), *plyr* (Wickham, 2011), *dplyr* (Wickham et al., 2020), *reshape* (Wickham, 2007), *lsr* (Navarro, 2013), *ggplot2* (Wickham, 2016), and *lmPerm* (Wheeler and Torchiano, 2016).

The number of subjects required in each group was *a priori* determined with a power analysis (Champely et al., 2020) with an effect size of 0.96, and an alpha of 0.05. Results showed that 18 individuals were required per group and per sex to achieve a power of 0.80.

To detect the presence of outliers, we used a Multivariate Model Approach using Cook's distance. Subjects having a Cook's distance four times greater than the group mean were considered as outliers and discarded from further analyses (Kannan and Manoj, 2015). We identified six outliers from different groups. To assess the normality of data distribution, we looked at the distribution of residuals (Q–Q plot).

As parametric assumptions were not met, we used non-parametric tests. In not-injected condition, to determine whether the testing Day (two levels: day 1 and day 3) and Sex (two levels: female and male) affected animacy preference, we performed a permutation test using *F*-test probabilities. A similar test was conducted for the IOP- and T_3-injected conditions, to determine whether the treatments (two levels: IOP/T_3 and respective vehicles) and Sex (Two levels: female and male) affected animacy preference.

To determine whether the preference was statistically different between groups within each condition (not-, IOP-, T_3-injected), we conducted two-sample Wilcoxon tests. To examine whether each group had a significant preference for either stimulus, we conducted one-sample Wilcoxon tests against chance level (50%). We calculated Cohen's *d* (*d*) for each Wilcoxon test performed.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of the University of Trento OPBA and by the Italian Health Ministry.

AUTHOR CONTRIBUTIONS

EL, BL, TM, GV, and EV: conceptualisation. EL, BL, TM, and GV: methodology. BL: software. EL and BL: formal analysis, investigation, writing–original draft, and visualization. GV and TM: resources. GV, TM, and EV: writing-review and editing. GV: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ Advanced Grant ERC PREMESOR G.A. (n 295517) to GV.

ACKNOWLEDGMENTS

Authors are particularly thankful to Orsola Rosa-Salva for fruitful discussion and advices.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.675994/full#supplementary-material

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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.

frontiers in Neural Circuits

MINI REVIEW
published: 29 July 2021
doi: 10.3389/fncir.2021.716605

Neuroendocrine Mechanisms Underlying Non-breeding Aggression: Common Strategies Between Birds and Fish

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Aggression is an adaptive behavior that plays an important role in gaining access to limited resources. Aggression may occur uncoupled from reproduction, thus offering a valuable context to further understand its neural and hormonal regulation. This review focuses on the contributions from song sparrows (*Melospiza melodia*) and the weakly electric banded knifefish (*Gymnotus omarorum*). Together, these models offer clues about the underlying mechanisms of non-breeding aggression, especially the potential roles of neuropeptide Y (NPY) and brain-derived estrogens. The orexigenic NPY is well-conserved between birds and teleost fish, increases in response to low food intake, and influences sex steroid synthesis. In non-breeding *M. melodia*, NPY increases in the social behavior network, and NPY-Y1 receptor expression is upregulated in response to a territorial challenge. In *G. omarorum*, NPY is upregulated in the preoptic area of dominant, but not subordinate, individuals. We hypothesize that NPY may signal a seasonal decrease in food availability and promote non-breeding aggression. In both animal models, non-breeding aggression is estrogen-dependent but gonad-independent. In non-breeding *M. melodia*, neurosteroid synthesis rapidly increases in response to a territorial challenge. In *G. omarorum*, brain aromatase is upregulated in dominant but not subordinate fish. In both species, the dramatic decrease in food availability in the non-breeding season may promote non-breeding aggression, via changes in NPY and/or neurosteroid signaling.

Keywords: neurosteroids, territoriality, food intake, testosterone, estradiol, songbird, aromatase, electric fish

Edited by:
Gervasio Batista, Regeneron Pharmaceuticals, Inc., United States

Reviewed by:
John C. Wingfield, University of California, Davis, United States
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Received: *28 May 2021*
Accepted: *06 July 2021*
Published: *29 July 2021*

Citation:
Quintana L, Jalabert C, Fokidis HB, Soma KK and Zubizarreta L (2021) Neuroendocrine Mechanisms Underlying Non-breeding Aggression: Common Strategies Between Birds and Fish. Front. Neural Circuits 15:716605. doi: 10.3389/fncir.2021.716605

INTRODUCTION

In all vertebrate classes, agonistic behavior is an adaptive social behavior that plays an important role in gaining access to limited resources. Arising early in animal evolution, aggression strongly impacts survival and fitness of individuals, and thus both aggressive behavior and its physiological regulation are under strong evolutionary pressures. This review focuses on two neuroethological models, the song sparrow (*Melospiza melodia*) and the weakly electric banded knifefish

(*Gymnotus omarorum)*, and their contributions to understanding the neuroendocrinology of agonistic behavior, particularly territorial aggression.

Although bony fish originated 400 MYA, while birds just 150 MYA, neuroanatomical and functional studies indicate that the neural circuits that regulate social behavior are highly conserved across vertebrates and play similar roles in the regulation of social behaviors (O'Connell and Hofmann, 2012). Originally described in mammals (Newman, 1999), the social behavior network (SBN) consists of reciprocally connected brain regions located in the forebrain, midbrain and hindbrain. More recent work suggests that a broader social decision-making network, which also includes the mesocorticolimbic reward system, regulates adaptive social behaviors in response to different contexts or stimuli. Birds and teleost fish, as well as reptiles and amphibians, all contain this social decision-making network that is homologous with the mammalian counterpart and has similar activation patterns in similar social contexts (O'Connell and Hofmann, 2012). These common features enable comparative studies in different species to establish general principles in the regulation of social behavior, such as aggression, among vertebrates. Both song sparrows and banded knifefish display territorial aggression throughout the year. Although aggression is generally more common in the breeding season, ecological pressures can also lead to territorial aggression in the non-breeding season, a behavior that is displayed in these two species, as well as in mammals (Jasnow et al., 2000, 2002; Trainor et al., 2006). Non-breeding territorial aggression offers a novel context to further understand the underlying mechanisms of aggression.

NON-BREEDING TERRITORIAL BEHAVIOR

Many animals carefully evaluate the cost–benefit ratios of agonistic interactions since such encounters are very costly in terms of time, energy, and potential injuries. In many species, individuals establish dominant-subordinate relationships to minimize the costs of protracted aggression. The dynamics of aggression are well-studied in both song sparrows and banded knifefish. Both display robust territorial aggression during the non-breeding season.

Melospiza melodia is common throughout North America. In the Pacific Northwest, where the climate is humid maritime, song sparrows are sedentary and exhibit year-round territoriality (except briefly during molt) (Wingfield and Hahn, 1994). Aggressive behavior in this species has been widely studied in the field. In a simulated territorial intrusion (STI), a live caged conspecific decoy and song playback are placed in the subject's territory for 10 or 30 min (Heimovics et al., 2013). During an STI, territorial males exhibit robust and stereotyped aggressive displays that are easily quantifiable. The number of songs, number of flights near the decoy, time spent within 5 m of the decoy, and closest approach to the decoy are recorded as indicators of aggressiveness (Heimovics et al., 2013). Similarly, in a laboratory-STI paradigm, the subject cage is placed adjacent to the decoy cage (with or without conspecific song playback) and the number of barrier contacts and time in proximity to the decoy cage are recorded. In both field and laboratory, males show similar behavioral responses year-round during the STI, although the persistence of aggression after the STI (when the stimuli are removed) is reduced during the non-breeding season (Wingfield, 1994). This reduction of persistence in the non-breeding season is energetically advantageous for these small songbirds (∼25 g body mass) at a time when temperatures are low, days are short, and food is scarce.

Gymnotus omarorum inhabits Uruguay, where the climate is humid subtropical. It displays year-round territorial aggression in both males and females, and non-breeding intrasexual aggression is robust and easily quantifiable (Batista et al., 2012; Silva et al., 2013; Quintana et al., 2016). In laboratory settings, the acquisition and defense of territories in non-breeding *G. omarorum* are mediated by agonistic encounters (Perrone et al., 2019). During dyadic encounters in a neutral arena, fish engage in rapid escalating conflicts that resolve in <3 min, with the establishment of a clear dominant/subordinate status. The only known predictor of contest outcome is body size. Agonistic behavior in *G. omarorum* is subdivided into three distinct phases, each with characteristic behaviors. First is a brief evaluation phase that ends with the first attack. Second is a contest phase characterized by overt aggression, where attacks of both contenders correlate positively, showing escalation. Last is a post-resolution phase where the dominant may continue attacking while the subordinate fish retreats without retaliation (Batista et al., 2012; Zubizarreta et al., 2015). In *G. omarorum* contests, subordinates display electric signals in a sequential pattern: first interrupting their electric discharge, then emitting transient electric communication signals in "chirps" and finally, adopting a lower post-resolution discharge rate (Batista et al., 2012; Perrone and Silva, 2018).

Why do animals display territorial aggression in the non-breeding season? It has been proposed that this behavior may arise to secure breeding sites for future reproduction, for shelter, and/or to ensure food resources. In sedentary bird populations in mid to high latitudes, such as song sparrows, territorial aggression in the non-breeding season increases survival by allowing access to food to meet the large energetic costs during cold winters. This seems especially important in hatch-year males, where individuals that gain territories in their first autumn have a higher overwinter survival rate than those that do not (Arcese, 1989). In these latitudes, non-breeding birds face multiple factors impacting metabolism, including reduced food availability, reduced foraging time due to shorter day lengths and inclement weather, and depletion of energy reserves to endure longer overnight fasts during low temperatures (Heimovics et al., 2013). Metabolite profiling reveals non-breeding male song sparrows exhibit lower fat deposition and higher fatty acid oxidation compared to breeding birds (Fokidis et al., 2019). This is consistent with a shift toward a catabolic state with an increased reliance on stored fat reserves, and this could amplify the need for non-breeding aggression to maintain access to a replenishing food supply.

In teleost fish, year-round territoriality also seems to be related to ensuring foraging grounds. Tropical damselfishes establish

well-defined year-round territories on corals (Brawley and Adey, 1977; Wallman et al., 2004) where they cultivate algae as a main food source (Lobel, 1980; Sammarco et al., 1983). When fish are not reproductively active, both sexes are highly territorial, fiercely defending their food source (Karino and Kuwamura, 1997; Hata and Umezawa, 2011). *Gymnotus omarorum,* from mid-latitudes, has year-round territoriality that may be due to its need to forage given its extremely high basal metabolic requirements. These animals continuously sense the world around them by producing and receiving electric discharges, a process that is energetically very costly (Markham et al., 2016). Fish that are physically larger also discharge electrical signals of higher amplitude (Caputi and Budelli, 1995) which may contribute to the need for larger foraging grounds. A field study in which the determinants of non-breeding spacing were explored during the winter shows that body size, but not sex, correlates positively with territory size (Zubizarreta et al., 2020a). Oxygen, a limiting physico-chemical variable in aquatic ecosystems, also correlates with territory size. Higher levels of dissolved oxygen may enable fish to defend large territories because their capacity for aerobic respiration is enhanced. The energetic requirements, and thus foraging needs, are probably the same in both sexes during the non-breeding season, and this may explain why territory sizes in the wild are not different between males and females (Zubizarreta et al., 2020a).

NPY: MEDIATOR OF A SEASONAL ENVIRONMENTAL CUE PROMOTING NON-BREEDING AGGRESSION?

In mid to high latitudes, photoperiod is the most robust environmental factor regulating life cycles. Nevertheless, food availability can be a supplementary cue that allows for year-to-year flexibility (Perfito et al., 2008). Social behavior is intimately linked to feeding at the behavioral level. Moreover, neuropeptides involved in food intake are expressed in the SBN across vertebrates (Fischer and O'Connell, 2017). Among these neuropeptides, the orexigenic neuropeptide Y (NPY), a 36-amino-acid amidated peptide, is particularly important. NPY is extremely well-conserved throughout vertebrate evolution with only a single amino acid differing between mammalian and avian NPY, and 83–85% homology in primary structure between birds and teleost fish (Chartrel et al., 1991; Blomqvist et al., 1992; Larhammar et al., 1992, 1993; Larhammar, 1996). The entire NPY signaling system over the 450 MYA of gnathostome (jawed vertebrate) evolution appears to be under strong stabilizing selection, resulting in structural conservation. Furthermore, other orexigenic neuropeptides, such as orexin, are also well conserved in vertebrates (Zendehdel and Hassanpour, 2014). Investigations into NPY function in bird and fish species have shown that the injection of this peptide stimulates feeding (Kuenzel et al., 1987; Richardson et al., 1995; Strader and Buntin, 2001; Davies and Deviche, 2015; Chen et al., 2016) reviewed in Matsuda et al. (2012) and Volkoff (2019). Fasting, on the other hand, increases NPY gene expression (Boswell et al., 2002; Yang et al., 2018; London and Volkoff, 2019a,b), and suppressing NPY decreases food intake (Chen et al., 2016), reviewed in Matsuda et al. (2012). NPY also regulates aggression and/or dominance/subordination in fish (Doyon et al., 2003; Filby et al., 2010; Baran and Streelman, 2020) and mammals (Karl et al., 2004; Emeson and Morabito, 2005; Lischinsky and Lin, 2020). Collectively, these studies demonstrate important physiological and behavioral functions of NPY, thus making it a good candidate for mediating the environmental factors that promote non-breeding territorial aggression in both fish and birds.

In both the song sparrow and banded knifefish, NPY in the SBN might be involved in non-breeding territorial aggression (Mukai et al., 2009; Fokidis et al., 2019; Eastman et al., 2020). In *M. melodia*, NPY immunoreactive cell bodies are found in some regions of the SBN (infundibulum and ventromedial hypothalamus) and the ventral tegmental area (VTA) (Fokidis et al., 2019), whereas fibers are ubiquitous in the SBN. NPY fibers are also present in the nucleus tractus solitarius, which contains specialized neurons that directly respond to changes in extracellular glucose and/or free fatty acids (Mizuno and Oomura, 1984; Blouet and Schwartz, 2012). Thus, NPY might integrate the SBN with metabolic information provided by these glucostatic and lipostatic neurons. Non-breeding song sparrows show elevated NPY in several regions of the SBN, compared to breeding sparrows (Fokidis et al., 2019). Song sparrows challenged with 30 min of STI upregulated gene expression for the NPY-Y1 receptor in the hypothalamus in the non-breeding season, but not in the breeding season (Mukai et al., 2009), suggesting that NPY signaling may respond quickly to changes in the social environment but only during the non-breeding season.

In *Gymnotus omarorum* non-breeding males have NPY transcripts in the POA. Fish dyads that competed over territory and established social hierarchy were subjected to transcriptomic profiling of the POA, and genes related to food intake were robustly clustered according to social phenotype. Dominants, which had acquired the territory through agonistic behavior and displayed exclusive access to its shelter and surrounding area, upregulated NPY and galanin transcripts. Subordinates, which remained in the periphery of the tank avoiding the dominant male, upregulated transcripts of the anorexigenic molecule cholecystokinin (Eastman et al., 2020). This matches a report in other teleost fish which upregulate orexigenic genes (galanin in particular) in dominant fish (Renn et al., 2008). In all, these results support a role for NPY in non-breeding territorial aggression. NPY may be a mediator signaling the seasonal decrease of food availability and promoting the mechanisms specifically underlying aggression in the non-breeding season, a hypothesis that will be tested in the future.

NEUROESTROGENS AS KEY REGULATORS OF NON-BREEDING AGGRESSION

Aggressive behaviors that occur outside of the breeding season suggest a role for non-gonadal regulatory mechanisms (reviewed in Jalabert et al., 2018). The independence of non-breeding aggression from gonadal androgens has been well established. In both *M. melodia* and *G. omarorum*, aggression occurs when the gonads are regressed. Furthermore, gonadectomy in the non-breeding season does not affect contest outcome,

dynamics, aggression levels, or submissive displays (Wingfield, 1994; Jalabert et al., 2015). Thus, gonadal hormones are not necessary for the expression of aggressive behavior during the non-breeding season in these species. In addition, as in other species that display non-breeding aggression, circulating androgens do not increase in response to territorial challenges (Hau and Beebe, 2011; Vullioud et al., 2013; Ros et al., 2014). However, estrogens have a prominent role in the regulation of non-breeding territorial aggression in both species. Aromatase inhibitors reduce non-breeding aggression in both *M. melodia* and *G. omarorum* (Soma et al., 1999, 2000a,b; Jalabert et al., 2015; Zubizarreta et al., 2020b). Direct actions of androgens, the substrate of aromatase, have been ruled out, as androgen receptor antagonism has no effect on non-breeding aggression (Sperry et al., 2010; Zubizarreta et al., 2020b). The effects of aromatase inhibition are rescued by concurrent estradiol replacement in *M. melodia* (Soma et al., 2000b). In both species, estrogens affect behavior in less than 90 min, which suggests non-genomic actions, most probably produced by locally synthesized steroids.

The brain is an important source of estrogens that promote non-breeding territorial aggression. In *M. melodia*, aromatase mRNA and enzymatic activity are present in the SBN during the non-breeding season (Soma et al., 2003; Wacker et al., 2010). Brain-derived estrogens might be synthesized from precursors such as progesterone or dehydroepiandrosterone (DHEA). Although circulating progesterone levels are similar year-round, progesterone in the SBN is higher in the non-breeding season. This neural progesterone might provide substrate for neural androgen and estrogen synthesis (Jalabert et al., 2021). Circulating levels of DHEA are higher than those of testosterone in the non-breeding season (Soma and Wingfield, 2001), and DHEA can be metabolized in the brain into active androgens and estrogens (Pradhan et al., 2008). In the non-breeding season, a territorial challenge rapidly increases the activity of brain 3β-HSD, an enzyme that converts DHEA to androstenedione (Pradhan et al., 2010). This suggests there is a local increase of aromatizable androgens, which may lead to a rise in local estrogen production. In *G. omarorum*, preliminary results show that estrogens are exclusively brain derived in the non-breeding season in both males and females. Moreover, transcriptomic data from the POA show that aromatase and other steroidogenic enzymes are expressed in the non-breeding season. Males that acquired a stable dominant status after an agonistic encounter show increased brain aromatase transcripts. Conversely, subordinate males show increased expression of transcripts involved in the conversion of androgens away from estrogens and toward non-aromatizable androgens (Eastman et al., 2020).

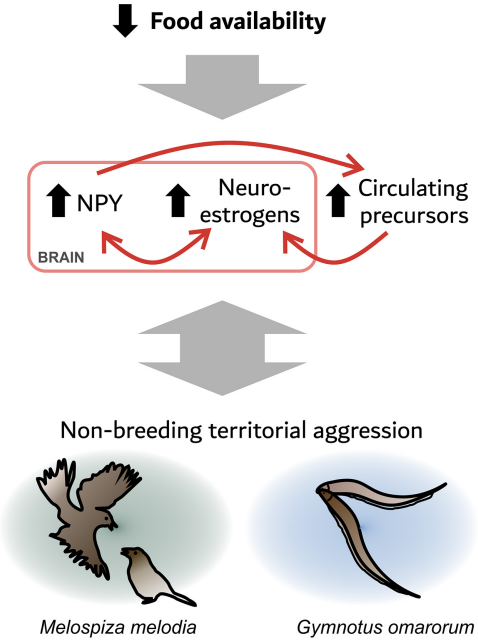

FIGURE 1 | Food availability may be an environmental factor that modulates the expression of territorial aggression through neuropeptide Y (NPY) and neurosteroid signaling. A seasonal decrease in food availability increases NPY in the social behavior network (SBN). NPY may stimulate non-breeding territorial aggression directly, or via the production of neuroestrogens. In addition, a decrease in food availability may increase circulating precursors to neuroestrogens. Agonistic encounters also affect the neuroendocrine state as dominants show an increase in NPY and aromatase expression, which may reinforce the defense of the foraging territory.

A HYPOTHESIS ON THE REGULATION OF NON-BREEDING AGGRESSION

Many studies link food intake physiology and sex steroids. For example, in the gymnotiform *Brachyhypomus gauderio*, long-term food restriction increases circulating androgens, as well as electric signaling in response to social challenge (Gavassa and Stoddard, 2012). In the zebra finch (*Taeniopygia guttata*), an acute fast decreases plasma testosterone levels, but increases plasma DHEA levels and estrogen levels in the VTA and periaqueductal gray (Fokidis et al., 2013). These areas contain aromatase (Shen et al., 1995) and NPY (Fokidis et al., 2019). Furthermore, fasting increases agonistic behavior in this otherwise gregarious species (Fokidis et al., 2013). In fish, NPY is present in key neuroendocrine regulatory centers, such as the POA, and is regulated by sex steroids (Peng et al., 1994). In turn, NPY has seasonal actions on gonadal sex steroid production through its stimulation of pituitary gonadotrophins (Kah et al., 1989; Kalra and Crowley, 1992; Peng et al., 1994; Yaron et al., 2003). Collectively, these data suggest an evolutionary conserved relationship between food intake and sex steroids that is mediated at least partly by NPY signaling. These observations suggest the hypothesis that decreased food availability during winter increases brain NPY signaling, which stimulates neuroestrogen synthesis and thus aggression. NPY might also affect aggression *via* other mechanisms, such as serotonin neurotransmission (Karl et al., 2004; **Figure 1**). In addition, agonistic encounters affect NPY and neurosteroid signaling, reinforcing the defense of the foraging territory. The similarities between the two species

highlighted here might be relevant for understanding non-breeding territorial aggression in other species.

AUTHOR CONTRIBUTIONS

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

FUNDING

This work was supported by the Canadian Institutes of Health Research Project Grant 169203. Facultad de Ciencias, Universidad de la República, Uruguay. Comisión Sectorial de Investigación Científica, UdelaR, Uruguay, Grant Grupos I + D (#883158). Programa de Desarrollo de las Ciencias Básicas, Uruguay.

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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.

The reviewer JW declared a past co-authorship with the authors CJ and KS to the handling editor.

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frontiers in Neural Circuits

REVIEW
published: 19 August 2021
doi: 10.3389/fncir.2021.713105

Vocal and Electric Fish: Revisiting a Comparison of Two Teleost Models in the Neuroethology of Social Behavior

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The communication behaviors of vocal fish and electric fish are among the vertebrate social behaviors best understood at the level of neural circuits. Both forms of signaling rely on midbrain inputs to hindbrain pattern generators that activate peripheral effectors (sonic muscles and electrocytes) to produce pulsatile signals that are modulated by frequency/repetition rate, amplitude and call duration. To generate signals that vary by sex, male phenotype, and social context, these circuits are responsive to a wide range of hormones and neuromodulators acting on different timescales at multiple loci. Bass and Zakon (2005) reviewed the behavioral neuroendocrinology of these two teleost groups, comparing how the regulation of their communication systems have both converged and diverged during their parallel evolution. Here, we revisit this comparison and review the complementary developments over the past 16 years. We (a) summarize recent work that expands our knowledge of the neural circuits underlying these two communication systems, (b) review parallel studies on the action of neuromodulators (e.g., serotonin, AVT, melatonin), brain steroidogenesis (*via* aromatase), and social stimuli on the output of these circuits, (c) highlight recent transcriptomic studies that illustrate how contemporary molecular methods have elucidated the genetic regulation of social behavior in these fish, and (d) describe recent studies of mochokid catfish, which use both vocal and electric communication, and that use both vocal and electric communication and consider how these two systems are spliced together in the same species. Finally, we offer avenues for future research to further probe how similarities and differences between these two communication systems emerge over ontogeny and evolution.

Keywords: electric fish, vocal fish, mochokid catfish, social behavior, neuromodulators, hormones, communication, neural circuit

OPEN ACCESS

Edited by:
Ana Silva,
University of the Republic, Uruguay

Reviewed by:
Laura Quintana,
Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), Uruguay
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Received: *21 May 2021*
Accepted: *12 July 2021*
Published: *19 August 2021*

Citation:
Dunlap KD, Koukos HM, Chagnaud BP, Zakon HH and Bass AH (2021) Vocal and Electric Fish: Revisiting a Comparison of Two Teleost Models in the Neuroethology of Social Behavior. Front. Neural Circuits 15:713105. doi: 10.3389/fncir.2021.713105

INTRODUCTION

The neuroendocrine mechanisms underlying social behavior are daunting in their complexity. They involve many interconnected brain regions whose activities are regulated through dozens

of neuroactive chemical signals acting over timescales ranging from milliseconds to years. Faced with this complexity, researchers have sought simple systems that have relatively few components whose interactions can more easily be quantified, and that can serve as models to guide studies in more complex systems. Among vertebrates, two of the most successful models have been the neural circuits underlying social communication in vocal and weakly electric fish.

In 2005, two of us (Bass and Zakon, 2005) reviewed the behavioral neuroendocrinology of distantly related teleost groups (see Nelson et al., 2016) that produce either vocalizations or electric organ discharges (EODs) and compared how their communication systems have both converged and diverged during their parallel evolution. Put briefly, both vocal and electric communication rely on hindbrain pattern generators that are relatively simple and that drive, in a one-to-one fashion, activation of peripheral effectors organs (the vocal muscles surrounding the swim bladder or the muscle-derived cells of the electric organ called electrocytes) to generate pulse-like signals. The frequency and timing of these sounds or EODs vary by sex and male phenotype (e.g., type I and II male morphs of sonic midshipman fish), and such variations are regulated largely by hormones acting as modulators in a coordinated but independent manner at multiple loci in the motor circuit.

Here, we revisit this comparison and review what has been learned in the intervening 16 years. We only briefly summarize the basics of each system since many comprehensive reviews have been published (Dunlap et al., 2017; Feng and Bass, 2017; Bass et al., 2019; Metzen, 2019). Instead, we focus on several key neural and endocrine processes that have been researched recently in both teleost systems and make direct comparisons to highlight how these analogous communication systems have evolved similar and different mechanisms. First, we summarize recent work that expands our knowledge of the neuroanatomy of circuits underlying these two communication systems. Second, we highlight several parallel studies of hormone and neuromodulator actions on these circuits. Third, we review transcriptomic studies that illustrate how contemporary molecular methods have elucidated the genetic regulation of social behavior in these two groups of fish. Finally, we describe recent studies of mochokid catfish that produce both vocal and electric signals and consider how these two systems can be spliced together and regulated in the same species.

BRIEF OVERVIEW OF VOCAL AND ELECTRIC SIGNALING IN FISH

The use of sound and EODs as social signals has evolved in distantly related teleost groups. For details of the phylogenetic relationships of groups described in this review, we refer the reader to Nelson et al. (2016).

Vocal Fish

Vocalization is widespread in teleost fishes (Rice et al., 2020), including some species of African electric fish (mormyroids). Our understanding of the neural mechanism underlying fish vocalization comes largely from a single group that includes toadfish and midshipman (Nelson et al., 2016). Toadfishes (order Batrachoididiformes) include close to 80 species of vocal fish found in temperate, subtropical and tropical seas that build nests in shallow waters to reproduce (Greenfield et al., 2008). Males produce their vocal signals mostly at night to attract mates and guard nests (only males provide parental care). Their vocalizations are generated by the rapid contractions (∼100 Hz at ∼16°C) of muscles attached to the walls of the swim bladder (**Figure 1A**).

Use of the term vocal to describe some groups of sound-producing fish was first adopted for toadfishes based on developmental and functional characters that they share, in particular, with birds, including: an effector organ dedicated to sound production, sound-producing muscles innervated by occipital (hypoglossal) nerve roots originating from motoneurons in the same caudal hindbrain location, premotor-motor circuitry with developmental origins in the same hindbrain compartments (rhombomeres), and a vocal midbrain center that gates descending input from the preoptic area-anterior hypothalamus to hindbrain pattern-generating circuitry (Bass et al., 1994; Bass, 2014). Vocal fish share some of these characters with non-avian tetrapods as well (see Bass, 2014).

Most neuroethological research on vocal fish has taken advantage of two prominent features of the highly vocal plainfin midshipman, *Porichthys notatus*: seasonal changes in vocal behavior and alternative reproductive tactics (ARTs). Plainfin midshipman have two adult male reproductive morphs, type I and type II (Brantley and Bass, 1994). The hormonal and behavioral characters of the two male morphs diverge, while type II males and females converge. Type I males guard nests in the intertidal zone and acoustically court females at night with a multi-harmonic advertisement call known as a "hum" that lasts up to 2 h in duration and repeats throughout an evening of courtship. Type II males are smaller and neither guard nests nor produce advertisement calls. Instead, they sit near nest openings or within type I male nests where they satellite or sneak-spawn attempting to fertilize eggs. Also, like females, type II males only produce agonistic grunts (Brantley and Bass, 1994). Other non-behavioral characters (e.g., vocal muscle and motoneuron size) are also uncoupled from gonadal sex (reviewed in Bass, 1996; Feng and Bass, 2016) and may be selected upon as dissociable units. This allows for labile patterning of somatic, neural and hormonal characters over evolutionary time and gives rise to divergent intrasexual phenotypes (Goodson and Bass, 2000a; also see Bass, 1996; Lee and Bass, 2006). Although early studies showed that type I and II males follow distinct developmental trajectories (see Bass, 1996) and have non-overlapping mating tactics, later field studies revealed that small, presumably younger (see Bass, 1996), type I males act like type II cuckolders when they do not assume nest ownership, i.e., they sneak or satellite spawn (Lee and Bass, 2004, 2006).

Electric Fish

Weakly electric fish are tropical and subtropical freshwater fish, with independent evolutionary lineages in South America (order

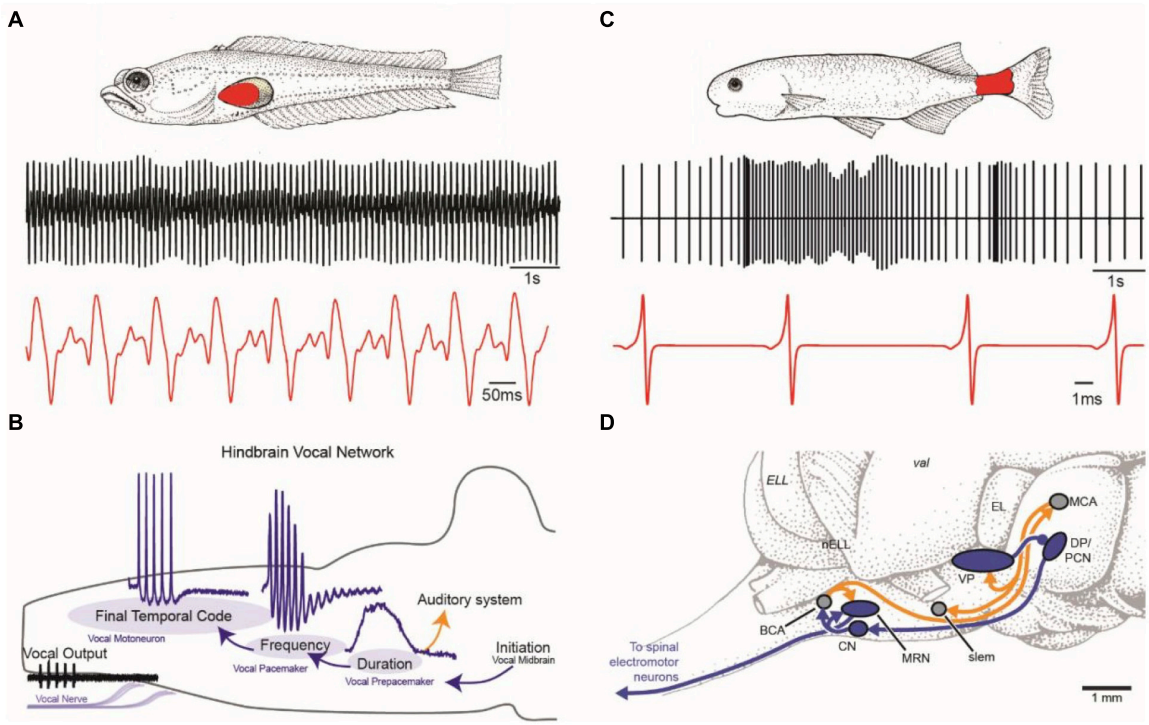

FIGURE 1 | Vocal and weakly electric communication signals and pattern generating neural circuitry. **(A)** Plainfin midshipman (*Porichthys notatus*) generate sound by contracting paired muscles attached to walls of the swim bladder (red, top). Male advertisement hum with characteristic amplitude modulation shown on two timescales (bottom). **(B)** Schematic in sagittal plane showing hindbrain vocal pattern generator (blue) and corollary discharge (orange) pathways of midshipman and other toadfishes that includes three topographically separate nuclei, each coding for a different vocal attribute (adapted from Chagnaud et al., 2011). **(C)** Weakly electric mormyrids use an electric organ located in the caudal peduncle (red, top) to produce a pulsatile electric organ discharge (EOD) shown on two timescales (bottom). **(D)** Schematic in sagittal plane showing EOD pattern generating circuitry (blue) and corollary discharge pathways (orange) of mormyrid fish. BCA, bulbar command-associated nucleus; CN, command nucleus; DP, dorsal posterior thalamic nucleus; EL, exterolateral nucleus; ELL, electrosensory lateral line lobe; MRN, medullary relay nucleus; MV, medioventral nucleus; MCA, mesencephalic command-associated nucleus; OB, olfactory bulb; PCN, precommand nucleus; slem, sublemniscal nucleus; tel, telencephalon; val, valvula of the cerebellum; VP, ventroposterior nucleus. (Panels **C** and **D** adapted from Baker et al., 2013 with permission from the Journal of Experimental Biology).

Gymnotiformes) and Africa (order Mormyriformes) (Bullock and Heiligenberg, 1986). Together these groups contain about 500 species across both continents. Most species produce weak electric discharges from modified muscle cells, electrocytes, located in the electric organ of the tail, and they detect these discharges through specialized electroreceptors located across the body (**Figure 1C**). They emit their EOD continuously, and in many species, they enhance their EOD at night, when they are most active.

Weakly electric fish use their EOD for sensing objects around them (electrolocation), but more relevant to this review, it is their primary modality of social communication (electrocommunication). The EOD conveys information about the sex and motivational state of an individual. For example, in most species, males and females differ in the frequency or wave form of their continuous EOD, and, during aggression and courtship, they produce brief frequency and/or amplitude modulations of the EOD (e.g., chirps and rises) that last milliseconds to seconds (reviewed in Dunlap et al., 2017).

Comparison

One major advantage of studying both vocal and electric fish is that their communication behaviors can be readily characterized by a finite set of easily quantified physical attributes. In both modalities, the signals vary in frequency (repetition rate), duration, and frequency/amplitude modulations, and these signal parameters commonly differ by sex and vary according to social context (Caputi et al., 2005; Bass et al., 2015). However, communication signals differ between vocal and electric fish in at least three ways. First, vocal fish intermittently produce their signals with important variation in the call duration while electric fish continuously generate their signals. Second, vocal fish produce their acoustic signals only for communication while electric fish use their EOD for the dual functions of electrocommunication and electrolocation. Finally, electric fish can generate salient variation in the waveform of their signal while the vocal signals vary little in waveform.

In general, the frequency of the signal in both groups is established by a hindbrain pattern generator. Modulations of this

baseline rhythm, such as variations in frequency or call duration, arise from midbrain inputs to the pattern generator. As pointed out previously (Bass and Zakon, 2005), the two systems differ in the role of the effector organ (vocal muscle or electric organ) in shaping the signal. In vocal fish, the vocal muscles do not modulate the waveform, but in electric fish, the electrocytes of the electric organ play a crucial role determining the shape of the signal (e.g., the number of phases and the duration of each phase). Below, we further compare the neural circuits underlying these two communication systems.

NEURAL CIRCUITS UNDERLYING VOCAL AND ELECTRIC SIGNALING

Comparisons of the neural circuitry in vocal and electric fish date back to the pioneering work of M.V.L. Bennett and colleagues in the 1960s and 1970s (Bennett, 1971a,b), who documented the electrotonic coupling between motoneurons in both systems. Over time, researchers revealed further similarities in structure-function organization shared by the pattern generating circuits underlying vocal and electric signal production (Bass, 1986, 1989; Bass and Baker, 1997; Bass and Zakon, 2005). This included the salient role of temporal precision in vocal muscle and electric organ activation, the location of pattern generating circuitry in the hindbrain near the spinal cord boundary, and the co-evolution of vocal motor and electromotor systems with their respective sensory systems to enhance sensory-motor coupling. Here, we further compare the pattern generating circuits between vocal and electric teleosts given the most recent studies of neural mechanisms for generating and perceiving communication signals.

Neural Circuitry Generating Vocal and Electric Signals

In signal generation, both vocal and electric modalities require precision in the temporal and spatial domains and sufficient energy for conspecific communication (**Figures 1A,C**). Because sound degrades in amplitude in the aquatic medium, especially in shallow water, most vocal fish face conditions unfavorable for long distance communication. Similar constraints exist for electric signals, which attenuate spatially to an even greater degree (Brenowitz, 1986). The solution in both modalities for extending their communication range is to generate high amplitude signals by synchronizing the oscillations of cells in the effector organs (muscle fibers of the vocal muscles and electrocytes of the electric organ) (Bennett et al., 1967b; Bass and Baker, 1990). Such synchrony is achieved in both systems by several specializations, including reduction in the number of motoneurons innervating the effector organ or by coupling motoneuron activation *via* presynaptic inputs and/or gap junctions (Bennett et al., 1967b; Bennett, 1971a; Bass and Baker, 1990; reviewed in Caputi, 2020). An extreme example of reduced central control is in electric catfish (*Malapterurus),* which have a single bilateral pair of motoneurons, each one innervating several millions of ipsilateral electrocytes (Bennett et al., 1967a; Bennett, 1971a).

The production of both vocal and electric signals relies on activating neurons at high frequencies (∼50–1100 Hz). Thus, a potential problem in both systems is erratic, spontaneous firing, which would disrupt synchrony. As one adaptation to prevent such unregulated activity, motoneurons in both systems have low input resistance, and thus require coherent synaptic input to fire action potentials. In this way, the motoneurons may be considered "followers." Recent studies of vocal fish (Chagnaud et al., 2021) and other vertebrate vocal (Lawton et al., 2017) and locomotor systems (Song et al., 2016; Matsunaga et al., 2017), however, suggest that the influence of motoneurons on premotoneurons *via* gap junctions gives them greater importance in patterning the activity of the effector organ than merely following premotor input (reviewed in Barkan and Zornik, 2019). Furthermore, motoneurons in vocal and electric systems are adapted to phasic input, preferentially firing at the onset of intracellular current influx. This makes them ideally suited to respond to short pulses of current flux and repetitive activity (Chagnaud et al., 2012). This adaptation clearly facilitates high frequency oscillatory-like firing, another common feature of motoneurons in both vocal and electric modalities.

If motoneurons are followers, who do they follow? In vocal fish and gymnotiform electric fish, neurons with pacemaking capabilities project directly (vocal) or indirectly (electric) to the motoneurons. In vocal fish, pacemaker neurons show intrinsic properties enabling voltage-dependent oscillatory behavior, but the pacemaker neurons themselves do not generate rhythms in the absence of synaptic input (Chagnaud et al., 2011). By contrast, in electric fish, relay neurons receive input from pacemaker neurons and "relay" patterning information to the motoneurons (Grant et al., 1986; Carlson, 2002). In one group of electric fish, the apteronotids, electromotor neurons have intrinsic rhythmic firing independent of sensory or midbrain input; their axons form the electric organ itself and are the source of the EOD as they lack the muscle-derived electric organ found in other electric fish (Dye and Heiligenberg, 1987; Shifman et al., 2020). This marked difference between vocal and electric fish in pacemaker circuitry correlates directly with how they control signal duration. While vocal fish modify the duration of different call types, electric fish instead mainly modulate the brief pauses between discharges. The duration of pauses can last from milliseconds to seconds, but because the EOD is used for electrolocation as well as electrocommunication, this system is never fully "turned off."

Several studies describe a variety of inputs to the vocal (Forlano et al., 2014; Rosner et al., 2018; Timothy and Forlano, 2020) and electromotor (Borde et al., 2020) pattern generating circuits in the hindbrain (**Figures 1C,D**). One interesting aspect of a recent study investigating the neurophysiological correlates of such inputs is the identification of gap junction coupled, glycinergic neurons within the vocal circuit (Chagnaud et al., 2021). These neurons are interesting in light of an early study (Pappas and Bennett, 1966) that provided evidence for inhibitory action onto vocal motoneurons. A neurophysiological study (Chagnaud et al., 2021) revealed the importance of glycinergic input to motoneurons in synchronizing the vocal motor output. In addition, this study showed that gap junctional coupling is essential to activate these glycinergic neurons and

that vocal premotor neurons are not only excited by gap junctional coupling (Pappas and Bennett, 1966; Bass and Baker, 1990), but that gap junctional coupling is indeed sufficient to activate premotoneurons (Chagnaud et al., 2021). Such coupled glycinergic neurons could also contribute to the temporal patterning of EODs in the hindbrain pattern generator, but this remains to be investigated.

Neural Circuitry for Reception of Vocal and Electric Signals

In toadfish, vocal communication depends on the detection of sound waves by inner ear otolith organs, especially the saccule (Fay and Popper, 2012). The hearing range of fishes is generally limited to <1 kHz, except in species with accessory organs (e.g., Weberian ossicles) that permit a higher frequency detection (Braun and Grande, 2008). In vocal fish, auditory neurons, especially those in the hind- and midbrain, encode vocalization attributes such as frequency content (e.g., encoded as best frequency), patterns of amplitude and frequency modulation, and the onset and overall duration of sound waves (Bass and Lu, 2007; Fay and Edds-Walton, 2008). Sound-producing and sound-perceiving circuits are not fully separated, as vocal-auditory coupling at different levels of the auditory system ensures that the latter is informed about one or more acoustic characters (Weeg et al., 2005; Chagnaud and Bass, 2013). For example, the vocal pattern generator in midshipman fish (**Figure 1B**) relays information about vocal duration from a prepacemaker nucleus to a separate hindbrain population that directly innervates the auditory epithelium of the inner ear (Chagnaud and Bass, 2013).

In electric fish, similar information is coded at peripheral and central levels. Electrosensory neurons have response properties similar to auditory neurons of vocal fish. The electroreceptors are tightly tuned to the dominant frequency in the fish's EOD. In species where the males and females differ in EOD frequency, the tuning of electroreceptors show corresponding sexual differences. As with the vocal system, information about ongoing EOD activity is transmitted to sensory structures from the motor command system (**Figure 1D**) *via* either peripheral reafference (gymnotiformes, *Gymnarchus*) or central corollary discharge (mormyrids) pathways (Perks and Sawtell, 2019; Fukutomi and Carlson, 2020). Extensive literature dating back to the early 1960s (Bennett, 1971b; Heiligenberg, 1977) documents this electrosensory processing, often with comparisons to audition, and was recently reviewed elsewhere (Carlson, 2004; Caputi, 2017; Carlson et al., 2019).

Despite coding for different behaviors, the vocal and electric pattern generators thus share several fundamental features such as oscillatory activity, synchrony and neural precision on the motor patterning side, as well as feature extraction of sensory stimuli and a strong connection between the motor and the sensory circuits. Future studies are needed to evaluate whether those shared general attributes are reflected in individual neurons by employing similar ion channels in the neurons coding for these two modalities.

NEUROMODULATORY AND HORMONAL REGULATION OF COMMUNICATION AND SOCIAL BEHAVIOR

The previous review comparing these two teleost models (Bass and Zakon, 2005) emphasized how steroid hormones act on the underlying neural circuits described above to achieve long-term changes (days to months) in communication behavior associated with season, sex and male phenotype. Since then, much work has focused on how other hormones and modulators work in combination (or in parallel) with steroids to regulate social behavior on more rapid time scales, ranging from minutes to days. Below, we summarize studies on how serotonin, AVT, melatonin and melanocortins influence communication and social behavior and how steroids can regulate these circuits through newly described mechanisms. These recent studies reinforce previous work showing how hormones coordinate the responses to predictable changes in the physical environment (e.g., behavioral responses to diel and annual cycles). In addition, these recent studies underscore the role of hormones and neuromodulators in coordinating the response to unpredictable and dynamic social environments.

Serotonin

Most communication signals are specific to social context. In the vocal and electric modalities, this specificity is achieved by modifying particular signal attributes (e.g., duration, spectral content, amplitude or frequency modulation). Neuromodulators acting at select loci in neural circuits contribute to such plasticity in signal production. In frog (Rhodes et al., 2007; Yu and Yamaguchi, 2010; Kelley et al., 2020) and bird vocal systems (Wood et al., 2011), serotonin (5HT) is one such modulator. The organization of the serotonergic system is highly conserved among teleost fishes (Lillesaar et al., 2007; Lillesaar, 2011; Lillesaar and Gaspar, 2019), and the widespread distribution of serotoninergic neurons in brains of this more ancestral vertebrate group suggests that 5HT may have played an important role in the evolution of neuroendocrine mechanisms regulating vertebrate communication behavior.

Vocal Fish

Although no one has yet investigated the behavioral or physiological effects of 5HT on vocal production in teleosts, several studies, primarily in toadfish, have described the distribution of 5HT and the projections of serotonergic neurons in vocal control regions of the brain, from higher order centers in the fore- and midbrain down to the pattern generator in the hindbrain (Rosner et al., 2020; Timothy and Forlano, 2020). Serotonergic projections to vocal-associated neurons are well identified, especially in neurons connected to the vocal pattern generator. However, we have only indirect evidence of such projections to vocal-associated neurons in higher brain areas. Such evidence could be demonstrated by combining 5HT immunocytochemistry with immediate early gene expression during vocal activity, as described previously for catecholaminergic neurons (Petersen et al., 2013).

In their recent study of 5HT distribution in the midshipman brain, Timothy and Forlano (2020) found that all currently known regions within the forebrain vocal-acoustic complex and the midbrain acoustic complex (each containing several brain nuclei) are characterized by serotonergic presence. By taking advantage of extensive transneuronal transport, investigators have mapped the hindbrain vocal pattern generator in toadfishes. Application of either neurobiotin or biocytin to a single vocal nerve leads to labeling of vocal motoneurons as well as premotor populations of pacemaker and prepacemaker neurons (Bass et al., 1994; Chagnaud and Bass, 2014). This feature likely depends on extensive gap junction coupling between neurons (see Bass et al., 1994; Chagnaud et al., 2011, 2012). Both 5HT immunoreactive somata and fibers are present in the vocal motor nucleus (VMN) (Rosner et al., 2018, 2020; Timothy and Forlano, 2020), which consists mainly of motoneurons innervating the vocal muscles (Bass, 1985). Transneuronally coupled neurons were not 5HT-positive (and thus likely not coupled *via* gap junctions). Since the VMN synchronizes motoneuronal firing and thereby plays a large role in determining call amplitude (Chagnaud et al., 2012), 5HT in the VMN may contribute to the modulation of call amplitude, which is a distinctive feature of toadfish vocalizations.

The neurons of the vocal pacemaker nucleus (VPN) that code for the fundamental frequency of toadfish vocalizations (Chagnaud et al., 2011) are also characterized by 5HT projections to the somata and to the VPN dendritic tree. Since frequency modulation is especially prominent in some vocalizations, 5HT could also act on call pulse repetition rate/fundamental frequency. The third main component of the toadfish vocal pattern generator, the vocal pre-pacemaker (VPP), also receives 5HT-ir projections, and 5HT ir-positive neurons are located in its immediate vicinity. 5HT may modulate call duration, which is regulated by the VPP (Chagnaud et al., 2011). Due to the major differences in call durations between vocalizations, which range from a few milliseconds to hours, neuromodulators could participate in generating such call diversity.

The well-mapped distribution of serotonergic neurons in the vocal motor system strongly suggests that 5HT acts at many loci to independently modulate different features of the acoustic call. However, it will be important to follow these neuroanatomical studies with corresponding physiological studies that demonstrate the full effects of 5HT. The description of 5HT distribution in vocal associated areas bears the caveat that neurons in some of these brain areas (e.g., POA or periaqueductal gray) are also known to be associated with other behaviors.

Electric Fish

In contrast to the thorough neuroanatomical description of the serotonergic system in vocal fish, the distribution of 5HT has not been investigated as deeply in electric fish. However, many physiological studies in gymnotiform electric fish using agonists and antagonists of serotonin receptors have demonstrated that 5HT is a widespread modulator of electric signaling and social behavior, exerting its influence on both the production and reception of the EOD at many different circuit levels (Zubizarreta et al., 2012; Marquez and Chacron, 2020). Depending on the context and the species, 5HT can modify EOD pulse amplitude and shape, EOD modulations, "chirps," as well as the electrosensory perception of the EOD. Moreover, the overall outcome of dominance interactions is heavily influenced by 5HT, and species differences in aggressive behavior correlates with evolved differences in the serotonergic system.

In *Brachyhypopomus pinnicaudatus*, males normally increase EOD duration (repolarization of the second phase) and EOD amplitude as they enter the dark phase of the light cycle (Stoddard et al., 2007). When presented with a conspecific male, these parameters increase even more so, perhaps as a way of exaggerating the range and "masculinity" of their signal. This exaggeration of the circadian oscillation is mimicked with peripheral injections of 5HT, which act within minutes *via* 5HT2 and/or 5HT1A receptors to increase EOD amplitude and duration (Stoddard et al., 2003; Allee et al., 2008). However, *in vitro* 5HT application directly to isolated electrocytes and the spinal cord has no effect (Markham and Stoddard, 2005), suggesting that 5HT acts centrally, perhaps through regulating pituitary secretion of melanocortins (ACTH and alpha-MSH), which then act directly on electrocytes (see section "Regulation of Diel Patterns of Signaling"). Both the pre-optic area (POA) and hypothalamus, whose activity influences pituitary secretion, densely express 5HT (Johnston et al., 1990) in the neuron terminals, and this may represent an endogenous pathway for serotonin regulation of the EOD via melanocortins.

At higher levels in the neural circuit, 5HT appears to exert an inhibitory action on EOD modulations during aggressive interactions. In *Apteronotus leptorhynchus*, the midbrain pre-pacemaker nucleus (PPn-C) initiates the production of "chirps" – rapid frequency/amplitude modulations of the EOD – *via* monosynaptic inputs to the pacemaker nucleus (PN). These chirps, especially the short duration type 1 chirps, are produced most vigorously during male aggression. Males injected intracerebrally with 5HT reduce their chirping (Maler and Ellis, 1987), and females, which chirp much less than males, have much greater expression of 5HT in the PPn-C (Telgkamp et al., 2007). In addition, among females, subordinate individuals have more 5HT in the PPn-C than did dominants. Pharmacological manipulations indicate that this inhibitory action of 5HT on aggressive chirps is mediated through 5HT2 receptors (Smith and Combs, 2008). Interestingly, 5HT may act through 5HT1A receptors to increase the production of type 2 chirps, which are produced by males during courtship. Together, these studies thus indicate that 5HT acts on the PPn-C to contribute to sexual differences and context-specific expression of chirps.

Several sets of studies have demonstrated that, in addition to inhibiting the production of chirps used in same-sex aggression, 5HT simultaneously enhances perception of same-sex stimuli (Deemyad et al., 2013; Marquez and Chacron, 2020). Using fast-scan cyclic voltammetry, Fotowat et al. (2016) showed that 5HT is released in the electrosensory-lateral line lobe (ELL) of the hindbrain in response to stimuli mimicking a conspecific male. Experimental elevation of local 5HT enhances the sensitivity of pyramidal neurons in the ELL and promotes burst firing of these neurons (Deemyad et al., 2013; Márquez et al., 2013). 5HT likely increases pyramidal cell excitability by binding to 5HT2 receptors (Larson et al., 2014) and downregulating the potassium

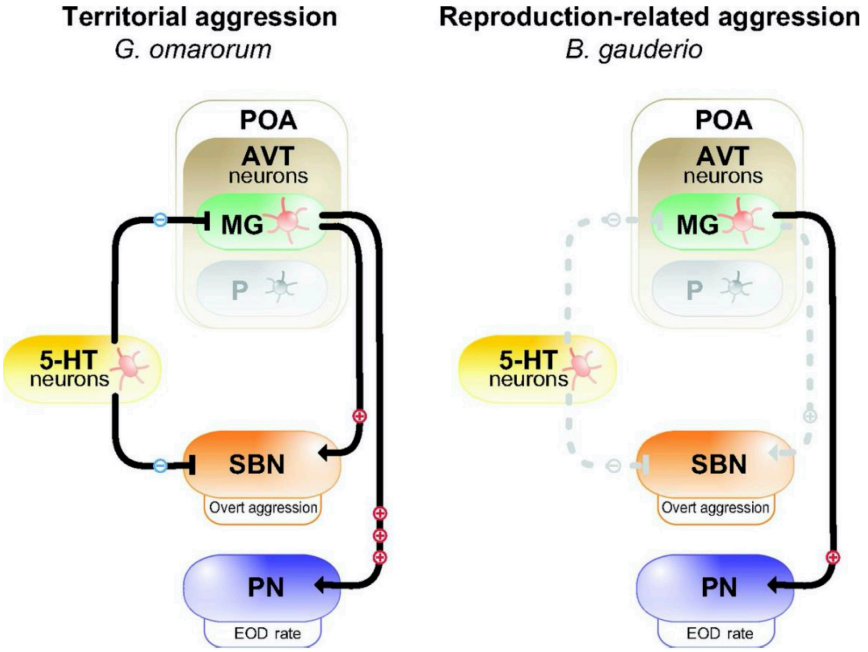

FIGURE 2 | Model for the neuromodulation of aggression by AVT and 5-HT in dominants of two species of electric fish with different forms of aggression. In dominant *Gymnotus omarorum* **(left)**, which displays territorial aggression year round, AVT magno-gigantocellular neurons (MG) in the pre-optic area (POA) strongly activate (+++) the pacemaker nucleus (PN) to increase EOD rate, and weakly active (+) overt aggression through the social behavioral network (SBN). 5-HT neurons inhibit (–) overt aggression. In dominant *Brachyhypopomus gauderio* **(right)**, which shows only reproduction-related aggression during the breeding season, AVT-containing MG cells of the POA weakly (+) increase EOD rate, but have no effect (dashed line) on overt aggression. 5-HT has no effect on aggression. The parvocellular cells (P) in the POA do not participate in regulating aggression in dominants of either species. Reproduced/adapted with permission from the Journal of Experimental Biology, Silva et al. (2013).

channels that contribute to the spike afterhyperpolarization. The same 5HT treatments that enhance this electrosensory sensitivity simultaneously inhibit chirp production (Deemyad et al., 2013). Thus, the authors of this work proposed that, overall, 5HT serves a "shut up and listen" function that minimizes aggression during same-sex interactions and contributes to social subordination.

The inhibitory action of 5HT on aggression typical of many vertebrates is exhibited in the interspecific comparison of two electric fish species (**Figure 2**; Zubizarreta et al., 2012; Silva et al., 2013). *Gymnotus omarum* is especially aggressive, and both males and females quickly attack intruders in the non-breeding as well as the breeding season. Associated with this high level of aggression, basal 5HT activity levels in the telencephalon of *G. omarum* are relatively low, and these levels fall even further in both combatants following staged encounters (Zubizarreta et al., 2012). Aggression is inhibited by the 5HT agonist 8-OH-DPAT, indicating that 5HT likely acts through 5HT1A receptors. By contrast, *Brachyhypopomus gauderio*, which is overall less aggressive and exhibits aggression only by males in the breeding season, has relatively high telencephalic 5HT activity. Following territorial disputes, 5HT activity increases but only in subordinates. The anti-aggressive actions of 5HT in this species does not occur *via* 5HT1A receptors. These species differences in the regulation of aggression indicate that evolutionary changes in the serotonergic system may have contributed to species diversification in patterns of social behavior.

Comparison

The serotonergic systems of vocal and electric fish have been studied largely through different approaches: neuroanatomical in vocal fish and physiological/behavioral in electric fish. Nonetheless, 5HT appears to have widespread influence on the communication behavior of both groups. In vocal fish, 5HT likely acts directly on nuclei in the hindbrain pattern generator as well as indirectly through fore- and midbrain inputs. In electric fish, it acts on the midbrain prepacemaker nuclei or on the hypothalamic regulation of melanocortins to regulate the production of electrocommunication signals. In addition, 5HT modifies neural activity in the electrosensory-lateral line lobe of the hindbrain to enhance electrosensory perception. Anatomical evidence of serotonergic projections in the auditory processing nuclei in vocal fish (Timothy and Forlano, 2020) suggest that auditory perception might be similarly affected. Finally, it appears that, among electric fish, the diversity of serotonergic receptors contributes to species differences in overt aggressive behavior as well as social communication.

Arginine Vasotocin

Across vertebrates, social behavior is greatly influenced by the nonapeptide arginine vasotocin (AVT) and its homologs, and evolution within the AVT system has likely contributed to behavioral diversification among vertebrates (Goodson, 2008, 2013). Consistent with this general trend among vertebrates, AVT

has potent effects on the social behavior in vocal and electric fish, and variations within the AVT system contribute to inter- and intra-specific differences in social behavior.

Vocal Fish

In vocal fish, the action of AVT has been studied most in an electrophysiological preparation in which AVT can be applied to specific brain regions while monitoring "fictive" output from the vocal nerves (Goodson and Bass, 2000a). In type I males of the plainfin midshipman, AVT decreases fictive call duration when it is injected directly into the preoptic area (POA) and anterior hypothalamus while application of an antagonist of the V1a AVT receptor increases call duration. When injected into the midbrain, AVT decreases the number of calls without affecting duration. In contrast to these actions in type I males, AVT has no effect on the vocal motor output of type II males and females. Instead, another nonapeptide, isotocin (an oxytocin homolog), exerts a potent inhibitory effect on vocal output. Immunoreactive AVT and isotocin fibers are found in neurons of many fore- and midbrain regions that influence vocal production, including the POA, the periaqueductal gray and the paralemniscal midbrain tegmentum. Interestingly, there appears to be no labeling in the hindbrain regions that are most directly related to vocal production (Goodson and Bass, 2000b; Goodson et al., 2003).

Although fish of different sex and morphotype show divergent responses to experimental manipulations of these nonapeptides, they show similar nonapeptide distribution in brain. This suggests that the different responses to exogenous AVT is likely attributable to differences in the density or distribution of their receptors.

Electric Fish

In electric fish, AVT modifies both agonistic behavior and electric signal production, and the specific effect varies widely by sex, dominance status, and species (Bastian et al., 2001; Perrone et al., 2010). In male *Apteronotus leptorhynchus*, AVT injection inhibited the production of aggressive chirps (type 1), however, this same treatment stimulated production of male courtship chirps (type 2). AVT had no apparent effect on chirping in females (Bastian et al., 2001). Thus, in this species, the action of AVT is specific to both sex and signal type. The mechanism and site of AVT action on chirping is unknown, however, AVT has been localized in the POA (Johnston and Maler, 1992) and there are abundant known connections between the POA and the PPn-C, the brain region that controls chirping.

In the gregarious species *Brachyhypopomus gauderio*, in which aggression is naturally confined to the breeding season, AVT administration to males during the breeding season increased diurnal EOD rate, which is a signal characteristic of dominant males (**Figure 2**; Perrone and Silva, 2016). Double labeling for AVT and an immediate early gene, FOS, showed that many neurons in the POA that express AVT become active specifically when a male is exposed to a female (Pouso et al., 2019). AVT neurons project from the POA to the PN, where AVT binds to V1a receptors to increase firing rate of pacemaker neurons (Perrone et al., 2014; Pouso et al., 2017). These studies demonstrated a positive effect of AVT on male sexual signaling. Interestingly, in this species, AVT had little effect on overt aggression (i.e., fighting) (Perrone and Silva, 2016).

In the solitary species *Gymnotus omararum*, which displays aggression year-round and mostly in the context of territorial disputes, the effect of AVT is notably different than in the gregarious *Brachyhypopomus gauderio* (**Figure 2**). AVT administration has little effect on basal EOD rate (Silva et al., 2013; Perrone and Silva, 2018). However, it modifies the production of submissive electric signals in a status-dependent manner: AVT increases submissive signaling in subordinates while showing no effect in dominants. As an additional example of species-specific actions, AVT increases the motivation for overt aggression in *Gymnotus*, but has no effect on overt aggression in *Brachyhypopomus*. Although these two electric fish species differ markedly in their AVT regulation of electrocommunication and aggression, they show no apparent differences in the distribution of AVT in the brain (Pouso et al., 2017). Thus, just as in vocal fish, variation in the behavioral response of electric fish to AVT is likely due to the variation in the distribution of receptors.

Comparison

It is clear from studies on both vocal and electric fish that, while AVT is an important regulator of social behavior, its effects are highly context-dependent; its actions vary considerably in intrasexual (plainfin midshipman, *Gymnotus*), intersexual (*Apteronotus*) and interspecific (*Gymnotus* vs. *Brachyhypopomus*) comparisons. Vocal and electric fish both have AVT receptors sensitive to V1a receptor antagonists in the neural circuitry underlying social communication. However, in both teleost models, behavioral differences (intrasexual and interspecific) are not related to any corresponding differences in AVT distribution in the brain.

In addition to these similarities, there is an apparent difference as well. In vocal fish, AVT tends to inhibit production of communication signals, acting at the level of the fore- and midbrain. In electric fish, it inhibits production of some signals (Type I chirps in *Apteronotus*) but stimulates other signals (type II chirps in *Apteronotus*, EOD rate in *Brachyhypopomus*, submissive signaling in *Gymnotus*). Finally, in vocal fish, AVT neurons are not found in the vocal control nuclei of the hindbrain, but, in at least some electric fish, AVT neurons are located in the hindbrain pattern generator as well as within the fore- and midbrain.

Regulation of Diel Patterns of Signaling

Both vocal and electric fish are socially most active at night and emit their communication signals in pronounced daily cycles. Several sets of studies in both groups have explored the role of melatonin acting in the brain or melanocortins acting in the periphery in regulating these diel cycles. In diverse vertebrate taxa, melatonin is released from the pineal gland in the dark phase of the photic cycle and serves as the main time-regulating hormone. Melanocortins [e.g., adrenocorticotropin (ACTH) and alpha-melanocyte stimulating hormone (alpha-MSH)] are secreted from the pituitary into the blood where they coordinate daily cycles in peripheral tissues.

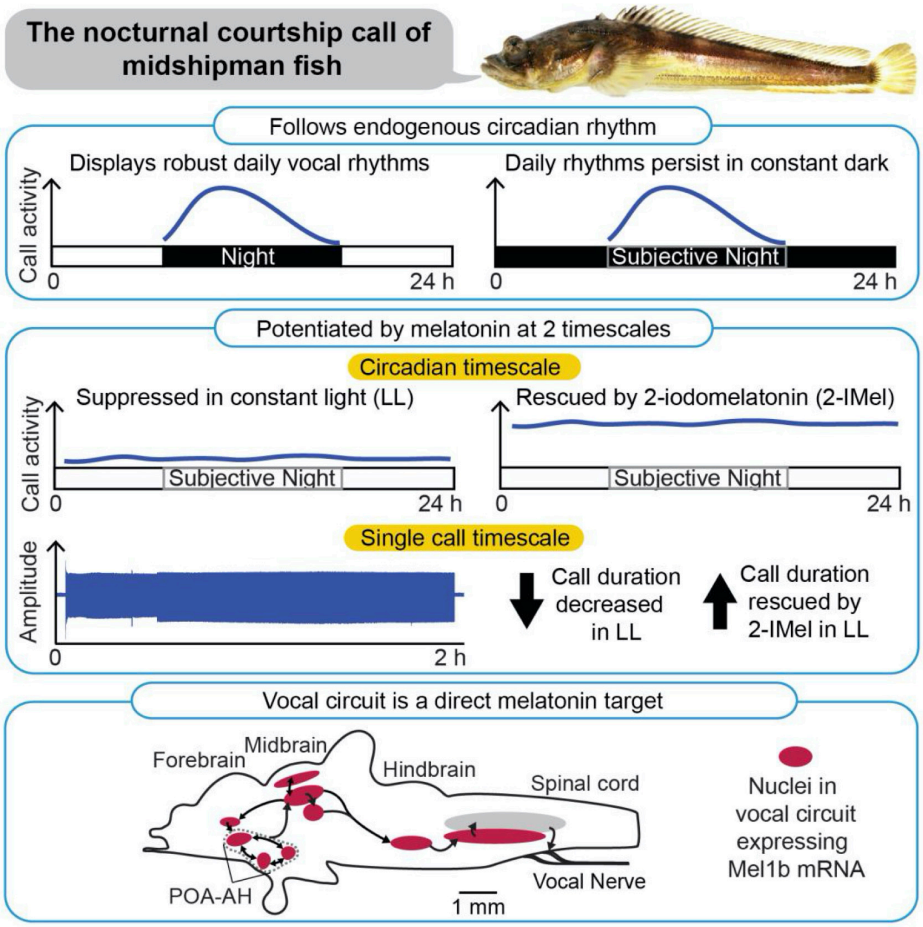

FIGURE 3 | Patterning of social acoustic signaling in the nocturnally breeding, plainfin midshipman fish (*Porichthys notatus*) at multiple timescales, from day–night rhythms to call temporal properties. **(Top)** Courtship vocalizations exhibit an endogenous circadian rhythm under constant dark conditions. **(Middle)** Courtship vocalizations are suppressed under constant light (LL), but systemic delivery of melatonin analog (2-IMel) rescues their daily occurrence (circadian timescale), including their duration (single call timescale). **(Bottom)** Sagittal view of midshipman brain (rostral is to the left) depicting robust expression of melatonin receptor Ib mRNA transcripts in evolutionarily conserved neuroendocrine and vocal networks, including the preoptic area-anterior hypothalamus (POA-AH). From Feng and Bass (2016).

Vocal Fish

As nocturnal fish, male plainfin midshipman broadcast advertisement calls repeatedly throughout the night. Feng and Bass (2016) demonstrated that this rhythmic display of courtship vocalization is synchronized by light conditions (**Figure 3**). Beginning with fish housed in a 15L: 9D light cycle that mimics the photic conditions in which the fish normally vocalize, they then transferred one group of fish to constant dark (DD) and another group to constant light (LL) conditions. DD fish displayed humming behavior during the subjective night, demonstrating an endogenous circadian rhythm. However, this cycle was disrupted in LL fish (Feng and Bass, 2016).

Subsequent studies showed that the nocturnal increase in vocalization is mediated by endogenous melatonin (Feng and Bass, 2014, 2016; Feng et al., 2019). DD fish stopped calling when the endogenous actions of melatonin were blocked pharmacologically (Feng and Bass, 2014). Conversely, LL fish resumed their cycles of humming when treated with a melatonin analog (Feng and Bass, 2016). The light-dependence of vocal behavior is paralleled by the *in vivo* excitability of the underlying neural circuits. In an intact neurophysiological preparation where fictive calls were evoked through electrical microstimulation of midbrain nodes in the vocal network, constant darkness decreased the threshold for evoking calls and increased call duration. These effects were reversed by melatonin receptor antagonists. By contrast, constant light decreased excitability and call duration, measures that were reversed by treatment with melatonin agonists (Feng and Bass, 2014).

Additional *in situ* hybridization studies demonstrated that melatonin receptor (mel1B) has widespread distribution within the brain, including in the hindbrain pattern generator circuit [i.e., the VPP, the vocal pacemaker (VPN)] and in fore- and midbrain nuclei (e.g., POA, the periaqueductal gray) that contributes to vocal production (**Figure 3**; Feng et al., 2019). While this distribution of receptors indicates that melatonin could act directly on the vocal circuitry, it is important

to note that these receptors also colocalize with other key neurochemical regulators of vocal production (e.g., steroid hormones, aromatase, and AVT), and that melatonin likely works in combination with these molecules to regulate diel and perhaps annual cycles of calling behavior (see Feng et al., 2019).

Together, the above behavioral, neurophysiological and neuroanatomical studies support the hypothesis that the stimulatory effect of darkness on vocalization is mediated by endogenous melatonin. One curiosity is that, although melatonin stimulates vocal behavior, fish vocalize most vigorously during the summer, when the duration of night and the period of elevated melatonin levels are short. This suggests that, in these nocturnal fish, the magnitude of melatonin secretion or sensitivity of the circuits to melatonin, rather than the duration of melatonin secretion, drives seasonal changes in calling behavior. As the summer breeding season approaches, fish migrate from deep, cold water to the warmer waters of the intertidal zone (Feng and Bass, 2014). This temperature increase may enhance nocturnal melatonin secretion or potentiate its effects in the vocal motor circuitry.

Electric Fish

Because electric fish can navigate, locate prey and communicate using their electrosensory system alone, they can perform most of their activities in complete darkness, and virtually all species examined are highly nocturnal (Bullock and Heiligenberg, 1986). Additionally, in several species, multiple features of their EOD (e.g., frequency or amplitude) are enhanced at night. Such changes enable them to sample their environment more frequently, expand the range of their signal and, in some cases, exaggerate the "maleness" of their signal. Conversely, a daytime decrease in these parameters lowers the substantial energetic cost of electrogenesis during the period when they are less apt to use electrolocation and electrocommunication (Salazar and Stoddard, 2008; Salazar et al., 2013).

Several species that emit a pulse discharge show nocturnal increases in EOD rate (Zupanc et al., 2001; Silva et al., 2007; Stoddard et al., 2007; Migliaro and Silva, 2016). Such rhythms are maintained even in constant photic conditions in the laboratory, indicating an intrinsic circadian organization in the activity of the hindbrain pacemaker. In one species, *Gymnotus omarorum*, this circadian rhythm in the EOD persists in field conditions even after controlling for diel changes in light, temperature and locomotion. However, these field studies also indicate that social interactions help synchronize the diel changes in EOD rates (Migliaro et al., 2018).

The nocturnal increase in EOD rate is likely mediated through endogenous fluctuations in melatonin, since a melatonin receptor antagonist eliminates the rhythm (Migliaro and Silva, 2016). Because melatonin receptor distribution has not yet been mapped, it is unknown whether melatonin binds directly to neurons in the pacemaker nucleus or whether it acts indirectly at other sites or through other neurochemical mediators. However, it is unlikely that endogenous AVT fluctuations participate in circadian patterns of EOD rate, since AVT receptor antagonists have no effect on this rhythm.

In addition to these EOD rate changes originating in the hindbrain pacemaker, EOD amplitude also fluctuates in a circadian pattern, indicating that the biophysical properties of the electrocytes in the periphery also cycle daily (Stoddard et al., 2006). In *Sternopygus*, the day–night cycle in EOD amplitude recorded from intact fish is paralleled by daily fluctuations in action potential amplitude of electrocytes measured *in vitro*. Electrocytes harvested at night generate higher amplitude action potentials than those harvested from the same individual during the day (Markham et al., 2009b). This diel cycle in action potential amplitude (and thereby EOD amplitude) is accomplished at the cellular level by trafficking sodium channel proteins between the electrocyte membrane during the night and back into intracellular vesicles during the day. Changes at all these levels – whole organism EOD, electrocyte excitability and ion channel trafficking—can be accomplished within minutes by *in vivo* and *in vitro* treatment with adrenocorticotropic hormone (ACTH), which is known to fluctuate in a circadian pattern in many teleost fish. Thus, researchers have traced the hormonal regulation of circadian changes in the communication behavior of these fish to identified subcellular processes that underlie the output of the peripheral effector organs.

In *Brachyhypopomus*, EOD shape along with EOD amplitude vary in a circadian pattern (Stoddard et al., 2007). Specifically, the second phase of the EOD, which is already broader in males than in females, becomes even broader at night. Thus, males further masculinize their EOD during periods when they are most engaged in social behavior. In a manner similar to that of *Sternopygus*, melanocortins (alpha MSH and ACTH) in *Brachyhypopomus* act directly on electrocytes *via* a cAMP/PKA phosphorylation pathway that regulates the electrocyte biophysics, in this case by altering kinetics of both voltage-gated sodium and potassium channels (Markham and Stoddard, 2005; Markham et al., 2009a).

Comparison

These studies over the past decade have demonstrated that melatonin likely plays a crucial role in diel rhythms of social signaling in both vocal and electric fish. Both groups show circadian patterns of signaling behavior that can be modified by manipulation of melatonin levels. In vocal fish, melatonin appears to act primarily on the duration of calls while in electric fish it acts on the EOD rate. Because midshipman fish live in the temperate zone and inhabit shallow intertidal waters during the breeding season, they experience seasonal changes in daylength and thus seasonal changes in melatonin may act in combination with reproductive hormones to regulate annual cycles of signaling behavior. By contrast, electric fish generally live in tropical regions where daylength changes are less detectable. Consequently, they may be less likely to use melatonin for regulating annual cycles of signaling. In electric fish, diel changes in signaling are also regulated by melanocortins as well as melatonin, while melanocortins have not been examined in vocal fish. These hormones act peripherally to control diel patterns in EOD amplitude and shape by acting on regulation and trafficking of ion channels in the electrocyte membrane.

Aromatase

One focus of a previous comparison between vocal and electric fish (Bass and Zakon, 2005) was the role of sex steroids produced by the gonads in long-term regulation of communication behavior during the breeding season. Since then, studies in both vocal and electric fish have demonstrated that rapid metabolism of steroids by the brain can be an important regulator of social behavior during the breeding season and, in electric fish, the non-breeding season as well. In these recent studies, the emphasis has been on the distribution and action of the steroidogenic enzyme aromatase in the brain. Aromatase converts testosterone to estradiol, and in doing so, it influences the local production and action of steroids on neural circuits controlling behavior. Compared to other vertebrates, teleost fish, including vocal and electric fish, have exceptionally high brain levels of aromatase (Forlano et al., 2006), suggesting that the brain is an important site of steroid metabolism.

Vocal Fish

In plainfin midshipman, aromatase is prominently expressed in brain regions controlling vocal production, including the POA, anterior and ventral tuberal nuclei in the forebrain, the periaqueductal gray in the midbrain, and the vocal pre-pacemaker nucleus (VPN) and VMN in the hindbrain (reviewed in Forlano et al., 2015). Aromatase expression varies across seasons, sexes and male phenotype, indicating a regulatory role in vocal signaling (reviewed in Shaw, 2018). Aromatase increases in the POA and VMN in females during the pre-nesting period and in type II males during the nesting period. Moreover, aromatase activity in the hindbrain is higher in both females and type II males than in type I males.

These differences in aromatase expression correspond to differences in vocal behavior and its response to exogenous steroids. In type I males, intramuscular injection of the non-aromatizable androgen, 11-ketotestosterone (11KT) rapidly increased fictive call rate in type I males, while in females and type II males, estradiol (E2) and testosterone (T) rapidly facilitated the production of sex and morph specific calls. When aromatase was inhibited pharmacologically with fadrozole (FAD), only females showed disruptions in call duration (Remage-Healey and Bass, 2004, 2007; Shaw, 2018). This suggests that aromatase plays a key role in regulating sex and morph specific behavior by shifting local steroid concentrations toward estrogenic pathways or away from androgenic pathways in females. All three morphotypes have elevated circulating testosterone levels in the breeding season, but type II males and females have the highest levels and do not exhibit humming calls. (Only type I males show elevated circulating levels of 11KT). Aromatase is found abundantly in type II males and females may increase local estrogen concentration in the VMN, and thereby inhibit vocal activity (Forlano et al., 2005; Fergus and Bass, 2013; reviewed in Shaw, 2018). Alternatively, high aromatase levels in females may decrease VMN testosterone levels that appear crucial for supporting the production of humming vocalizations (Schlinger et al., 1999; Forlano and Bass, 2005a,b; reviewed in Shaw, 2018).

Brain aromatase may influence perception as well as the production of vocal signals. During the breeding season, females enhance the sensitivity of their auditory system to match the dominant frequency in the male advertisement call (Sisneros, 2009; reviewed in Shaw, 2018). Experimental treatment with either estradiol or testosterone induced this same shift in the auditory system (Sisneros et al., 2004; Shaw, 2018). The auditory nerve ganglion, located adjacent to the sensory epithelium of the inner ear's saccule, expresses high levels of aromatase (Forlano et al., 2005; reviewed in Shaw, 2018). These observations suggest that aromatase increases local levels of estradiol in the ganglion *via* conversion of circulating testosterone, which then diffuses to the saccule to induce a cascade of events that shift the tuning of saccular hair cells (also see Rohmann et al., 2013).

Electric Fish

In contrast to vocal fish, electric fish show more ambiguous evidence for a direct effect of aromatization on the brain nuclei controlling signal production or reception. In a transcriptomic of *Apteronotus leptorhynchus*, Smith et al. (2018) found abundant aromatase transcripts in the hindbrain pacemaker nucleus (PN) that drives the continuous EOD and sets its discharge frequency. However, Shaw and Krahe (2018), using *in situ* hybridization, found no aromatase mRNA in the PN. These contrasting findings may result from different methods or from different gonadal states of the subjects. The midbrain prepacemaker nucleus (PPn-C) that regulates chirping behavior lacks aromatase mRNA, but the forebrain nuclei (e.g., the ventral subdivision of the ventral telencephalon, the POA and lateral hypothalamus) that influence electric signaling express abundant aromatase, suggesting that local estrogen production could indirectly affect communication by acting on higher order inputs to the electrocommunication circuitry (Shaw and Krahe, 2018).

Although it is not clear whether aromatase plays a prominent role in the regulation of electrocommunication, it clearly participates in the regulation of the unusual non-breeding aggressive behavior of the electric fish *Gymnotus omarorum*. In the past, aggression has been typically studied in the context of male competition for resources and mates during the breeding season, when elevated androgens produced by the gonads act on neural circuits in the brain (reviewed in Cunningham et al., 2012; Fuxjager et al., 2017). However, such reproduction-related male aggression is only one form of aggression. In some species, including *G. omarorum*, males display aggression during the non-breeding season as well as the breeding season, and females display aggression as well as males (Batista et al., 2012; Silva et al., 2020). In both of these unusual forms of aggression, circulating androgens are at low levels (Quintana et al., 2016). This non-breeding season male aggression and female aggression raise the question of how this behavior is regulated through mechanisms other than gonadal androgens. Several recent sets of studies suggest that such aggression is likely regulated through aromatization of extra-gonadal androgen into estrogen (Jalabert et al., 2015).

While *G. omarorum* shows aggression in both the breeding and non-breeding season, the underlying mechanisms appear to vary seasonally. During the non-breeding season, male aggression is unaffected by castration, and dominant and subordinate

males do not differ in plasma levels of 11-KT. Thus, non-breeding aggression is independent of androgens or any other gonadal signal. However, treatment with an aromatase inhibitor, FAD, rapidly (within 30 min) decreases aggression, indicating that production of estrogens in the brain act through quick non-genomic mechanisms to regulate non-breeding aggression (Jalabert et al., 2015). In contrast to this non-breeding aggression, aggression during the breeding season most likely depends on the more typical hormonal regulation: high circulating androgen levels originating from the gonads increase aggression with a time course of hours to days. Thus, while the aggressive behavior of males is similarly high all year, the underlying hormonal control mechanisms change seasonally (Quintana et al., 2016).

Female agonistic behavior during the non-breeding season depends on aromatase in a manner similar to that in males. FAD treatment to females rapidly inhibits overall female aggression. Notably, treatment with an androgen receptor antagonist does not affect aggressive levels, at least over the timescale of minutes (Zubizarreta et al., 2020). These studies indicate that estrogen originating in the brain regulates aggression in females as well as males. None of these aromatase-dependent changes in overt aggression are accompanied by changes in electric signaling (Zubizarreta et al., 2020).

Comparison

Studies of aromatase have expanded our notions of how steroids regulate social behavior in these two teleost groups. In vocal fish, such studies have helped explain how steroids can have rapid effects on vocal behavior by the local production and rapid action of estrogens, especially in females. In electric fish, these studies have helped explain the regulation of female aggression and non-breeding male aggression. However, while there is abundant neuroanatomical and behavioral evidence for a direct action of aromatase on the vocal nuclei of vocal fish, the evidence for a direct action in the electrocommunication system is still equivocal.

Social Regulation of Steroids and Communication Behavior

Before 2005, several studies in these two systems focused on how seasonal changes in the *physical* environment stimulate steroid production, which then had long-term actions on the nervous system to cause seasonal changes in reproductive behavior and signaling. More recently, two sets of studies have demonstrated that specific features of the *social* environment can induce steroid secretion and consequent changes in social behavior. One commonality in these studies is that they demonstrate that in both electric and acoustic modalities, exposure to communication signals alone is sufficient to induce steroid-dependent changes in behavior.

Vocal Fish

As male toadfish gradually populate nesting sites during the breeding season, the calling of one male can induce calling in neighboring males. Field experiments showed that non-calling males can be induced to call within 48 h by exposing them to a nearby calling male (Remage-Healey and Bass, 2005). Such social exposure elevated plasma 11KT levels without affecting plasma cortisol. Subsequent fieldwork demonstrated that audio playbacks of male calls were sufficient to elicit this behavioral and hormonal response. This response was only elicited by acoustic stimuli that replicated the naturally occurring advertisement call ("boatwhistle") and not by less realistic acoustic stimuli. Further studies showed that experimentally increasing 11KT levels in non-calling males by feeding them food pellets embedded with 11KT increased call rate and duration within 20 min (Remage-Healey and Bass, 2006). Underwater audio playbacks induced an increase in both call rate and duration, implying a separate effect of auditory stimulation on call duration (Remage-Healey and Bass, 2005). The rapid effect of this treatment along with companion neurophysiological studies indicated that androgens exert their action through a non-genomic mechanism (Remage-Healey and Bass, 2006). Together these studies support a model of social regulation of communication in which acoustic features of the natural call cause an increase in androgen secretion which then potentiates calling by acting rapidly on nuclei of the vocal network.

Electric Fish

In the wild, male electric fish, *Apteronotus leptorhynchus*, emit chirps when intruder males enter their territory and compete for mates (Henninger et al., 2018). In the laboratory, long-term exposure of a male to a nearby conspecific male potentiated chirping over a time course of 4 days (Dunlap et al., 2002). Under these conditions, a male's overall chirp rate decreased over time, but when presented with a standardized synthetic electric signal that mimics a conspecific male, the focal male chirped at greater rates, indicating that the underlying neural circuitry becomes sensitized to stimuli. Such long-term social interactions simultaneously increased plasma cortisol without affecting androgens. Experimental treatments that increased cortisol in isolated males potentiated chirping while pharmacologically blocking cortisol receptors in socially exposed males decreased chirping (Dunlap et al., 2011). These hormonal manipulations indicate that cortisol causally contributes to socially induced changes in chirping behavior (Dunlap et al., 2013).

In addition to their effect on chirping behavior, social exposure and cortisol treatment increased the addition of newborn neurons in the PPn-C, the brain region that regulates chirping (Dunlap et al., 2006). While the precise mechanism by which this neurogenesis potentiated chirping is not known, the temporal and regional specificity of the effect strongly suggests that it contributes to socially induced, cortisol-dependent changes in chirping behavior (Dunlap et al., 2013). Experimental presentation of electrocommunication signals alone was sufficient to induce these changes in the neurogenesis and behavioral output of the PPn-C, but a simple electrical sine wave of the same frequency was ineffective (**Figure 4**; Dunlap et al., 2008). Thus, like in vocal fish, this behavioral change can be elicited with stimuli in a single modality, and only when these stimuli quantitatively mimic the natural communication signal.

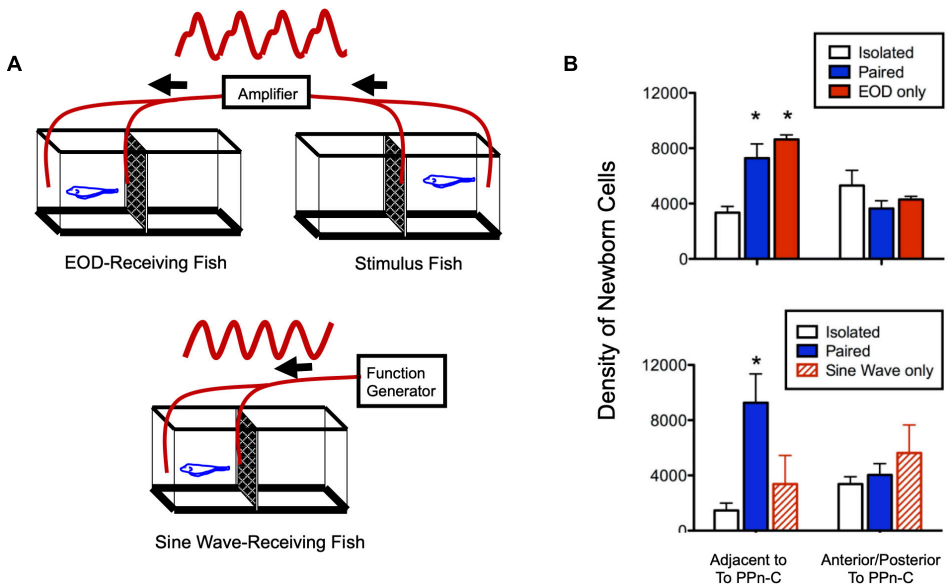

FIGURE 4 | Social electric signals stimulate neurogenesis in the electrocommunication circuitry. **(A)** Experimental set up in which fish received a real, one-way social electric signal from a conspecific (top) or an artificial sine-wave stimulus (bottom). **(B)** Fish exposed to direct paired social interaction or EOD stimulus alone have equivalently higher levels of neurogenesis compared to isolated fish in a neurogenic zone adjacent to the brain region controlling chirping (prepacemaker nucleus, PPn-C), but not in a neighboring control region (top). Fish receiving an artificial sine-wave stimulus show levels of neurogenesis equivalent to isolated fish (bottom). Asterisks indicate significantly different than isolated group. Adapted and modified from Dunlap et al. (2008).

Comparison

Studies in both vocal and electric fish have thus identified specific components of social signals that are effective in causing steroid-mediated changes in social behavior. However, these studies have focused on different behavioral contexts, timescales, steroids and neural mechanisms. In vocal fish reproductive signaling, social stimuli rapidly elevate androgen levels which activate the vocal-motor circuits within minutes through non-genomic pathways. In electric fish aggressive signaling, social stimuli elevate cortisol levels which promote new cell formation in the PPn-C to modify the output of the electrocommunication over the course of days.

CONTRIBUTIONS OF MOLECULAR STUDIES TO THE NEUROETHOLOGY OF SOCIAL BEHAVIOR

Since the 2005 review (Bass and Zakon, 2005), many new molecular techniques, particularly in transcriptomic analysis, have enabled neuroethologists to probe genetic mechanisms underlying social behavior. While work at this molecular level is still in its infancy in both systems, several studies have demonstrated new ways that the neural circuitry underlying vocal and electric communication are regulated over both physiological and evolutionary timescales.

Vocal Fish

Recent studies using contemporary transcriptomic techniques in midshipman have examined how the neuropeptide galanin, acting in the preoptic area (POA), influences neuroendocrine characters related to alternative reproductive tactics (ARTs). Both the POA and anterior hypothalamus (AH) of teleosts include neuronal populations comparable to populations of hormone-synthesizing neurons in the POA of birds and mammals. This region is referred to as the POA-AH in Tripp et al. (2018); but here it is referred to as the POA (for further discussion, see Tripp et al., 2020). Goodson and Bass (2000a) and Kittelberger et al. (2006) had previously provided strong neurophysiological evidence that the POA is a key node regulating expression of ART-related vocal behaviors in midshipman fish.

Propelled by advances in next generation sequencing technologies (RNA-seq), a recent transcriptomic study in midshipman fish revealed candidate genes related to hormone action in the POA that were specific to male morph (type I vs. type II) and behavior (nest-holding vs. cuckolding) (Tripp et al., 2018). Four genes – galanin, urocortin, corticotropin releasing hormone (CRH), and oxytocin receptor – showed highest expression levels in courting type I males, which provide parental care, compared to both type I and type II cuckolders, which do not provide parental care. Two other genes, thyrotropin and growth hormone, showed the highest expression levels in cuckolding type I and II males compared to courting type I males.

The well-described influence of galanin on social behavior (including parental and sexual behavior) in rodents (Bloch et al., 1996; Park and Baum, 1999; Moffitt et al., 2018) inspired Tripp and Bass (2020) to follow up the transcriptomic analysis with two subsequent studies. The first study mapped the distribution of galanin throughout the brain using a midshipman-specific galanin antibody and revealed a sex difference in the number of galanin-containing somata in the POA and the density

of galanin-labeled fibers, especially in the midbrain and the hindbrain (both values were greater in both male morphs than in females) (**Figure 5**; Tripp and Bass, 2020). The results supported the earlier transcriptome study, showing that the POA has the largest population of galanin-containing somata in the brain (see references in Tripp and Bass, 2020 for other teleosts).

In the second study, Tripp et al. (2020) used the galanin antibody together with a marker for neural activity, phosphorylated S6 protein (pS6; see Knight et al., 2012), to determine whether morphotype or vocal behavior correlated with activation of galanin-containing neurons. They found a far greater proportion of active galanin-containing POA neurons in courting type I males than in females and cuckolding type I and type II males. Moreover, this greater fraction of active galanin neurons was found only when courting type I males were in the nest with gravid females and not when they were either guarding previously fertilized eggs or defending the nest against type I or II cuckolders (Tripp et al., 2020). Thus, the activity of these galanin-containing neurons is specific to both morphotype and behavior. The results are consistent with earlier studies using microarray and RNA-seq analyses of whole brain samples in other teleost species with (bluegill sunfish *Lepomis macrochirus*; Partridge et al., 2016) and without (African cichlids, *Astatotilapia burtoni*, formerly *Haplochromis burtoni*; Renn et al., 2008) ARTs and suggested a role for galanin in the regulation of divergent patterns of social behavior among males. The lack of increased activation of galanin-containing neurons during egg care by type I males is consistent with the results in a study of dendrobatid poison dart frogs showing a similar lack of response in species with uniparental (male or female) care, whereas one species with biparental care shows increased galanin-POA neuron activation during parental care (Fischer et al., 2019). In aggregate, these studies suggest that POA-galanin neurons (1) have a conserved role in reproductive-related behaviors in lineages as divergent as fish, amphibians and mammals, and (2) are one of the neural substrates contributing to the evolution of ARTs among teleosts and perhaps other vertebrates (see Tripp et al., 2020 for further discussion).

These investigations point to exciting new research directions to pursue in the future, for example those investigating interactions between galanin and other hormone signaling systems recognized in the initial transcriptome study (Tripp et al., 2018). How might those signaling mechanisms change the intrinsic and network properties of neuronal networks driving social behaviors, such as reproductive-related vocalization in midshipman fish?

Electric Fish

In electric fish, molecular work has focused mainly on the circuitry that generates the EOD (Pn, spinal electro-motoneurons [EMNs], and EO), which are evolutionarily novel structures. The earliest studies, begun before the widespread use of transcriptomics, took a candidate-gene approach focusing on species differences in expression and sequence of a muscle-expressing, voltage-gated sodium channel (*scn4a*) gene. This gene duplicated in an ancestral teleost, and eventually one paralog (scn4aa) shifted its expression from muscle to the evolutionary novel, muscle-derived EO in both mormyroids and gymnotiformes (Zakon et al., 2006; Arnegard et al., 2010; Paul et al., 2016). There, it evolved rapidly and likely contributed to the underlying species differences in EOD.

Subsequent transcriptomic studies assess differences in gene expression between muscle and EO more broadly (Gallant et al., 2014, 2017; Nagel et al., 2017). One recent study utilized the rapidly radiating mormyrid genus *Paramormyrops* to identify a gene for structural elements of the EO that vary across species and might be the basis for species differences in EOD waveform (Losilla et al., 2020). Transcriptomes of mormyrid EOs also revealed a gene for a voltage-gated potassium channel (*kcna7a*) that is expressed at high levels (**Figure 6**; Swapna et al., 2018). Like the sodium channel gene *scn4aa*, this gene is expressed in muscle of other fish but shifted its expression into the EO in the ancestor of mormyrids and underwent a burst of rapid evolution. This channel evolved a novel region that shortens action potential duration, thereby shaping the extremely brief EODs characteristic of many mormyrid species.

Most work on the evolution of electric signaling in the gymnotiformes comes from the family Apteronotidae. Apteronotids are interesting for a few reasons. First, they have a neurogenic electric organ, that is formed by the axons of EMNs. Second, as mentioned above, their EMNs are spontaneously active and synchronized by descending inputs from the Pn. Third, they have strong sex differences in EOD frequency and the direction of sexual dimorphism differs across species. In a transcriptomic analysis of the PN, Smith et al. (2018) identified a number of genes that are differentially expressed between two species of apteronotids with species differences in EOD range and the direction of sexual dimorphism. These include genes for steroid receptors and enzymes in steroidogenic pathways, as well as various ion channels that likely control the continuous firing frequency of PN neurons. Thompson et al. (2018),identified a novel voltage-gated Na+ channel (*scn4ab1*) expressed in the EMNs that resulted from a gene duplication within apteronotids. This channel has amino acid substitutions that prevent it from closing completely. Continuous Na+ influx through this leaky channel leads to spontaneous firing of the EMNs.

Just recently, researchers have begun using molecular analysis to examine how evolutionarily novel regions of the brain originated and how these new sensory and motor regions interface with the existing brain regions controlling social behavior (e.g., hypothalamic nuclei). As a start to this endeavor, Eastman et al. (2020) examined gene expression in the hypothalamus of a gymnotiform pulse-type species, *Gymnotus omarorum*. As mentioned above, this species is highly aggressive, even in the non-breeding season, and shows strong dominance-submissive relationships when paired in the laboratory. In this study, gene expression in the POA was assessed and a number of genes (such as somatostatin) and genes associated with sex steroid synthesis (aromatase) or metabolic processing (e.g., Cyp450) were differentially expressed between dominant and submissive animals.

The diversity of electric fish life histories and communication within each lineage and, especially the fact that numerous

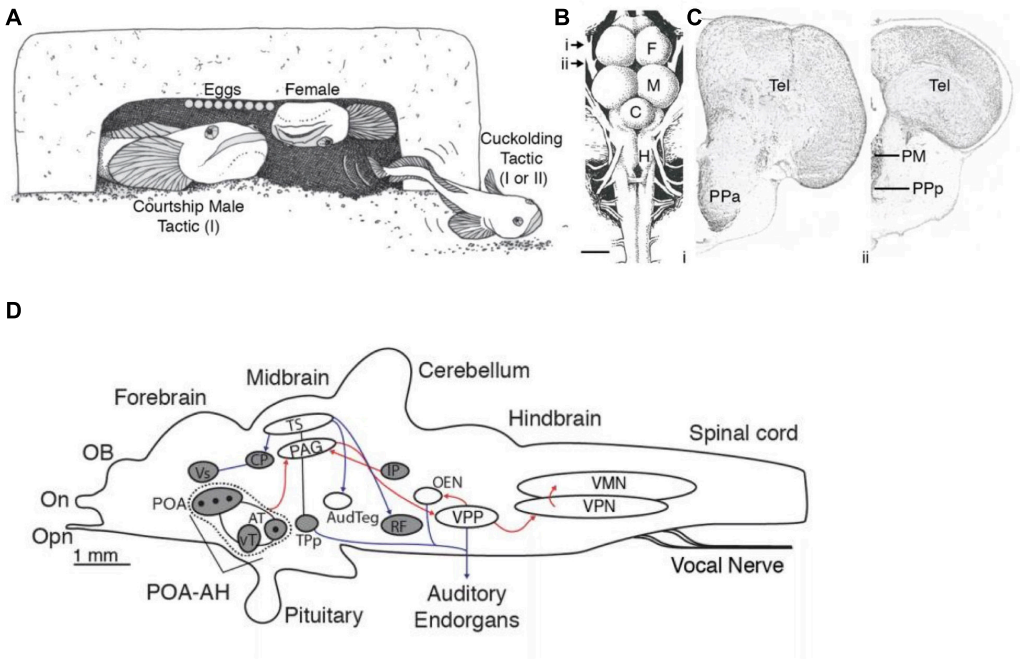

FIGURE 5 | Alternative male reproductive morphs, preoptic area and galanin expression in the vocal circuitry of plainfin midshipman, *Porichthys notatus.* **(A)** Male midshipman exhibit alternative patterns of reproductive tactics. Type I males guard nests under rocky shelters, from where they broadcast a long duration (up to 2 h), multi-harmonic advertisement call known as a hum to attract females for spawning. Type II males do not exhibit these behaviors, but instead satellite (as shown here) or sneak (within the nest) spawn trying to steal egg fertilizations from resident type I male. Type I males that are small in body size also sneak or satellite spawn when they are unable to have their own nest. See text for more details. **(B)** Dorsal view of midshipman brain. Arrows indicate level of sections shown to the right. Scale bar represents 1 mm. **(C)** Transverse sections through midshipman brain at rostral (i) and caudal (ii) levels of the preoptic area (see panel **B**). **(D)** Distribution of immunoreactive galanin expression differs between male and female midshipman. Figure shows sagittal view with major nuclei in the auditory and vocal systems. Black dots indicate location of Gal-ir somata. Shading indicates brain regions having Gal-ir fibers in females and both male morphs, whereas unshaded regions contain Gal-ir fibers in both male morphs that are greatly reduced or absent in females. Red and blue lines indicate connections within the vocal and auditory systems, respectively. Arrowheads show direction of connections. Lines without arrowheads indicate reciprocal connections. C, cerebellum; F, forebrain; H, hindbrain; M, midbrain; PM, magnocellular preoptic area; PPa, anterior parvocellular preoptic area; PPp, posterior parvocellular preoptic area; Tel, telencephalon. Adapted from Tripp and Bass (2020) and Tripp et al. (2020).

lineages evolved electroreception and electrogeneration, provides a richness for future mining using transcriptomic approaches, and the number of additional molecular techniques available for these groups is rapidly increasing (Pitchers et al., 2016).

VOCAL AND ELECTRIC: THE NEUROETHOLOGY OF DUAL COMMUNICATION SYSTEMS IN CATFISH

Mochokid catfishes offer a unique opportunity to reveal general principles underlying the organization of different communication systems because this speciose taxon includes species that produce either vocal signals or weakly electric discharges (ED) using the "same" neural circuitry and muscle (**Figure 7**; e.g., Hagedorn et al., 1990; Baron et al., 1994). In some especially intriguing species, a single individual can produce both vocal and electric signals from the same peripheral effector organ (Hagedorn et al., 1990). Some mormyrid fish are both weakly electric and vocal, but unlike mochokids, they use completely different organ systems to generate each type of signal (Bass, 1986; Crawford and Huang, 1999).

Organization of Motor System in Catfish

Both vocal and electric signals are generated by the muscle associated with the elastic spring system (ESS), which evolved originally as a sonic swim bladder mechanism (Boyle et al., 2014). The ESS is composed of a neural circuit in the caudal hindbrain and the elastic spring apparatus (ESA) in the periphery. The ESA consists of the protractor muscle connecting a process of the fourth vertebra, the Müllerian ramus, and the swim bladder wall (Parmentier and Diogo, 2006). Contraction of the protractor muscle at pulse repetition rates of ∼100 Hz vibrates the swim bladder to generate sounds in a manner similar to the muscle surrounding the swim bladder of toadfishes.

Electrogenic catfish have smaller Müllerian rami compared to their vocal relatives (Kéver et al., 2021) and marked differences in the ultrastructure of the protractor muscle. In sound-producing species, this muscle has many myofibrils organized into highly ordered sarcomeres like other skeletal muscles, while in ED producing species, the muscle is largely missing this pattern

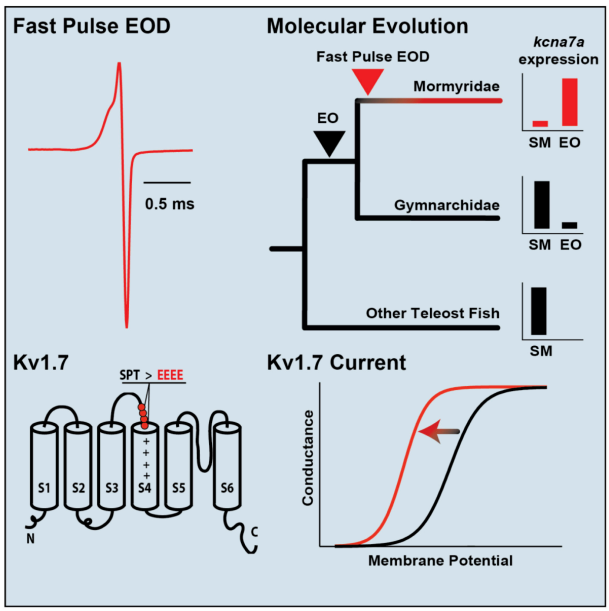

FIGURE 6 | Specialization of a voltage-gated potassium channel for tuning species-specific EOD duration. **(Top, left)** The mormyrid *Brienomyrus brachyistius* produces a brief EOD pulse about half a millisecond in duration. **(Top, right)** the potassium channel gene, kcna7a (which makes a potassium channel called Kv1.7), is expressed in skeletal muscle (SM) in most teleosts (black), but it has shifted its expression to the electric organ (EO) in mormyrids (red). **(Bottom, right)** Sensitivity of Kv1.7 channel to voltage shown by a sigmoidal curve of membrane potential vs. conductance. The sensitivity of Kv1.7 in *B. brachyistius* (red) is shifted to the left (i.e., is activated at a more hyperpolarized voltage) than in other teleosts (black). **(Bottom, left)** The cause of the leftward shift in the membrane potential-conductance curve is due to the evolution of four negatively charged glutamates (EEEE) that replaced three neutral amino acids (SPT, serine, proline, threonine) in an ancestral mormyrid. This has occurred above the voltage-sensing part of the potassium channel (S4) and indicated by the plus signs. Adapted and modified from Swapna et al. (2018).

of contractile elements (Boyle et al., 2014), with myocytes that resemble the independently evolved myogenic electrocytes of gymnotiform and mormyrids. When the protractor muscle of an ED producing species is treated with an acetylcholine antagonist, the amplitude of the ED is greatly diminished, indicating that the ED is indeed produced by activation of these modified muscle cells. Thus, the same muscle appears to function for sound production or electric discharge (Boyle et al., 2014).

The protractor muscle is innervated by the hindbrain motor nucleus, which receives input from several premotor neural populations (Hagedorn et al., 1990; Ladich and Bass, 1996; Kéver et al., 2020, 2021). The overall organization of this circuit is similar to the comparable circuit in toadfishes (see section "Neural Circuitry Generating Vocal and Electric Signals" above): a medially fused nucleus with large motoneurons and surrounding premotoneurons.

Many species of mochokid catfish produce either vocal or electric signals. However, some, such as *Synodontis eupterus*, can produce both vocal and electric signals in different phases of social behavior. In *S. eupterus,* the ESS phenotype (e.g., the density of myofibrils in the protractor muscle and the length of the Mullerian ramus) is intermediate between vocal-only or electric-only species (Hagedorn et al., 1990; Boyle et al., 2014; Kéver et al., 2021). In addition, the neural circuit that controls the ESS, has a larger pool of motoneurons compared to the homologous circuit in closely related species that produce only sonic or only electric signals. Thus, while this dual signaling species has evolved an "intermediate" peripheral signaling organ, it has simultaneously evolved greater motor control by the brain.

At higher levels in the control circuit, little is known about the neurochemical identity of the transneuronally mapped premotoneurons (i.e., excitatory, inhibitory or modulatory) or how the intrinsic and network properties of neurons contribute to motor patterning of protractor muscle output, whether sonic or electric. Behavioral and EMG recordings from the protractor muscle, however, indicate precise bilateral synchronous contractions, with high repetition rates suggestive of superfast muscles in the vocal species (Rome et al., 1996; Rome, 2006). A study investigating differences between the intrinsic properties of motoneurons of a vocal and ED fish showed that indeed motoneurons are adapted to such precise firing (Kéver et al., 2020). Electrophysiological studies like those carried out in toadfishes are needed to better understand the function of the individual network components.

Evolutionary Patterns in Vocal and Electric Communication in Catfish

The available phylogenetic evidence suggests that vocal signaling is the ancestral condition among mochokid catfishes (Kéver et al., 2021). However, many of the investigated species in the genus *Synodontis* appear to have transitioned partly or entirely to electric signaling (see above). The ability to communicate with multiple channels might be selectively advantageous to both sender and receiver. But what selection pressures favored a full transition from vocal to electric or the ability to generate both signaling modalities? While all fishes can apparently hear sounds, only some (including catfishes) have the capability to detect weakly electric fields (Andrianov and Ilyinsky, 1973; Peters and van Wijland, 1974; Knudsen, 1976a,b; Bullock and Heiligenberg, 1986; Peters and van Ieperen, 1989). Thus, communication in the electric modality would offer a more "hidden" form of communication and limit detection by non-electroreceptive predators. Environmental factors could further favor such transitions. As suggested by Kéver et al. (2020), clear water environments could favor the more cryptic ED system since the combination of acoustic and visual signals in clear water would make them especially conspicuous to predators. In addition, the two modalities differ considerably in their effective communication distance: EDs are short range signals while acoustic signals are far ranging (Heiligenberg, 1977; Brenowitz, 1986; Rogers and Cox, 1988; Bass and Clark, 2003). EDs could thus be favored for close range communication (<1 m), while vocal signals could be favored for longer-distance communication (>1 m). While many questions about mochokid catfish remain unexplored, they might offer insights to fundamental issues

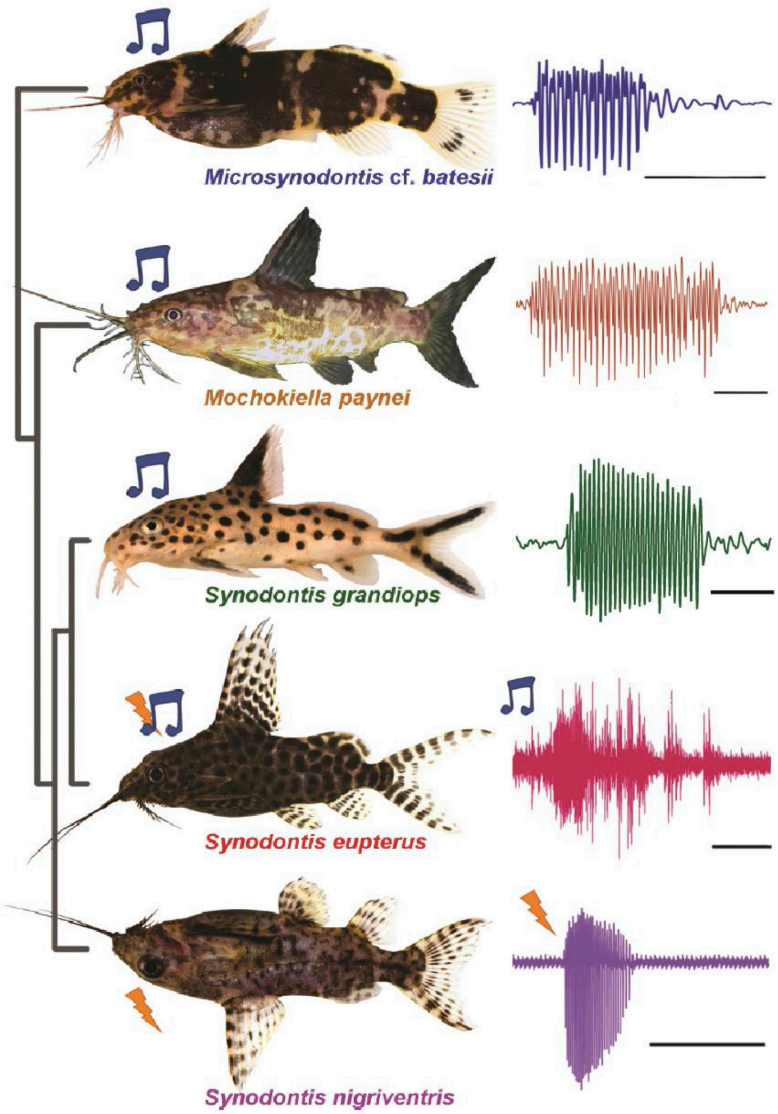

FIGURE 7 | Mochokid catfish produce either vocal and/or weakly electric discharges. **(Left)** Photographs of five different mochokid species (blue note symbol denotes species that produce sound signal; lightning symbol denotes species that produce electric signal). Species studied so far are either sonic (e.g., top three), generate both types of signals (e.g., *S. eupterus*; only sonic signal shown here) or generate only electric discharges (e.g., *S. nigriventris*). **(Right)** Examples of waveforms of signals produced by the elastic spring apparatus. Scale bar is 50 ms for top four species shown and 500 ms for *S. nigriventris*. Modified and adapted from Kéver et al. (2020) and Kéver et al. (2021).

in comparative neuroethology, such as the developmental and evolutionary origins of novel communication channels among vertebrates.

FUTURE DIRECTIONS

Through over 50 years of intensive research in these two systems, we are now well aware of the many commonalities and differences in the regulation of circuits underlying vocal and electric communication in teleost fish. Still, the usefulness of this comparison would be advanced by future research in several specific areas. For example, more thorough mapping of neuromodulator receptors in electric fish, particularly receptors for 5HT and melatonin, would enable us to compare the neuromodulatory regulation of electromotor and electrosensory systems with the better mapped receptor systems of vocal fish. On the other hand, additional research on the physiological actions of 5HT on vocal circuitry and behavior and the behavioral actions of 5HT and AVT on aggressive behavior would allow for better comparison with similar published studies in electric fish. Currently, there is no information on the role of adult neurogenesis in the regulation of social behavior in vocal fish and little information on the role of galanin in the social behavior

in electric fish; future research in both these areas will allow for interesting comparisons. Finally, although the role of light and melatonin in regulating daily cycles of social behavior have been examined in both species, very little is known in either system about their role in seasonal cycles of behavior.

Mochokid catfish with both vocal and electrogenic systems raise particularly interesting questions about the regulation of the neural circuitry underlying social communication. Do the same modulatory systems that regulate the more ancestral vocal system also regulate the more derived electrogenic system? Do the modulators act to control the same temporal patterning in both systems? In species that produce both vocal and electric signals, are neuromodulators involved in switching between these dual modalities? Comparative neuroanatomical, physiological and behavioral studies among mochokid catfishes offer many opportunities to investigate these questions.

While research on these important gaps in our understanding of vocal and electric communication in adult fish should be pursued, we also believe there is great promise in examining the ontogeny of neural circuits in these systems as well. Further application of transcriptomic methods, including single cell RNA sequencing, would allow us to characterize how certain cell types within a circuit (for example, pacemaker vs. vocal/electro- motoneurons) differ in gene expression. These techniques could similarly describe how the novel, highly specialized cells involved in vocal and electric communication diverge from ontogenetically and phylogenetically homologous tissue types (e.g., the transition from skeletal muscle to sonic muscles or electrocytes). Parallel developmental studies could trace when and how these differences arise during ontogeny. Recent methods in spatial transcriptomics (Waylen et al., 2020) could resolve gene expression differences in closely apposed cell types within the developing neural circuits. Finally, targeted genetic manipulations [e.g., using CRISPR (Constantinou et al., 2019) for loss-of-function studies or transgenics for gain-of-function studies] could then demonstrate which genetic differences contribute causally to the divergence in neuronal phenotype within a neural circuit or the emergence of novel cell types during evolution.

Catfish with dual-modality signaling systems offer a particularly interesting model for addressing how vocal and electric communication systems are constructed in other teleosts. Are similarities in the neural circuits that generate these different signals in the same individual attributable to a shared tissue origin or common developmental processes? How do differences in their vocal and electrogenic circuits emerge ontogenetically? In the broadest sense, these and other future investigations of vocal and electric fish offer great promise for those seeking to uncover mechanisms underlying the evolution and development of vertebrate social behaviors.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and editing of the manuscript.

FUNDING

This work was supported in part by funding from the NSF (165664) to AHB.

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The handling editor declared a past co-authorship with one of the authors HZ.

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frontiers in Neural Circuits

OPINION
published: 29 September 2021
doi: 10.3389/fncir.2021.731691

Using Optogenetic Dyadic Animal Models to Elucidate the Neural Basis for Human Parent–Infant Social Knowledge Transmission

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Keywords: social learning, optogenetics, dyadic EEG, parent–infant, neural synchrony

INTRODUCTION: SOCIAL LEARNING ACROSS SPECIES

Healthy early development depends on a warm reciprocal relationship between parent and offspring, where parent and infant interact in close temporal co-ordination as if engaged in a "dyadic dance" of glances, gestures, smiles and words (Stern, 1985; Gianino and Tronick, 1988). Most, if not all, early learning takes place during these well-choreographed social exchanges, which support cultural knowledge transmission from parent to offspring using verbal and non-verbal forms of communication and behavioural modelling. Such vicarious knowledge transmission through social interaction (rather than direct experience) is known as social learning (Bandura, 1971; Csibra and Gergely, 2009). Tomasello (2014) argues that human mastery of these "second-personal social relations" (Darwall, 2006)—in which social partners share and create *joint* knowledge, intentionality and goals—has accelerated the rise of the human species through "cultural intelligence" (Herrmann et al., 2007).

One important and early developing form of social learning is social referencing. Here, a social partner's actions and emotions are used to form one's own understanding of a situation and guide behaviour (Feinman, 1982). Two main forms of social referencing are commonly recognised. *Instrumental* social referencing—also termed observational learning—refers to the use of others' actions to shape behaviour (cf. Bandura's Bobo doll experiment; Bandura, 1992), as occurs during imitation. *Affective* social referencing refers to the use of others' emotional expressions for event appraisal (Campos, 1983; Hornik and Gunnar, 1998). Affective social referencing - the focus of this article - is well-studied in human infants (Feinman, 1982; Hornik and Gunnar, 1998; Clement and Dukes, 2016), and develops over the first year of life. By 10–12 months of age, infants begin to seek information from others in novel situations and use this information to regulate their own affect and behaviour (Feinman et al., 1992). For example, human infants at this age will avoid crossing a short visual cliff (Sorce et al., 1985), show less interaction with toys (Gunnar and Stone, 1984; Hornik et al., 1987) and be less friendly to strangers when their mothers show negative emotion toward these objects or individuals as compared to neutral or happy emotional expressions (Feinman and Lewis, 1983; Feinman et al., 1986). Such social knowledge transmission from parent to offspring is therefore crucial during early life in helping infants to safely explore and learn about their physical and social environments.

An analogous rudimentary form of social learning occurs in animal species such as mice, an example of which is the social transmission of food preferences (STFP). When a naive mouse interacts with a social partner who has eaten a novel flavoured food, this social interaction confers familiarity with the flavour, and the naive mouse will now eat more of the familiarised food than completely new food (Galef, 2003; Wrenn, 2004). Crucially, as in human subjects, the learning

OPEN ACCESS

Edited by:
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Reviewed by:
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University of Rennes 1, France

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Received: *28 June 2021*
Accepted: *31 August 2021*
Published: *29 September 2021*

Citation:
Leong V, Ham GX and Augustine GJ (2021) Using Optogenetic Dyadic Animal Models to Elucidate the Neural Basis for Human Parent–Infant Social Knowledge Transmission. Front. Neural Circuits 15:731691. doi: 10.3389/fncir.2021.731691

of food preferences occurs through face-to-face social interaction: when the naive mouse sniffs the breath, face and whiskers of the demonstrator mouse. During murine development, this form of social learning underpins intergenerational transmission of food choices between adult mice and pups, allowing weanlings who are exploring their food options to learn vicariously about safe foods (and avoid eating poisoned foods) that their elders have experienced (Silverman et al., 2010). Therefore, in both human and murine species, knowledge transmission through social interaction with adult caregivers plays a vital role in shaping the developing youngling's understanding of the world and how to interact successfully with it. However, much still remains unknown about the neural mechanisms and processes that support this form of vicarious social learning.

NEURAL SYNCHRONY: AN EVOLUTIONARILY CONSERVED MECHANISM FOR SOCIAL KNOWLEDGE TRANSMISSION?

Neuronal oscillations are observed across many species and support basic processes in information encoding, memory and attention. In humans, perception relies on neural oscillatory processes in the cortex that shape our conscious experience (Buzsaki, 2006). Research suggests that the oscillatory phase of neural activity at the time a stimulus occurs may relate to the excitability of cortical neuron populations and to the magnitude of event-related responses elicited by the stimulus (Lakatos et al., 2008; Busch et al., 2009; although see Ruzzoli et al., 2019). Accordingly, perceptual stimuli that are delivered during a high-excitability phase of neural oscillations are more likely to be detected and encoded than stimuli that arrive at a low-excitability, inhibitory phase (Busch et al., 2009; Mathewson et al., 2009). Extending this conceptual framework to the social (dyadic) domain, the phase of on-going neural oscillations in the child's brain may similarly determine the efficacy of capturing information from their social partner "in the moment." However, because social interaction is an active process, rather than a passive one, this presents the possibility that social partners may actively modulate each other's neural state, using salient social cues (like gaze or touch) to transiently reset the phase of their partner's neural oscillations. For example, a parent may initiate eye contact to reset the phase of her child's neural oscillations to match her own oscillations, triggering a short-term increase in parent-child neural synchrony. During this brief state of high interpersonal synchrony, parents' and infants' neural receptivity periods are mutually well-aligned in time or "coupled." This allows pieces of information delivered by the parent (e.g., spoken

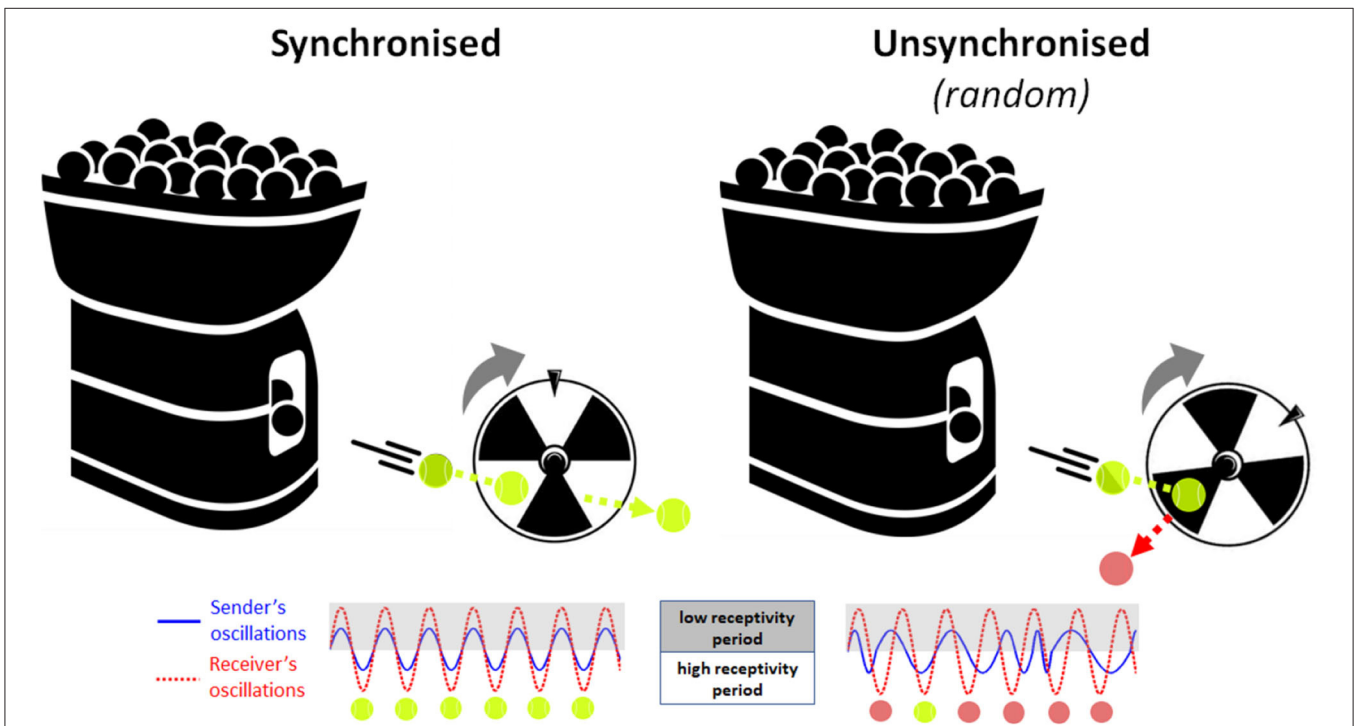

FIGURE 1 | Analogical illustration of the Learning through Interpersonal Neural Coupling (LINC) hypothesis. Here, the tennis ball machine (information sender) attempts to fire balls through the blades of a rotating wheel (information receiver). (Left) If the timing of ball firing is well-synchronised to the rotation pattern of the wheel, then every ball passes successfully between the blades every time. (Right) Conversely, if the balls are fired randomly without reference to the phase of the turning wheel, then many balls will hit the blades and bounce out and only a few will pass through by chance. Therefore, synchronisation permits all the information (balls) to be safely transmitted from the sender (machine) to the receiver (wheel), where the timing of ball firing and wheel rotation represent the neuronal oscillation patterns of the information sender (blue solid line) and the information receiver (red dotted line), respectively.

words) to be presented at optimal times for encoding (learning) by the infant "receiver." As a simple analogy, imagine a scenario where a tennis ball machine fires balls repetitively through a rotating turbine wheel (see **Figure 1**). If the timing of ball firing is well-synchronised to the rotation pattern of the wheel, then every ball passes successfully between the blades every time. Conversely, if the balls are fired randomly without reference to the phase of the turning wheel, then many balls will hit the blades and bounce out and only a few will pass through by chance. Therefore, synchronisation is the key to transmitting all the information (balls) from the sender (machine) safely through to the receiver (wheel), where the timing of ball firing and wheel rotation represent the neuronal oscillation patterns of the sender and receiver, respectively. This two-brain synchronisation model of social learning, or *Learning through Interpersonal Neural Coupling (LINC) Hypothesis* predicts that social learning is "gated" by interpersonal neural synchronisation, and that transient states of synchronisation are achieved through the use of social signals that reset the phase of on-going oscillations.

In human adult-infant dyads, recent dyadic-electroencephalography (EEG) studies have shown that during social interaction, adult-infant neural oscillation patterns can indeed become transiently synchronised (Leong et al., 2017; Santamaria et al., 2020; Wass et al., 2020). Consistent with the LINC Hypothesis, it has recently been found that stronger neural synchronisation between human mothers and their infants (as measured by an index of phase-locking) does indeed predict a higher likelihood of successful affective social referencing by infants (Leong et al., 2019). Further, natural increases in interpersonal phase synchronisation are associated with the use of social teaching signals such as eye contact and prosodically enhanced maternal speech (Leong et al., 2017, 2019), which suggests that such social signals may indeed increase interpersonal synchronisation through mechanisms such as oscillatory phase-resetting. However, the non-invasive constraints of human infant studies prevent a deeper interrogation and understanding of the exact neural structures and circuits that generate interpersonal synchrony. Further, the correlational nature of human infant studies does not permit causal inference of whether neural synchrony is *necessary* for social learning or merely a meta-phenomenon of the process—a long-standing debate in the field of two-person neuroscience.

A PRECISION TOOL FOR STUDYING SOCIAL LEARNING NEURAL MECHANISMS: THE DYADIC OPTOGENETIC MOUSE MODEL

Although interpersonal neural synchrony was documented first in humans, this mechanism may in fact be evolutionarily conserved to subserve social interaction behaviour across human and non-human animal species. Recent animal research suggests that interbrain neural synchrony predicts a diverse set of social interaction behaviours in rodents (Kingsbury et al., 2019) and in bats (Zhang and Yartsev, 2019). For example, Kingsbury et al. (2019) performed microendoscopic calcium imaging between pairs of freely interacting mice and found that neural activity in the dorsomedial prefrontal cortex (dmPFC) was highly correlated between mice during social interaction. This strong adult-adult dyadic neural correlation was dependent on features of the ongoing social interaction rather than on shared sensory input from a common environment or concurrent behaviour. Further, dmPFC correlation between mice predicted their future social interaction patterns and dominance relationship. In a similar study with bats, Zhang and Yartsev (2019) used wireless electrophysiology to perform simultaneous recordings of neural local field potentials (LFPs) and spiking activity in pairs of spontaneously interacting bats. Both LFP power and spike activity were highly correlated between bats over multiple timescales, ranging from seconds to hours. Further, the degree of neural correlation covaried with the extent of social interaction between bats, spiking just before interactions were initiated. These initial animal studies indicate that socially induced synchronisation of neural activity between conspecifics may be a fundamental mechanism that drives and shapes social interaction patterns and preferences. Here, we specifically propose that interpersonal neural synchronisation supports social knowledge transmission across species.

If interpersonal neural synchronisation is in fact causally necessary for social learning, then targeted manipulation of neural synchronicity within the dyad should also influence the success of social learning. Optogenetic methods provide an optimal way to test this causal link. Optogenetics is a revolutionary technology that permits genetically defined, light-based control of neural circuits, providing unparalleled spatial, temporal and genetic resolution for the study of neural and cognitive mechanisms in living organisms (Boyden et al., 2005; Kim et al., 2017). In this approach, transgenic animals (e.g., rats or mice) express light-gated ion channels, pumps or receptors [i.e., opsins such as channelrhodopsin-2 (ChR2) and halorhodopsin] in specific types of neurons. The activity of these neurons can be selectively increased (photostimulation) or decreased (photoinhibition) by exposure to light of the appropriate wavelength, allowing experimental control of neural activity—and the cognitive functions that these neural circuits subserve—at the flick of a light switch. Animal models have long been used to study basic learning and social behaviour, but in recent years, optogenetic technology has increasingly been employed to study complex social behaviour in animals (e.g., anxiety, depression and aggression; Yizhar, 2012). In regard to the study of social learning, optogenetics may be employed to assess the success of transmission of food preference from a mouse dam to her pup (i.e., STFP) during either synchronous or asynchronous stimulation of parent and infant brain regions. Recently, Yang et al. (2021) demonstrated the feasibility of a dyadic optogenetic approach in pairs of freely interacting adult mice, through the use of implantable, miniaturised wireless stimulation devices. This is an important methodological advance as it permits precise control and experimental manipulation of interpersonal synchrony *at the neural source*, allowing the direct tests of causality on observed

social behaviour that will significantly advance understanding in our field.

FUTURE RESEARCH AVENUES AND CHALLENGES

If dyadic optogenetic technology can be successfully implemented in infant mice, parent-pup mouse optogenetic models could revolutionise the study of early social learning and be used to elucidate the precise neural pathways and mechanisms by which responsive caregiving and parenting behaviour act to scaffold early neurodevelopment and cognitive skills in offspring. These models can also be extended to study the aetiology of social developmental disorders such as autism, ADHD and other learning disabilities, as well as disorders of parent–child interaction and bonding, which occur during maternal depression and other forms of early life stress.

However, a dyadic optogenetic mouse model involving pups has never been created before, which presents new and significant technical challenges. For example, although the expression of channelrhodopsin and other optogenetic probes can be robust by age P21, this may impose a lower limit on the age at which pups may be tested and (depending on the exact promoter employed) may preclude the study of very early perinatal behaviour. Also, although lightweight head-mounted wireless devices (e.g., weighing as little as 20 mg) are now available (Montgomery et al., 2015), this still presents a significant load for very small pups and may impose restrictions on movement, feeding and other social interactive behaviour that would be of interest.

A second major challenge pertains to the design and selection of animal social experimental paradigms that are suitable for use with very young animals, and also closely parallel social behaviour in human infants, to permit meaningful comparison of cross-species data. For example, here we suggest that the social transmission of food preference in mice is a form of social learning that is analogous to social referencing by human infants. Although learning occurs through social interaction in both cases, the modality of information and its transfer (and therefore the sensorimotor pathways involved) are different. In the mouse paradigm, the information transmitted and learned is primarily olfactory (although auditory cues such as ultra-short-range high-frequency vocalisations may also be involved in shaping such social interactive behaviour; Warren et al., 2020) whereas human infants rely more on visual and auditory information from the caregiver's facial and vocal expressions, gestures and actions when performing social referencing (Sorce et al., 1985; Leong et al., 2019). This difference in perceptual processing pathways may be non-trivial when seeking to draw inferences from animal to human learning behaviour. It would be even more complex—and perhaps impossible—to draw extrapolations to higher social mental functions, such as theory of mind and other social mentalising abilities (e.g., inferring others' intentions, goals, beliefs), possibly limiting the utility of animal models in advancing understanding in these more complex areas of social cognition.

Nonetheless, the advent of dyadic optogenetic technology is a boon and could—within the next decade—fundamentally remake the landscape of developmental social neuroscience and neuropsychiatry.

AUTHOR CONTRIBUTIONS

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

FUNDING

This research is supported by the Ministry of Education, Singapore, under its Academic Research Fund Tier 1 [RG99/20 to VL and GA; RG152/18 (NS) to VL].

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 11 November 2021
doi: 10.3389/fnbeh.2021.733140

Spontaneous Visual Preference for Face-Like Stimuli Is Impaired in Newly-Hatched Domestic Chicks Exposed to Valproic Acid During Embryogenesis

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Faces convey a great amount of socially relevant information related to emotional and mental states, identity and intention. Processing of face information is a key mechanism for social and cognitive development, such that newborn babies are already tuned to recognize and orient to faces and simple schematic face-like patterns since the first hours of life. Similar to neonates, also non-human primates and domestic chicks have been shown to express orienting responses to faces and schematic face-like patterns. More importantly, existing studies have hypothesized that early disturbances of these mechanisms represent one of the earliest biomarker of social deficits in autism spectrum disorders (ASD). We used VPA exposure to induce neurodevelopmental changes associated with ASD in domestic chicks and tested whether VPA could impact the expression of the animals' approach responses to schematic face-like stimuli. We found that VPA impairs the chicks' preference responses to these social stimuli. Based on the results shown here and on previous studies, we propose the domestic chick as animal model to investigate the biological mechanisms underlying face processing deficits in ASD.

Keywords: autism spectrum disorder, face processing, social predispositions, brain development, sodium valproate

OPEN ACCESS

Edited by:
Gervasio Batista, Harvard University, United States

Reviewed by:
Özge Sungur, University of Marburg, Germany
Konstantin V. Anokhin, Lomonosov Moscow State University, Russia

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Specialty section:
This article was submitted to Individual and Social Behaviors, a section of the journal Frontiers in Behavioral Neuroscience

Received: *29 June 2021*
Accepted: *22 October 2021*
Published: *11 November 2021*

Citation:
Adiletta A, Pedrana S, Rosa-Salva O and Sgadò P (2021) Spontaneous Visual Preference for Face-Like Stimuli Is Impaired in Newly-Hatched Domestic Chicks Exposed to Valproic Acid During Embryogenesis. Front. Behav. Neurosci. 15:733140. doi: 10.3389/fnbeh.2021.733140

INTRODUCTION

Biological predispositions to orient to and preferentially learn about conspecifics are one of the earliest expressions of social behavior in vertebrates and are critical for survival. These elementary behavioral markers of social orienting are spontaneous, possibly hard-wired, mechanisms that bias visual attention to simple features of animate beings since the earliest minutes of life (Goren et al., 1975; Johnson et al., 1991). Human faces and schematic face-like patterns generate remarkable responses in typical developing neonates (Simion and Di Giorgio, 2015). More strikingly, the same abilities can be observed in newly-hatched chicks (Rosa-Salva et al., 2010; Rosa Salva et al., 2011) and visually naïve monkeys (Sugita, 2008, 2009). Other species have also been shown to respond to similar schematic configurations (Leopold and Rhodes, 2010), such that privileged face processing could be pervasive in vertebrates.

More importantly, it has been hypothesized that early disturbances of these social orienting mechanisms may be one of the earliest signs of social deficits in autism spectrum disorders (ASD) and might also contribute to the pathophysiology of these disorders by compromising, early on, the typical developmental trajectories of the social brain (Dawson et al., 2005; Johnson, 2005; Senju and Johnson, 2009; Johnson et al., 2015). In line with that, impairments in face and eye-gaze direction processing have been reported in infants at risk of ASD (Di Giorgio et al., 2016; Webb et al., 2017, for a critical discussion see also Jones and Klin, 2013; Shultz et al., 2018; Bradshaw et al., 2020).

Given the complexity of human social behavior and the limitations that human studies impose, animal models are instrumental in providing clues on the nature and origin of these crucial social orienting mechanisms and their role in atypical social development. Valproic acid (VPA) exposure has been extensively used in several animal models to reproduce ASD core symptoms (Bambini-Junior et al., 2014). Previous studies have shown that exposure to different doses of VPA during embryogenesis induces alterations of several aspects of social behavior in domestic chicks (Nishigori et al., 2013; Zachar et al., 2019). We used VPA exposure to induce neurodevelopmental changes associated with social deficits in domestic chicks and tested whether VPA could impact the expression of early approach responses to schematic face-like patterns. We found that VPA impairs the chicks' preference responses to these social stimuli. Based on the results shown here, we propose the domestic chicks as elective animal models to study these early-emerging neurobehavioral markers and to investigate the biological mechanisms underlying face processing deficits in ASD.

MATERIALS AND METHODS

Ethical Approval

All experiments were conducted according to the current Italian and European Community laws for the ethical treatment of animals. The experimental procedures were approved by the Ethical Committee of the University of Trento and licensed by the Italian Health Ministry (permit number 986/2016-PR).

Embryo Injections

Fertilized eggs of domestic chicks (*Gallus gallus*), of the Ross 308 (Aviagen) strain, were obtained from a local commercial hatchery [Agricola Berica, Montegalda (VI), Italy]. Upon arrival the eggs were placed in the dark and incubated at 37.5°C and 60% relative humidity, with rocking. One week before the predicted date of hatching, on embryonic day 14 (E14), fertilized eggs were selected by a light test, before injection. Chick embryo injection was performed according to previous reports (Nishigori et al., 2013; Sgadò et al., 2018). Briefly, a small hole was made on the egg shell above the air sac, and 35 μmoles of VPA (Sodium Valproate, Sigma Aldrich) were administered to each fertilized egg, in a volume of 200 μl, by dropping the solution onto the chorioallantoic membrane (VPA group). Age-matched control eggs were injected using the same procedure with 200 μL of vehicle (double distilled injectable water; CTRL group). After sealing the hole with paper tape, eggs were placed back in the incubator until E18, when they were placed in a hatching incubator (FIEM srl, Italy). Hatching took place at a temperature of 37.7°C, with 72% humidity. The day of hatching was considered post-hatching day 0 (P0).

Rearing Conditions

After hatching in darkness, 69 chicks (38 males and 31 females) were kept in the hatching incubator for 24 h before the experiment.

Apparatus and Test Stimuli

The test apparatus was a corridor, 45 cm long × 22.3 cm wide, made from wood and covered with opaque white plastic coating. The apparatus was divided in three sections (outlined on the apparatus floor), one central for positioning the animal, equidistant from the two stimuli, and two on the opposite side of the corridor, in proximity to the stimuli, considered the choice section. The stimuli were placed at the opposite side of the rectangular arena, on panels of light-filtering Plexiglas, lit by a 201 lumen LED placed behind the Plexiglas partition. The visual stimuli were previously described in Rosa-Salva et al. (2010). Briefly, they consisted of featureless face silhouette shapes, made of orange stiff paper (10 × 5.6 cm, see **Figure 1**) that contained internal features: three black squares (of side 1 cm), organized as an upside-down triangle for the schematic face-like configuration, or aligned vertically for the control non-social stimulus. Both stimuli were top-heavy configurations, having two elements in their upper part and one in their lower part.

Test Procedures

At postnatal day 1 (P1), about 24 h after hatching, chicks were transported in complete darkness to the test room and placed in the apparatus: positioning with respect to the test stimuli, as well as the left-right position of the stimuli in the apparatus, was counterbalanced across animals. The animals' approach responses were recorded using a camera placed on top of the apparatus, for the entire duration of the test (12 mins).

Statistical Analysis

We evaluated the absolute time spent in each section of the apparatus (face section, central section, and non-face section) and the effect of treatment and sex on these measures, using a mixed model considering treatment and sex as fixed between-subject factors and the time spent in each apparatus section as fixed repeated measures (within subject factor with three levels: face section, central section, and non-face section). The relative preference expressed for the two stimuli was also measured as a social preference index adjusted for the overall exploratory activity of the chicks during the test. This was calculated as the time spent in the choice section close to the social stimulus (schematic face-like configuration) divided by the total time spent in the two choice sections (face + non-face). Values of this ratio range from 1 (full choice for the social stimulus) to 0 (full choice for the non-social stimulus), where 0.5 represents the absence of

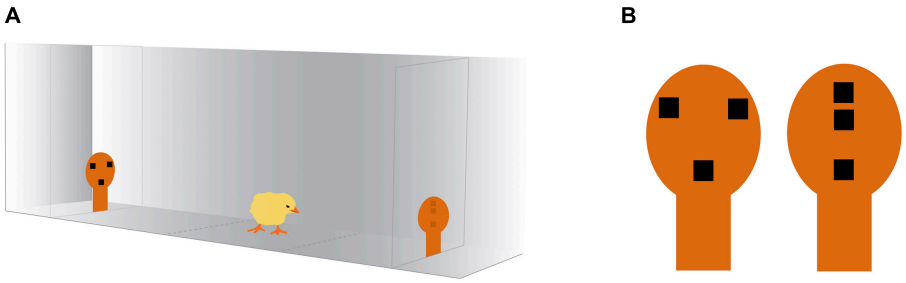

FIGURE 1 | Schematic illustration of the social preference test apparatus and the stimuli. **(A)** The chick was placed in the center of the arena and was free to approach either of the stimuli, placed at the two ends of the apparatus and lit by a 201 lumen LED. The chick's behavior was video-recorded from above. **(B)** The stimuli consisted of orange stiff paper silhouettes containing internal features resembling a face-like configuration (left) or a non-social control configuration (right). The chick image is courtesy of Openclipart (openclipart.org) under Creative Commons Zero 1.0 Public Domain License.

preference. Significant departures of the social preference index from chance level (0.5) were estimated by one-sample two-tailed *t*-tests. The number of chicks that first approached the two stimuli in the two treatment and sex groups was compared using two-sided Pearson's chi-square test. We assessed differences in behavioral activity measuring the time required to move to one of the choice sections (latency to choice) and the number of section switches (spontaneous alternations). Effect of Treatment and Sex on the social preference index, the latency to first choice and the spontaneous alternations was evaluated by multifactorial analysis of variance (ANOVA). Statistical analyses were performed with GraphPad Prism 9 and RStudio. Alpha was set to 0.05 for all tests.

RESULTS

To assess the effect of VPA on face perception, and avoid any possible influence of previous experiences in evaluating the chicks' approach to the stimuli, we excluded visual experience prior to the test. To obtain a better approach rate, we extended the duration of the test compared to the previous reports to 12 mins. Using this adapted paradigm, we tested 69 chicks (31 females, 38 males), 24 h after hatching.

We first analyzed the time spent by the animals in the choice sections of the apparatus (**Figure 2A**) using a mixed model analysis (see "Materials and Methods"). The results showed no significant main effect of treatment and sex on the time spent in the apparatus sections [treatment $F_{(1,\ 195)}$ = 4.812E-012, $p > 0.9999$; sex $F_{(1,\ 195)}$ = 1.084E-010, $p > 0.9999$], a significant main effect of the sections [apparatus sections $F_{(2,\ 195)}$ = 44.48, $p < 0.0001$] and a significant interaction of the treatment on the visited sections [treatment × apparatus sections $F_{(2,\ 195)}$ = 4.904, p = 0.0084]. No other significant interactions emerged [treatment × sex $F_{(1,\ 195)}$ = 9.114E-011, $p > 0.9999$; sex × apparatus sections $F_{(2,\ 195)}$ = 0.4469, p = 0.6403; treatment × sex × apparatus sections $F_{(2,\ 195)}$ = 1.287, p = 0.2784]. The Sidak multiple comparison test showed a significant effect of treatment on the time spent in the non-face chamber [$t_{(201)}$ = 2.421, p = 0.0335]. Thus, VPA treatment selectively increases the time spent by the animals attending the non-face stimulus.

To further evaluate the effect of treatment on the preference for the stimuli independent of the exploratory activity we also analyzed the effect of VPA exposure on the preference index (see "Materials and Methods"). We found a significant difference between the treatment groups in the preference index for the schematic face-like configuration stimulus [**Figure 2B**; treatment: $F_{(1,\ 65)}$ = 4.805, p = 0.0320; sex: $F_{(1,\ 65)}$ = 0.5745, p = 0.4512; treatment × sex: $F_{(1,\ 65)}$ = 2.652, p = 0.1083]. While vehicle-injected chicks significantly preferred the schematic face-like stimulus, VPA-exposed chicks did not display any significant preference for this stimulus compared to what expected by chance [**Figure 2B**; CTRL $t_{(32)}$ = 2.481, p = 0.0186; VPA $t_{(35)}$ = 0.3425, p = 0.7341; group mean: CTRL 0.6694 (95% CI: 0.5303–0.8085); VPA 0.4764 (95% CI: 0.3364–0.6164)]. We then analyzed the latency to express a choice and the number of section alternations after the first choice. We found a significant effect of treatment on the latency: VPA-injected chicks had a shorter latency to choice compared to controls [**Figure 2C**; treatment: $F_{(1,\ 65)}$ = 5.369, p = 0.0237 sex: $F_{(1,\ 65)}$ = 0.1881, p = 0.6660; treatment × sex: $F_{(1,\ 65)}$ = 0.1270, p = 0.7228; group mean: CTRL 339 s (95% CI: 275–403); VPA 234 s (95% CI: 172–295)]. Spontaneous alternations in the two choice sections did not significantly differ between treatment groups [**Figure 2D**; treatment: $F_{(1,\ 65)}$ = 1.941, p = 0.1683; sex: $F_{(1,\ 65)}$ = 0.0790, p = 0.7795; treatment × sex: $F_{(1,\ 65)}$ = 1.293, p = 0.2598; group mean: CTRL 5.091 (95% CI: 2.344–7.838); VPA 8.389 (95% CI: 5.077–11.70)].

The number of chicks that approached the face-like configuration as the first stimulus was not significantly different between treatment groups (Pearson's X_1^2 = 2.944, p = 0.0862; CTRL: face N = 21, non-face N = 12, VPA: face N = 15, non-face N = 20, data not shown).

DISCUSSION

Newborns of several vertebrate species exhibit rudimental knowledge about the typical appearance of animate beings that orients the young organisms' attention toward plausible social partners and caregivers. Several studies hypothesized that this mechanism contributes to create an early social bond with

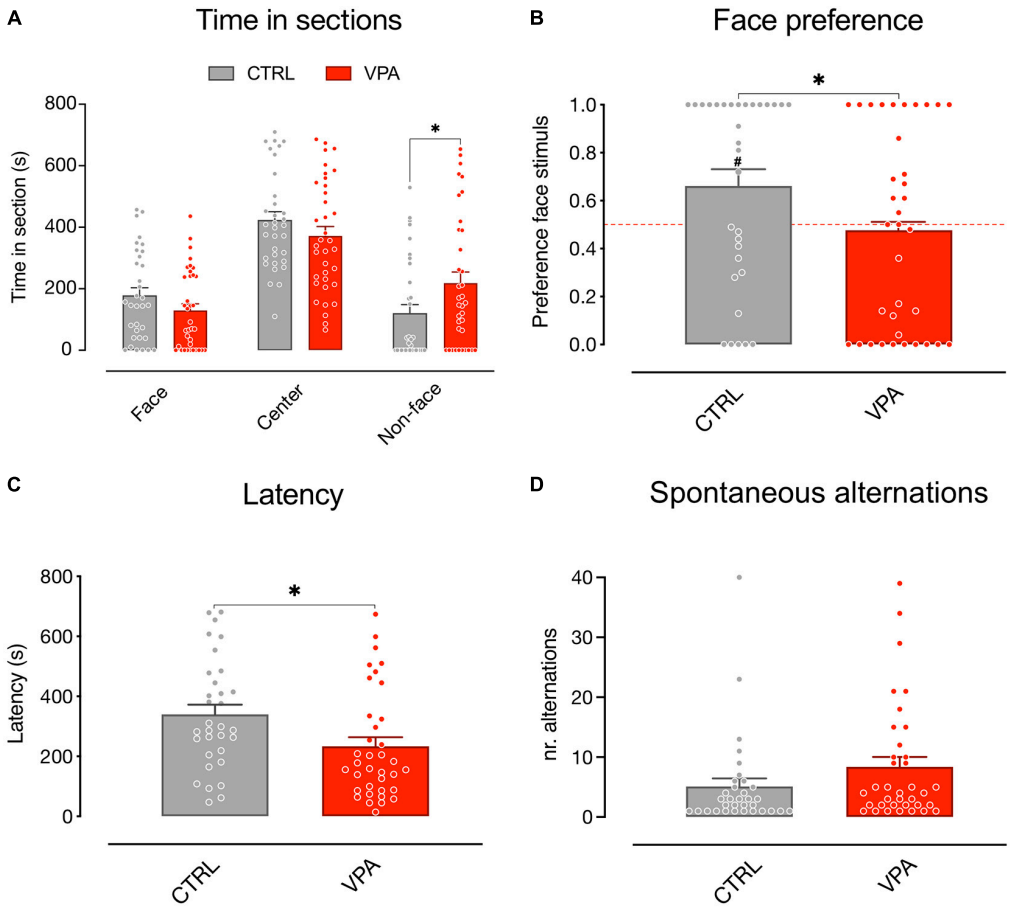

FIGURE 2 | Spontaneous visual preference test. Social preference test for schematic face-like (social) stimulus and non-social stimulus (see "Materials and Methods" for details). Bar graphs represent time spent in the choice sections **(A)** social preferences indexes **(B)**, latency to first choice **(C)**, and spontaneous alternations **(D)**. **(A)** Mixed model analysis on the time spent in the three apparatus sections (face, center, and non-face) considering treatment and sex as fixed between subject factors and the time spent in each apparatus section as fixed repeated measures, shows a significant difference in the absolute time spent in the three sections (not shown) and a significant interaction between treatment and time spent in each apparatus section, and no other main effect or interactions between the factors analyzed. Sidak multiple comparison test shows a significant effect of treatment on the time spent in the non-face chamber. **(B)** Analysis of variance of social preference indexes using treatment and sex as between-subject factors, revealed a significant main effect of treatment and no other main effects or interactions among the factors analyzed. One-sample *t*-test on preference indexes indicate a significant difference from chance level for the control group, but not for VPA-treated chicks. The number sign (#) indicate significant departures of the preference index from chance level (0.5), marked by the red line. **(C)** Behavioral activity during the test measured as latency to *express a* choice. Analysis of variance on time taken by the chicks to move in one of the choice sections using treatment and sex as between-subject factors, showing a significant effect of treatment and no other main effects or interaction. **(D)** Behavioral activity during the test measured as *sections* alternations. Analysis of variance on number of *alternations* between the three *sections*, using treatment and sex as between-subject factors, showing no significant main effect of treatment or sex, and no interactions. Data represent Mean ± SEM, $^{\#}p < 0.05$, $^{*}p < 0.05$.

caretakers and social companions (Johnson, 2005; Tomalski et al., 2009), an essential process for subsequent social and language development. Newborn babies, as well as non-human primates and domestic chicks, have been shown to express remarkable orienting responses to faces and schematic face-like patterns (Sugita, 2008, 2009; Rosa-Salva et al., 2010; Rosa Salva et al., 2011). Divergence from these early social interactions may induce a cascade of maladaptive trajectories culminating in atypical social abilities, such as those observed in ASD.

Predispositions in domestic chicks have been observed toward a variety of features of animate creatures and trigger preference responses to a very broad spectrum of representations: being them face-like configurations (Rosa Salva et al., 2011) biological motion (Vallortigara et al., 2005) or self-propelled motion (Rosa-Salva et al., 2016). Newly hatched domestic chicks express social preferences to features of animals belonging to other species, including potential predators, as shown by their innate preference toward a walking cat (Vallortigara et al., 2005) represented by point light displays or toward a taxidermized polecat (Rosa-Salva et al., 2019) or a human face (Rosa Salva et al., 2011). Similarly, face-naïve Japanese macaques spend equal time attending to humans and monkey faces and prefer both over inanimate objects (Sugita, 2008). This data shows that biological predispositions are clearly not species-specific, but include rudimental configurations shared across species to increase the chance of orienting toward other animals. In

the natural environment of a newly hatched organism, these other animals are most likely to be conspecifics (parents, siblings). Subsequently activated learning mechanisms, whose action is directed toward living creatures by the predispositions themselves, will provide the young animals with species-specific information on the appearance of their conspecifics [see also Morton and Johnson (1991) and Johnson (2005) for a broader discussion of the species-general nature of the representations underlying face-preferences in newborn babies and domestic chicks]. As to whether the face-like stimulus can be extended as a feature of conspecifics, studies show that the predisposed preference observed in newly-hatched chicks toward the stuffed hen or taxidermized newly-hatched chicks, mallard ducks or polecats, are indeed triggered by the head and neck region, suggesting a major role of face configurations in the head region (Johnson and Horn, 1988; Rosa-Salva et al., 2019; Miura et al., 2020).

Using the preference response to face-like stimuli as an evolutionarily conserved neurobehavioral marker and exploiting the advantages of animal models, we investigated whether these early-emerging social orienting mechanisms could be affected by a compound, VPA, known to interfere with development of the social brain. We examined the preference response toward schematic-face like configurations of animals whose pattern of brain development may have been altered by VPA, an anticonvulsant increasing the risk to develop ASD in humans. We found that VPA had a dramatic effect on the preference toward schematic-face configuration stimuli.

Previous studies have revealed a predisposed response to schematic face-like configurations in newly-hatched chicks, using both subjects imprinted on face-neutral stimuli and visually naïve subjects (Rosa-Salva et al., 2010; Rosa Salva et al., 2011, 2012). To assess the effect of VPA on face perception, and avoid any possible influence of previous experiences on the chicks' approach to the stimuli, we applied this latter experimental procedure, excluding visual experience prior to the test. Since dark reared animals are less active compared to chicks exposed to visual stimuli, to obtain a better approach rate, we extended the duration of the test compared to the previous reports. Increasing the test duration in our experiment contributed to heighten the approach response and the face preference, without introducing the potential influence of visual experience. We also noticed that the preference for the face-like stimulus was especially conspicuous in control females, which showed a remarkable preference level compared to all other groups [**Figure 3**; group mean preference index CTRL females 0.8029 (95% CI: 0.6425–0.9632), one-sample t-test $t_{(13)} = 4.081$, uncorrected $p = 0.0013$; group mean preference index VPA females 0.4318 (95% CI: 0.2064–0.6571); $t_{(12)} = 0.6419$, uncorrected $p = 0.5301$; group mean preference index CTRL males 0.5711 (95% CI: 0.3589–0.7832); $t_{(18)} = 0.7035$, uncorrected $p = 0.4907$; group mean preference index VPA males 0.5163 (95% CI: 0.3245–0.7081); $t_{(18)} = 0.1787$, uncorrected $p = 0.8602$]. However, given that no significant interaction between the factors sex and treatment emerged in our previous analysis, any difference between the two sexes observed here should be interpreted with caution. Notably, regardless of the sex of the chicks examined, VPA-exposed chicks spent significantly more time attending the non-social stimulus. This data is in line with what observed in other VPA models (Zhao et al., 2019) in which juvenile VPA-treated monkeys attended to non-social stimuli significantly more than their control siblings. Future studies will investigate the potential sex differences in the level of face-preference and in their susceptibility to VPA, suggested by some of our data, and clarify the mechanism of action of VPA on the development and expression of face preference in domestic chicks.

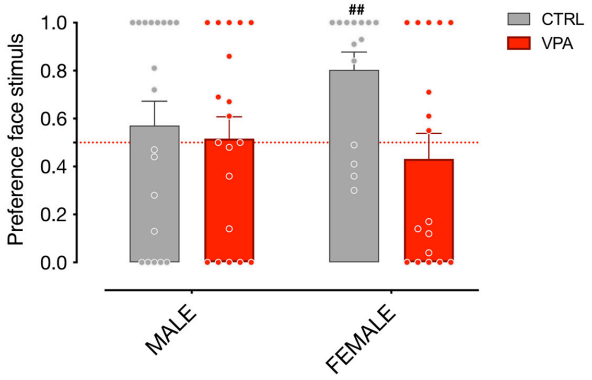

FIGURE 3 | Social preference index of sex groups. Bar graphs represent social preference indexes for males and females. One-sample t-test on preference indexes indicate a significant difference from chance level for female control animals, but not for males or VPA-treated chicks of both sexes. The number sign (#) indicate significant departures of the preference index from chance level (0.5), marked by the red line. Data represents Mean ± SEM, $^{\#\#}$uncorrected $p < 0.01$.

The reduced latencies observed in the VPA group, indicate that VPA exposure affects the visual preference for schematic face-configuration patterns without significantly hindering the chicks' motoric activity during the test. In line with that, previous studies from our lab have shown that VPA exposure, at the dosage used in this study, does not significantly affect motor behavior or discriminative abilities of simple artificial objects in domestic chicks (Sgadò et al., 2018).

A previous study has investigated the attentive behavior toward faces in VPA-exposed juvenile macaques (Zhao et al., 2019). Using eye-tracking analysis to measure the animals' attention to faces or scene containing conspecifics, the authors found that juvenile VPA-treated monkeys attended to non-social stimuli significantly more than their control siblings. However, the study did not specifically investigate the predisposed response of visually naïve animals to faces compared to a visually equivalent stimulus without social content. In this respect, our study is the first to analyze a very early predisposed response to faces in a visually naïve animal model of ASD.

Valproic acid is an anticonvulsant extensively used to treat epilepsy and bipolar disorders. VPA mechanism of action involves its direct inhibition of histone deacetylases (HDACs), interfering with normal deacetylation of chromatin and disrupting gene transcription at global scale, as well as

HDAC independent mechanisms (Sinha et al., 2021). Embryonic exposure to VPA is normally achieved by a single acute dose of VPA (ranging between 400 and 800 mg/kg in rodents) that induces a transient HDAC inhibition producing long lasting effects. Several studies suggest that embryonic VPA exposure affects neurogenesis (Kataoka et al., 2013; Lee et al., 2016; Sakai et al., 2018; Zhao et al., 2019; Cui et al., 2020; Sawada et al., 2021) and alters expression of several neurodevelopmental genes, involving serotonergic system development (Jacob et al., 2014; Messina et al., 2020) and excitation/inhibition imbalance (Rinaldi et al., 2007; Gogolla et al., 2009; Banerjee et al., 2013; Nagode et al., 2017). Given its antiepileptic pharmacological action, VPA has been shown to increase GABA levels in the brain, trough different mechanisms, acting on GABA transaminase and other enzymes linked to the metabolism of GABA (Johannessen, 2000), as well as trough inhibition of sodium channels (Abdelsayed and Sokolov, 2013). Despite extensive research investigating VPA pharmacological action and the genetic networks responsible for its effects on brain development, the biological mechanisms underlying the detrimental consequences of embryonic VPA exposure on social behavior in animal models are still unclear.

CONCLUSION

Altogether, this study and previous studies from our lab, demonstrate a detrimental effect of VPA, an anticonvulsant increasing the risk to develop ASD in humans, on the very early predisposed responses toward social stimuli in visually-naïve domestic chicks. Based on these results, we propose the domestic chicks as elective animal models to study these early-emerging neurobehavioral markers and to investigate the biological mechanisms underlying face processing deficits in ASD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of the University of Trento and licensed by the Italian Health Ministry (permit number 986/2016-PR).

AUTHOR CONTRIBUTIONS

PS conceived and designed the experiments and drafted the manuscript. AA and SP conducted the experiments. PS and OR-S analyzed the data. AA, PS, and OR-S wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by the University of Trento (intramural funds to PS and AA) and (OR-S) a grant from the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013) Grant ERC-2011-ADG_20110406, Project No: 461 295517, PREMESOR (PI Vallortigara).

ACKNOWLEDGMENTS

We thank Giorgio Vallortigara for his support, advice, and comments on the manuscript. Tommaso Pecchia for help with the experimental apparatus, Grazia Gambardella for administrative help and Ciro Petrone for animal facility management.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.733140/full#supplementary-material

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frontiers in Neural Circuits

OPINION
published: 18 November 2021
doi: 10.3389/fncir.2021.734474

The Parental Dilemma: How Evolution of Diverse Strategies for Infant Care Informs Social Behavior Circuits

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Keywords: parental care, neuroethology, neurogenetics, ethology, evolution

INTRODUCTION

Social relationships are cornerstones of human well-being and functioning in society, which is painfully apparent during the current global pandemic that has forced social isolation. Indeed, one of the most urgent problems highlighted by this crisis is the lack of available childcare for working parents. We are far from understanding how behavior is governed by external and internal forces, despite decades of intense scientific and lay interest. Social behavior may be examined using a range of methods, and indeed we (the authors) have opposite, but converging, perspectives that complement one another and offer an opportunity to re-examine how the researchers approach the study of social behavior. One view is a "behavior-based" study of social behavior, which values an understanding of natural history, ethological variability, and ecology using field based studies that maximize comparative approaches through the lens of evolution. The other is a "brain-based" study of social behavior, which values an understanding of neuronal circuits, connectivity, or gene expression using laboratory based studies that minimize variability. We believe the lack of overlap or cross-talk between these approaches is a barrier for the growth of our field and here we argue that these approaches are complementary and require insights gained from one other.

Among a range of naturalistic behaviors, parenting is exciting to study because these social relationships can be complex, multi-generational, and long-lasting. Parental care is also an evolutionary antecedent to other complex social behaviors, such as monogamy and eusociality (Queller, 1994; Numan and Young, 2016). Thus, exploring parenting can lead to mechanistic insights that are broadly applicable to many aspects of social behavior. Here, we use parenting as an example of how "behavior-based" and "brain-based" approaches to studying behavior can yield insights both into generalizable principles of how organisms function and alternative neural mechanisms of behavior that give rise to a diversity of behavioral strategies.

FROM DIFFERENT VIEWPOINTS: "BEHAVIOR-BASED" VS. "BRAIN-BASED"

Parental care has evolved independently across many taxa, highlighting the breadth of this behavior and the various ecological pressures that make this behavior more likely to increase offspring fitness. Parenting generally refers to behaviors that directly or indirectly benefit the young (Royle et al., 2012). For example, direct caregiving can consist of cleaning eggs, provisioning food to the young, carrying, retrieving, or grooming neonates, and huddling over newborns. Furthermore, indirect parenting behavior can include building and maintaining a nest, providing food for another parent, as well as protection of the young through defensive behaviors. While females are the sole providers

OPEN ACCESS

Edited by:
Joel D. Levine,
University of Toronto, Canada

Reviewed by:
Fadi A. Issa,
East Carolina University, United States
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Received: *01 July 2021*
Accepted: *27 October 2021*
Published: *18 November 2021*

Citation:
Autry AE and O'Connell LA (2021) The Parental Dilemma: How Evolution of Diverse Strategies for Infant Care Informs Social Behavior Circuits. Front. Neural Circuits 15:734474. doi: 10.3389/fncir.2021.734474

of care in the majority of mammals, males play more prominent roles in other vertebrate taxa, where most birds display biparental care and amphibians and fishes show a greater flexibility in who cares for offspring. Interestingly, social monogamy co-occurs with male involvement in offspring care in many birds and mammals, highlighting how ecology and neural mechanisms are likely intertwined to facilitate paternal care. The repeated evolution of parental behavior with many different forms begs the question of whether there are ecological and neural commonalities across these independent origins or whether different neurobiological mechanisms drive similar behavioral forms.

Investigating parental care with an ethological and evolutionary lens often centers around asking why such a behavior exists (its adaptive significance and how it evolved) and how such a behavior is regulated by physiology (from an ontogenetic and mechanistic perspective) (Tinbergen, 1969). Variations in social and parental care strategies are tightly linked to ecological resources in their environment (Wilson and Landry, 1975), making field studies an important part of understanding behavioral evolution. For example, the evolution of biparental care in burying beetles (*Nicrophorus*) is thought to have evolved in part due to the limited availability of carcasses in which they raise their families (Scott, 1998). Another example comes from poison frogs (Family Dendrobatidae), which have diversified in parental care strategies based partly on the size of nursery utilized to rear their young (Brown et al., 2008; Furness and Capellini, 2019). In most dendrobatid species, males guard egg clutches and then transport hatched tadpoles to pools of water. Female involvement in offspring care evolved twice within this clade when species shifted to tiny pools of water to avoid tadpole predation (Summers et al., 2008), which necessitated mothers to provision tadpoles with food in these resource-poor nurseries. Understanding the ecological challenges and opportunities that species face in their natural habitat is key to understanding why certain parental care strategies have (or have not) independently evolved or diversified among closely related species or species occupying similar habitats.

Repeated evolution or rapid diversification of parental care strategies sets the stage for comparative work on how behavior is governed at more mechanistic levels. This kind of research embraces variation within and across species at deep and shallow phylogenetic levels. Recently, RNA sequencing has been used to examine how brain gene expression changes with the onset of parental behavior in burying beetles (Parker et al., 2015), earwigs (Wu et al., 2020), stickleback fish (Bukhari et al., 2019), and poison frogs (Fischer and O'Connell, 2020), all highlighting key metabolic or immune system pathways that shift in gene expression as individuals transition to parenthood. More sophisticated sequencing approaches like phosphoTRAP, which allows the isolation of mRNA from active neurons (Knight et al., 2012), is becoming increasingly used in unusual animals to narrow down thousands of differentially expressed genes to a few hundred that could be targeted in functional manipulations. Although phosphoTRAP has been used to identify transcripts enriched with paternal behavior in dendrobatid poison frogs (Fischer et al., 2019), it lacks the cellular resolution of single cell sequencing. However, single cell sequencing requires well-annotated genomes and thus its use will lag behind genome sequencing efforts. Thus, the embrace of breadth often comes at the cost of depth in probing neuronal circuits, as many technologies used to functionally manipulate neural circuits are species specific (e.g., most viral vectors optimized for expression in mice) or are difficult to implement in most freely moving animals (e.g., electrophysiological recordings of neural activity). This lack of cross-species neurogenetic technologies requires one to either build the tools from scratch or ask questions that can be answered by measuring gene expression or neural activity and conducting follow-up pharmacological manipulations.

Modern neuroscience tools have enabled us to build on this critical foundation of behavior-based knowledge to dissect the underlying neurobiology in the context of laboratory experiments. Typically in these studies, a population of neurons is first identified and then it is subsequently asked how these cells contribute to behavior based on neuromodulatory pathways and neuronal connectivity. The advantage of this approach is that the role of a specific population of neurons may be studied and dissected, but the downside is that other potentially critical or redundant components of the behavior circuit may be overlooked. However, the concept of a neural circuit implies the activity of multiple neural populations, from the sensory inputs to integration with interoceptive cues followed by motor outputs and recurrent feedback. Thus, a single neural population or even two nodes of a circuit may function as part of numerous behavior circuits. As a result, some neural circuit manipulations may lead to behavioral impacts that are somewhat contradictory or complex. Therefore, it is important to connect the physiological properties of a cell type or population as precisely as possible with the manipulations being performed. Specifically with regard to dissecting circuits for complex behaviors such as social interactions, it is key to uncover how neurons respond to discrete aspects of the behavior including locomotion, novelty, social cues such as odor or vocalizations, as well as the motor output components.

Social behavior poses a unique challenge for quantification and manipulation of neural circuits in the laboratory due to the freely moving nature of the interactions. This is particularly the case for parental behavior which relies on interactions between adults and offspring in a simulated naturalistic environment. The advent of less invasive tools for these applications is opening up the possibilities for studying parenting. For example, neuropixel probes allow high-density electrophysiological recordings from multiple brain sites over protracted time periods (van Daal et al., 2021). Lightweight, wireless devices are also becoming available for optogenetic and pharmacological manipulations as well as recording neural signals, enabling greater flexibility in studying social behaviors (Barbera et al., 2019; Zhang et al., 2019). Finally, the ever-decreasing size of implants required for recording, combined with increasing ability to multiplex, will allow for recording and/or manipulating from multiple brain regions and allow a more fully integrated understanding of how neural circuits coordinate behavior (Yamawaki et al., 2016; Meng et al., 2018; Jennings et al., 2019). However, one caveat is that the majority of these tools have been designed for use in

mice and rats, which limits comparative studies (Stagkourakis et al., 2020). Another class of techniques propelling the field forward are single cell and single nucleus sequencing techniques which have revealed novel relationships among neural classes and are beginning to distinguish common and divergent genetic features among neurons active during specific behaviors (Moffitt et al., 2018; Lee et al., 2019). These approaches will lead not only to a more sophisticated understanding of neuronal cell-types involved in specific aspects of social behavior, but also enable more refined circuit manipulations using intersectional genetic targeting.

INTEGRATING PERSPECTIVES MOVING FORWARD

Understanding parenting behavior from both ethological and neurogenetic viewpoints is transforming how we understand circuit dynamics and trade-offs in behavior. While the preponderance of previous research has focused on studying the neural control of parenting behavior, overwhelmingly in mothers, we have an opportunity to learn about neural circuits for infant care by studying behavior in non-parents, including male and female alloparental behavior or even infanticide (Lukas and Huchard, 2014; Rogers and Bales, 2019). For example, ethologists have long studied parental care trade-offs associated with the dilemma between choosing offspring investment or infanticide (Hausfater and Hrdy, 2017; Ringler et al., 2017). Infanticide often occurs to destroy the young of a competitor, such as in male lions (Packer and Pusey, 1983) or poison frogs (Ringler et al., 2017). When environmental resources are scarce, parents may also choose to invest in specific offspring to the harm of others, like when poison frogs feed their younger tadpoles to older ones (Rojas, 2014). Neurogenetics experiments have shed light on the reciprocal interactions of care and infanticidal circuits, where circuit nodes highly active during parental care are often silent in infanticidal animals and vice versa, suggesting that the behavior circuits may be intertwined in order to tightly control the expression of behavior (Wu et al., 2014; Odaka et al., 2015). In mice, beautiful neurogenetics work has highlighted galanin as promoting parental care and inhibiting aggression (Wu et al., 2014; Kohl et al., 2018), while urocortin-3 seems to promote infanticide (Autry et al., 2021). In turn, these neurogenetics experiments have inspired more ethological work that has found that galanin contributes to parental care across a wide range of taxa (Fischer et al., 2019; Butler et al., 2020), but not all (Tripp et al., 2020). Thus, it appears as though highly conserved galanin circuits across animals may be evolutionarily tuned to promote the gains and losses of parental care in various taxa.

Moving forward, we call for more integration of ethological and neurogenetics approaches, whose reciprocal feedback enriches both the breadth and depth at which we understand the mechanisms of behavior and how these evolve and diversify. Neurogenetics allows extreme precision in understanding neural circuits while comparative work is necessary for understanding what neural circuit principles are generalizable across taxa vs. specific to a subset of species. Ultimately, it is behavior and physiology that are the substrates of natural selection and therefore understanding behavior in the context of the natural environment will be key to deciphering the purpose of any neural circuit. We believe that integration of both ethological and neurogenetic perspectives will open new experimental avenues, better define questions, and refine experimental approaches moving forward.

AUTHOR CONTRIBUTIONS

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

ACKNOWLEDGMENTS

The authors thank the editors for inviting their Opinion article and encouraging collaboration across disciplines. AEA is supported by a NARSAD Young Investigator Award, a Whitehall Foundation Award, and NIH Pathway to Independence Award R00-HD085188. LAO is a New York Stem Cell – Robertson Investigator and is supported by the National Institutes of Health (DP2HD102042), the National Science Foundation (IOS-1845651 and IOS-1827333), the Rita Allen Foundation, the McKnight Foundation, and Pew Charitable Trusts.

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frontiers in Neural Circuits

OPINION
published: 30 November 2021
doi: 10.3389/fncir.2021.769314

Investigating the Neurobiology of Abnormal Social Behaviors

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Keywords: social neuroscience, behavioral neuroscience, animal models, group behavior, abnormal social behavior, psychosocial illness

INTRODUCTION

Social interactions play a crucial role in our daily lives, well-being, and survival. For example, think about the last person you talked to, laughed or shared a meal with, and how it may have affected your mood. Now think about a recent group gathering, event or lab meeting and how it may have affected your actions or even your career. The term "social" is anchored in the processing of information that relates to other individuals and how we interact with them. Humans are social animals, yet many of us suffer from psychiatric illnesses that manifest in symptoms pertaining to social behaviors. These conditions include autism spectrum disorder (ASD), schizophrenia, depression, and social anxiety (Insel and Fernald, 2004; Frith, 2007; Báez-Mendoza et al., 2021b). Despite their prevalence, however, the etiologies behind such deficits are not well-understood and there are few effective treatments for them.

Discovering novel treatments for social deficit disorders requires a fundamental understanding of social behaviors and their neural substrates that are borne not only by observing neurotypical brains but also by describing aberrant behaviors caused by these disorders (Kennedy and Adolphs, 2012). Over the past century, abnormal social behaviors and their neurobiological underpinnings have been studied in humans and animal models, ranging from insects to non-human primates (O'connell and Hofmann, 2012). More complex behaviors have also been reduced to well-defined series of cognitive processes including (1) verbal and non-verbal communication, (2) interpreting others' feelings or intentions, and (3) social interactions. Such divisions have allowed researchers to take advantage of model species that specifically utilize one or more of these behaviors in their natural state, though no animal model can fully describe the complex neurological presentation of social deficit disorders displayed in humans. Nevertheless, many genetic animal models have been created that are well-suited to study certain aspects of these disorders and extrapolate the mechanisms that may underlie such behaviors. Although these models can allow for specific, well-defined phenotypes to be studied in detail, they do not truly capture the complex and multifaceted naturalistic behaviors that define most animal and human behaviors.

COMMUNICATIVE BEHAVIORS

Successful vocal and non-vocal communication between individuals plays a central role in the social behavior of many animal species (Krause et al., 2009). Communication facilitates the transfer of information between individuals, the identification of individuals or groups, and learning about the animals' environment. In humans, social communication includes verbal and non-verbal components (e.g., social touch, gestures, and facial expressions). Many forms of communicative dysfunction have also been studied across animal species, ranging from erroneous courtship in

OPEN ACCESS

Edited by:
Gervasio Batista,
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Reviewed by:
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New York University, United States
Bartul Mimica,
Princeton University, United States

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Received: *01 September 2021*
Accepted: *11 November 2021*
Published: *30 November 2021*

Citation:
Li SW, Williams ZM and Báez-Mendoza R (2021) Investigating the Neurobiology of Abnormal Social Behaviors.
Front. Neural Circuits 15:769314.
doi: 10.3389/fncir.2021.769314

drosophila (Yost et al., 2020), decreased chemo-signaling in zebrafish and avian species (Caro et al., 2015; Hoffman et al., 2016), atypical ultrasonic vocalizations in rodents (Jamain et al., 2008; Neunuebel et al., 2015; Léna and Mantegazza, 2019), and decreased imitation in the transmission of learned vocalizations in zebra finches (Garcia-Oscos et al., 2021). Additionally, recent development of transgenic non-human primates has culminated in studies finding strikingly similar autism-like verbal communicative dysfunctions in monkey models to human patients (Liu et al., 2016; Zhou et al., 2019). These studies have revealed fundamental neurobiological underpinnings of communicative behaviors and their disorders, including a host of neurocircuit and neurochemical processes involved in a myriad of various behaviors (Chen and Hong, 2018; Tang et al., 2020b).

Although these studies have largely taken advantage of the animals' inherent methods of communicating with one another, reductionist behavioral designs have primarily used either individual or dyadic interactions between animals in confined, artificial environments. While dysfunctional communicative behaviors in patients with social deficit disorders manifest in dyads, they are more prominently displayed in groups of individuals, such as in a classroom or within a sports team (Philip et al., 2012; Lord et al., 2020). Animals show communicative behaviors during group interactions in natural settings. Greeting rituals, where rodents take turns sniffing each other (Wesson, 2013), or where songbirds "tap dance" to each other (Ota et al., 2015), suggest the presence of neuronal representations of conspecifics and the utility of communication. These behaviors shape the animals' future decisions and their social network. Animals also typically forage in groups, utilizing communication to more effectively search for resources (Clark and Mangel, 1986).

Therefore, gaining a full picture of verbal and non-verbal communication must involve behavioral designs that include groups ($n > 2$) of interacting animals. Recent advancements in technology that, for example, can localize and characterize vocalizations in multiple animals (Fonseca et al., 2021) using telemetric technology, automated algorithms, and machine vision, have opened new doors to study communicative behaviors in more naturalistic contexts with groups of animals (Rose et al., 2021). Combining these techniques for automated detection and classification of vocalizations as well as high spatiotemporal resolution marker-less kinematic tracking technologies (Mathis and Mathis, 2020; Topalovic et al., 2020), could broadly expand the types of experiments that mimic social deficit pathology (Banerjee-Basu and Packer, 2010) across avian and mammalian species. While different degrees of complexity evoke distinct social-communicative behaviors and their dysfunction, more naturalistic experimental designs could allow us to gain a better understanding of the interplay between social context (the *where*), agency (the *who*), and the communicative behaviors (the *how*) that underlie social disorders.

EMPATHIC BEHAVIORS

Empathy refers to the ability of individuals to perceive the internal state of another individual (Smith, 2006) and plays a central role in how we socially interact with others. Individuals with ASD, for example, often struggle relating to the emotions of others, displaying diminished ability to identify the mental states of other individuals or to recognize emotive facial expressions (Baron-Cohen et al., 2000; Lockwood et al., 2016). The past half-century has yielded a golden age of psychosocial experimentations in animals, including non-human primates (Masserman et al., 1964; Bernhardt and Singer, 2012), rats (Church, 1959; Ben-Ami Bartal et al., 2011, 2014), and voles (Burkett et al., 2016), demonstrating the ability of different species to display empathy-like behaviors. These studies have indicated that empathic and prosocial tendencies are conserved across species. Interestingly, these behaviors are augmented when partnered with familiar conspecifics (Silk and House, 2011; Ben-Ami Bartal et al., 2014; Burkett et al., 2016), findings that suggest kin selection as a powerful driver for these phenotypes (Maynard Smith, 1964).

Decreased empathic behaviors have been recently evaluated in animal models using transgenic techniques and neural circuit manipulation. However, little is understood about what specific neural mechanisms related to empathic behavior are disrupted in social behavioral disorders such as ASD. Dysfunction in the medial prefrontal cortex and insula, however, is associated with diminished empathic behaviors in rodents (Rogers-Carter et al., 2018; Lee et al., 2021; Smith et al., 2021), non-human primates (Ballesta and Duhamel, 2015; Gangopadhyay et al., 2021), and humans (Bernhardt and Singer, 2012; Fan et al., 2014). While these studies normally focus on the welfare of all animals involved or animal pairs (Preston and De Waal, 2002; Decety and Svetlova, 2012), it has remained unclear what role specific brain areas or circuits play in ethologically meaningful empathic behaviors or how interindividual differences in personality traits, dominance, or sex affect them. For example, no benefit is associated with helping members of outgroups in some settings, prosocial behaviors may even be maladaptive due to competition for limited resources. Naturally occurring social interactions within groups can also involve empathic behaviors, such as coalition building. Therefore, a better understanding of the neurobiological mechanisms for these behaviors will benefit from longitudinal observations in groups of animals to better capture the group's dynamics (e.g., Rose et al., 2021), and to elucidate the relation between empathy and other social and non-social variables that culminate in strengthening or weakening of group-level behaviors such as social cohesion.

INTERACTIVE SOCIAL BEHAVIORS

Social interactions, particularly within groups, play a vital role in the behavior of most animal species and hold broad implications to fields of study in psychology, ecology, evolution, genetics, and neuroscience (Geng and Peterson, 2019; Matthews and Tye, 2019; Mohrle et al., 2020). We recently showed that the prefrontal cortex encodes signals related to specific others' behaviors, a finding only possible when testing the behavior of a group (Báez-Mendoza et al., 2021a). Yet, most of our understanding of social behavior has come from dyadic interactions, which fail to encompass important types of group

(or "high-order") social behavior (Couzin, 2009). While even solitary species display social interactive behaviors such as mating, aggression, and maternal care, species that live in groups display a profoundly more complex social repertoire (Silk and House, 2011). Studies have used standardized assays to quantify sociability and social interactive behavior in animals, such as the three-chamber and the social preference tasks, where interactions are evaluated by pairwise associations (Moy et al., 2004). Dysfunctional social interactions have, therefore, been typically defined by simple metrics such as diminished shoaling in fish (Ogawa et al., 2021), decreased interest in social stimuli in rodents (Lee et al., 2021), and impaired social play in monkeys (Zhou et al., 2019). Gaining a full understanding of social behavior and its underlying neurophysiology, however, requires approaches that access the dynamic interactions among freely behaving individuals and their naturalistic contexts within groups.

The study of naturalistic group behavior has benefited from recent advancements in wireless neuronal recording, inhibition, and stimulation technologies, as well as in computational methodologies that allow tracking the kinematics of multiple animals (Kim et al., 2013; Hultman et al., 2016; Pinti et al., 2018; Anpilov et al., 2020; Berger et al., 2020; Mathis and Mathis, 2020; Topalovic et al., 2020; Marx, 2021). While there is a growing understanding of the behavior of groups (Shemesh et al., 2013; Weissbrod et al., 2013; Harpaz and Schneidman, 2020), there is still little understanding of the neurobiological basis these behaviors (Anpilov et al., 2020; Kim et al., 2020; Tang et al., 2020a). Since animals need to be able to predict the consequences of their behavior on future social interactions in order to make decisions, experimentation of these interactions requires recognition of key social interactions in realistic contexts that captures social and environmental situations that occur in natural habitats (Couzin, 2009). From an evolutionary and ecological perspective, it is essential to relate the cognitive abilities of each species to their social challenges such as their unique environment and group states. Understanding the convergence of these measures upon social decision making, real-time social interactive behaviors, and social affiliation requires an interpretation of higher-level behavioral metrics beyond that of dyadic interactions.

DISCUSSION

The field of social neuroscience and experimentation in animal models has been extremely fruitful over the past decade, with an increasing emphasis on understanding interactions between pairs of animals under structured task settings. For example, using animal dyads, there has been an expanding understanding of the neural mechanisms and circuits that underlie interactive behaviors such as parenting, social approach, aggression, observational learning, and social bonding. While animal models are well-suited for studying specific, well-defined aspects of social behavior, there is also a need to use naturalistic and ethologically relevant assays that elicit the animals' innate behaviors and environments that organisms rely upon in the wild. More importantly, we need to integrate modeling techniques and experimental paradigms adapted from ecology to better understand the richness and complexity of social behavior and its disruption in psychosocial disease states.

In our search for biomarkers and treatment of social deficit disorders, we need to expand our repertoire of assays and approaches for studying social behavior. Psychosocial disorders often manifest across multiple dimensions including the ability to verbally or non-verbally communicate, interpret the feelings or intentions of others, and effectively interact (Lord and Bishop, 2015). Dysfunctional social behaviors can also be caused by diverse genetic or environmental factors that may differ across individuals and contexts. For example, little attention has been paid to real-world behaviors such as group living or unconstrained naturalistic interactions under which most animal species interact. These limitations, in turn, have made it difficult to interpret and translate data obtained from structured tasks into clinical practice. Therefore, to study and effectively treat these disorders, we need to combine generalizable behavioral measures, naturalistic behavioral paradigms, and telemetric recording techniques. This approach will capture the broader phenomenology of normal and abnormal social behavior. Advancements in social neuroscience will, therefore, likely bring about not only a shift in the way that we quantify social behaviors but also how we observe their neuronal dynamics in both humans and animal models.

AUTHOR CONTRIBUTIONS

SWL and RB-M drafted the manuscript. SWL, ZMW, and RB-M revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

SWL is supported by the Autism Science Foundation. ZMW is supported by NIH R01HD059852, NIH R01NS091390, and NIH U01NS123130 and RB-M is funded by an MGH-ECOR Fund for Medical Discovery Fellowship and a NARSAD Young Investigator Grant from the Brain and Behavior Research Foundation.

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frontiers in Neural Circuits

REVIEW
published: 03 December 2021
doi: 10.3389/fncir.2021.755093

Using Flies to Understand Social Networks

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Many animals live in groups and interact with each other, creating an organized collective structure. Social network analysis (SNA) is a statistical tool that aids in revealing and understanding the organized patterns of shared social connections between individuals in groups. Surprisingly, the application of SNA revealed that *Drosophila melanogaster*, previously considered a solitary organism, displays group dynamics and that the structure of group life is inherited. Although the number of studies investigating *Drosophila* social networks is currently limited, they address a wide array of questions that have only begun to capture the details of group level behavior in this insect. Here, we aim to review these studies, comparing their respective scopes and the methods used, to draw parallels between them and the broader body of knowledge available. For example, we highlight how despite methodological differences, there are similarities across studies investigating the effects of social isolation on social network dynamics. Finally, this review aims to generate hypotheses and predictions that inspire future research in the emerging field of *Drosophila* social networks.

Keywords: *Drosophila*, neurogenetics, social networks, pheromones, machine vision

OPEN ACCESS

Edited by:
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Harvard University, United States

Reviewed by:
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Received: *07 August 2021*
Accepted: *08 November 2021*
Published: *03 December 2021*

Citation:
Jezovit JA, Alwash N and Levine JD (2021) Using Flies to Understand Social Networks. Front. Neural Circuits 15:755093. doi: 10.3389/fncir.2021.755093

INTRODUCTION

Collective behavior can be defined as a manifestation of group-level patterns produced by simple interactions between individuals (Sumpter, 2010). Animals display a wealth of interesting collective behaviors such as migrating geese flying in V-shaped formation, flocks of starlings turning in unison, schools of fish splitting and reforming while outmaneuvering a predator, honeybees foraging, and the division of labor in ant colonies (Sumpter, 2010). How individuals organize these interactions depends on their social environment. Several factors, such as the composition and size of the group, alter the social environment and may affect expression of collective behaviors. The African migratory locust illustrates this phenomenon: crowded group conditions alter the morphology, physiology, and behavior of individual locusts, resulting in aggressive swarms (Gillett, 1973). Similarly, manipulating group composition in the fruit fly affects the mating behavior and cuticular hydrocarbon profile of individuals through differences in gene expression (Kent et al., 2008; Krupp et al., 2008; Billeter et al., 2012). These examples, easily seen by the naked eye, emphasize that interactions between individuals defines the social environment, and, in turn, the social environment influences the behavior of the collective group.

The relationship between individual interactions and collective behavior of animal groups can be studied in numerous ways. Simple informative assays have been developed that compute the

distance to an animal's nearest neighbor through static images or video sequences (Simon et al., 2012). More elaborate approaches involve tracking the identity and motion of animals in video recordings with machine vision software (Branson et al., 2009; Grover et al., 2009; Eyjolfsdottir et al., 2014; Crall et al., 2015; Wario et al., 2015; Robie et al., 2017), and this has inspired the application of machine learning algorithms to classify and predict various social behaviors (Kabra et al., 2013). Research on collective behavior of animals often converges on the theme that simple rules applied to pair-wise interactions drive emergent group structures (Mersch et al., 2013; Baracchi and Cini, 2014; Pasquaretta et al., 2016a). Although more remains to be uncovered about how animals form collective units, our understanding has progressed from experiments quantifying social interactions on an individual basis to social network analyses that emphasize the group as an entity.

Social network analysis (SNA) relies on statistical tools to identify patterns of interaction in groups and consequences of social structure (Krause et al., 2009). Applications of SNAs originated in the 1930s to study sociological factors of human populations (Moreno, 1934; Lewin, 1951; Wasserman and Faust, 1994; Scott, 2000). Later, SNAs were applied to studying exclusively the social structure of non-human primates (Sade, 1965; Fedigan, 1972; Pearl and Schulman, 1983; Sade et al., 1988; Kudo and Dunbar, 2001; reviewed in Brent et al., 2011). In the last 20 years, SNAs have been applied to various animals in the field and laboratory such as fish (Croft et al., 2004), birds (Boogert et al., 2014), insects (Otterstatter and Thomson, 2007; Formica et al., 2017; Stroeymeyt et al., 2018), and other mammals including spotted hyenas (Ilany and Akçay, 2016), elephants (Goldenberg et al., 2016), and giraffes (Shorrocks and Croft, 2009). Across this literature of animal behavior, a *social network* is defined as any number of nodes interconnected via social ties between them (Krause et al., 2009). *Nodes* are defined as social entities that represent an individual animal. *Edges* represent the connection between two nodes (social relationship or interaction), and these can be *weighted* or *unweighted* (see **Figure 1**). Unweighted networks are binary and consider only the presence or absence of an interaction between individuals. Weighted networks assign numerical values to all edges in the network, and these values typically reflect the strength or frequency of interactions between nodes. Weighted networks summarize the history and structure of the group and unweighted networks emphasize the distribution of interactions within the group, and each approach has different strengths and limitations. In a *directed network,* edges represent both the connection of nodes and the directionality of an incoming or outgoing interaction. In an *undirected network*, edges represent the sum of all interactions between a pair of nodes but does not take the direction of interactions into account (see **Figure 1**). Finally, a social network represents connections between nodes over time. Social networks may be *static*, meaning all connections between nodes over a period of time are represented in a single network that represents a history of social connections. Alternatively, *iterative* approaches to networks have been studied. *Iterative* refers to a process of generating multiple transient social networks over a set interval of time to measure dynamic social properties of animal groups (Schneider et al., 2012; reviewed by Blonder et al., 2012; Farine, 2017). Iterative networks offer opportunities to analyze how social connections and group-wide network properties change throughout time.

Both static and iterative social networks derive from pair-wise interactions, which are analyzed to assess pattern and structure. In some cases, network measures describe individual nodes, and in other cases qualities of the entire network. *Degree* is the number of edges connected to a single node. In a directed network, *in-degree* represents the sum of incoming interactions, and *out-degree* represents the sum of outgoing interactions from a single node. Every node in a network has these degree scores, and the *degree distribution* is used to characterize features of a network, such as whether it is random. In a weighted network, the *strength* of a node is calculated as the sum of the edges' weights connected to that node. Edges are often weighted by the number of interactions between nodes to emphasize short interactions or by the duration of interactions between nodes to emphasize longer social interactions (Bentzur et al., 2020). In a directed and weighted network, the *in-strength* is the sum of the incoming edge weights, and *out-strength* is the sum of the outgoing edge weights. The *density* of the network is defined as the number of actual connections between nodes divided by the maximum number of connections possible between nodes in the network. This measurement indicates how densely individuals are connected throughout the network. There are a variety of properties that measure different aspects of the network. Examples of these properties are listed and defined in **Table 1**.

Social network analysis provide researchers with a powerful tool that contributes to our understanding of mechanisms underlying collective behaviors. The aim of SNA across animals has been dedicated to understanding how ecology and evolution affect collective behavior. For instance, there is evidence that wild animals occupy consistent positions in social networks when introduced to new environments (Krause et al., 2017; Canteloup et al., 2020), and across changing seasons (Blaszczyk, 2018; Stanley et al., 2018b; Rose and Croft, 2020). Also, social network structures of animals analyzed in captivity are consistent with those studied in the wild (Brandl et al., 2019; Ripperger et al., 2019), suggesting that there is order to animal social groups that can be predictably recreated and measured using statistical approaches. Other factors of biological relevance are known to influence the social network position of animals such as age (Baracchi and Cini, 2014; Liao et al., 2018), development (Boogert et al., 2014; Brandl et al., 2019) and reproductive success (Oh and Badyaev, 2010; Formica et al., 2012). Social networks also map how a single animal is connected to the larger population and this can offer insight into probabilities of disease contagion (Sah et al., 2018). The ability to relate biological factors to social networks makes SNA an appealing means to further study animal behavior.

Traditionally SNAs were used to study animals in the field, but increasingly more work has emerged that apply SNA to animals in the laboratory. This shift is a result of recent advancements in the automated identification and tracking of individuals (Branson et al., 2009; Straw and Dickinson, 2009; Greenwald et al., 2015; Hong et al., 2015; Robie et al., 2017). Increased interest in applying SNAs to the genetic model organism, *Drosophila*

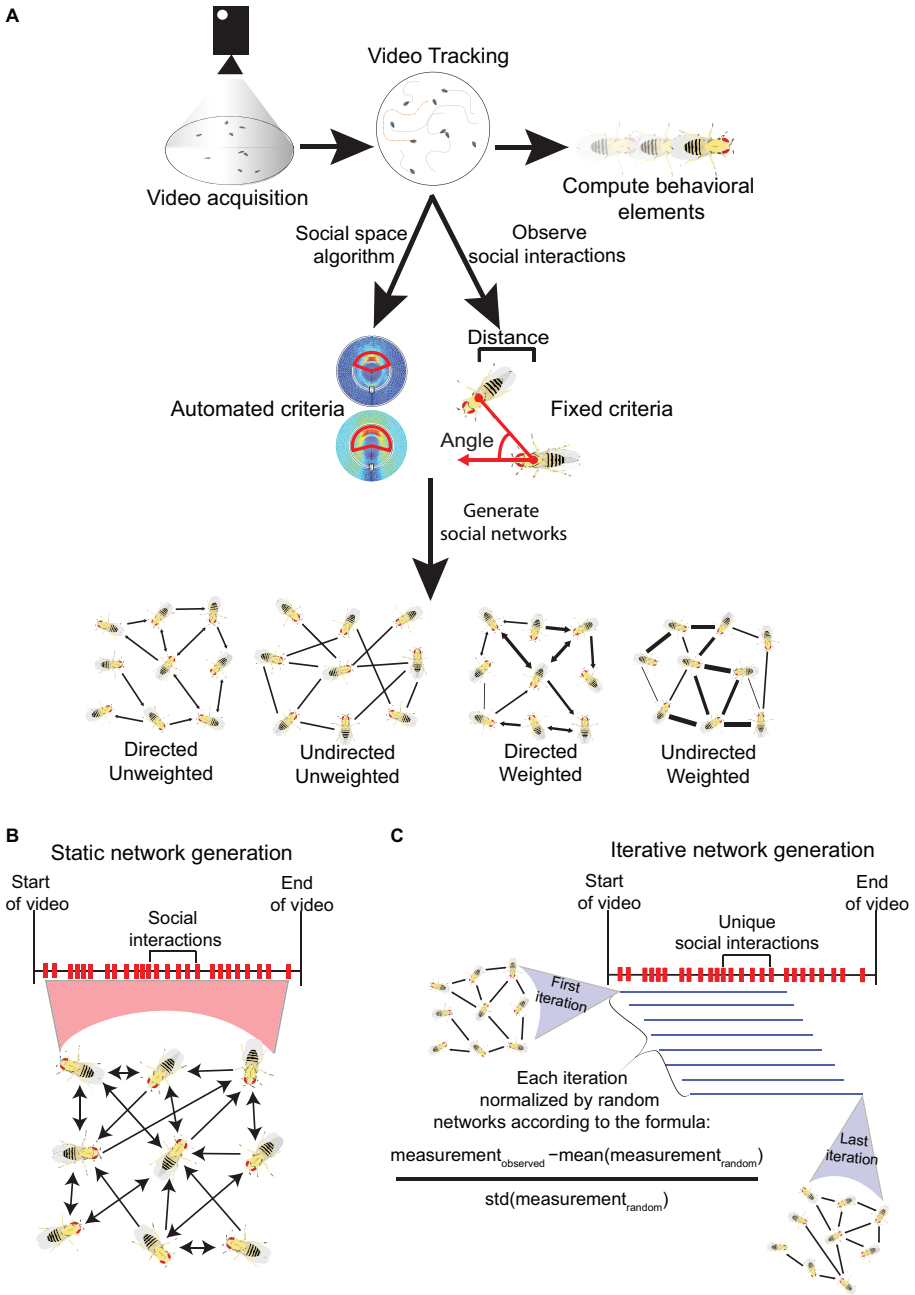

FIGURE 1 | Visualization of the methods involved in acquiring *Drosophila* social networks. **(A)** First videos with a specific number of flies confined in an arena are acquired and the position, orientation and identity of each fly is tracked with machine vision software (e.g., Ctrax). This information acquired from tracking can be used to calculate a variety of behavioral element measures such as the average locomotor activity of the flies. To generate networks, criteria that define a directed interaction are necessary. Typically, three parameters are used: (i) the angle connecting the center of the interactee fly relative to the interactor fly (shown with red arrows); (ii) the distance between the two flies' center of mass; and (iii) how long these conditions must be maintained for. The criteria can be defined manually, based on observation (fixed criteria) or automatically computed through a published algorithm (automated criteria; see Schneider and Levine, 2014). Once the criteria are selected, social networks can be generated each time they are met in the tracked videos. Networks can be computed with the following properties: (i) directed - the directionality of incoming or outgoing interactions are recorded; (ii) undirected – the directionality of interactions are not recorded; (iii) weighted – interactions are weighted to reflect the strength or frequency of interactions between nodes; (iv) unweighted - networks are binary and only consider the presence or absence of interactions between individuals. **(B)** Visualization of static networks, a conventional form of SNA where every observed social interaction within a video sequence is combined into a single, large social network that encompasses the entire history of social interactions. To avoid saturation of node connections, static networks can be weighted. **(C)** Visualization of the iterative network method (published by Schneider et al., 2012) where a variety of network iterations are generated throughout a single video sequence. Once a threshold number of unique interactions are observed, one iteration is generated. Each subsequent unique interaction creates a new iteration where the oldest interaction is removed. Each iteration is normalized to randomly generated networks with equal degree distributions. All iterations also have the same number of interactions. As a result, degree distribution and density are controlled through this method.

TABLE 1 | A list of common social network measurements defined by their both technical definition and their general applications.

Network measure	Definition	Application	References
Degree	Number of edges connected to a single node. In-degree refers to the number of interactions a node receives, and out-degree refers to the number of interactions a node outputs.	In all types of networks, degree informs how popular a single node is toward receiving and/or relaying connections.	Wasserman and Faust, 1994
Strength	In networks with weighted edges, strength is the sum of all edge weights connected to a node. In-strength refers to the sum of all edge weights a node receives, and out-strength refers to the sum of all edge weights a node outputs.	In weighted networks, strength informs overall how popular a single node is toward receiving and/or relaying connections relative to the weight of each connection.	Bentzur et al., 2020
Density	Proportion of actual connections in a network over the number of theoretically possible connections.	Measures to what extent the network connections are filled out between nodes.	Bentzur et al., 2020
Betweenness centrality	Number of shortest paths that traverse a node.	Measures how central a node is in a network for relaying information and maintaining the network cohesion.	Newman, 2010
Weighted closeness centrality	Calculated as inverse between the shortest path between two nodes, from one node to all other nodes in the network and weighted for number of connections among nodes.	Measures how central a node is in a network for relaying information and maintaining the network cohesion.	Pasquaretta et al., 2016a
Eigenvector centrality	Directly related to the number of contacts a node has and to the relative weight of the nodes to which it is connected.	Measures how central a node is in a network for relaying information and maintaining the network cohesion.	Pasquaretta et al., 2016a
Information centrality index	Calculated by combining all the paths present in a network and assigning a weight to them that is equal to the inverse of the path length.	It reflects the amount of information per individual contained in all possible paths that originate from and end with that individual.	Pasquaretta et al., 2016a
Clustering coefficient	A measure of how interconnected nodes are to one another.	Typically used to measure how cliquish nodes are in a network.	Newman, 2010
Modularity	A measure of how a network can be subdivided into clusters of sub-networks.	Typically used to measure how cliquish nodes are in a network.	Pons and Latapy, 2005
Assortativity	A measure of the homogeneity of the degree distribution of a network.	Distinguishes whether nodes in a network all have a similar degree.	Newman, 2010
Global efficiency	A measure of redundant pathways in the overall network and how efficient information can spread.	Distinguishes whether the overall network has shorter or longer paths between nodes.	Latora and Marchiori, 2001

melanogaster has surfaced. Although the number of these studies is currently limited, the research questions addressed are surprisingly diverse. Such studies also provide insight into the social diversity and group-level complexity of these 'simple' organisms. However, the SNA approach differs in these studies at the experimental, statistical, and conceptual levels. The aim of this review is to compare the scope, objectives, and methods of these studies, and attempt to draw parallels between them and the broader literature of animal social networks. In the process, we highlight the benefit of *Drosophila* insects toward studying complex social phenomena and we attempt to generate hypotheses and predictions that may inspire future experiments.

Drosophila SOCIAL NETWORKS

Social Space

Social network analysis relies on a concrete definition of social behavior to fill connections between nodes. This definition varies across animal species and the scope of the study. For example, social networks generated from animals in the field often considers individuals socially connected if they are found in a common geographical location (Goldenberg et al., 2016; Deng et al., 2017; Brandl et al., 2019). More precise animal interactions may be used to build social networks and examples include grooming or dominance interactions observed in a variety of mammals (Madden et al., 2009; Blaszczyk, 2018; Büttner et al., 2019). Social networks can also be produced from animals in the laboratory, based on precise social interactions observed or tracked in video sequences. Examples include physical contact between the antennae of ants (Blonder and Dornhaus, 2011), and the transfer of regurgitated food (trophallaxis) observed in bees (Gernat et al., 2018). What forms of social communication occur in *Drosophila*? Decades of investigation into the genetic, neurological, and physiological basis of social behavior in *D. melanogaster* offers the consensus that social communication involves various combinations of visual, acoustic, tactile, and chemosensory cues (von Schilcher, 1976; Agrawal et al., 2014; Bontonou and Wicker-Thomas, 2014). As we will discuss below, social networks in *Drosophila* are derived from physical encounters between conspecifics, like SNA in ants and bees (Blonder and Dornhaus, 2011; Gernat et al., 2018). In this section we discuss the *social space* of flies, defined as spatial criteria between the bodies of flies that approximate social interactions. This can be conceptualized as a physical space that once crossed, scores a social interaction. Also, we note that the terminology in the field is not consistent. Social distance is used by some authors (Simon et al., 2012; Brenman-Suttner et al., 2020) and social space by others (Schneider and Levine, 2014; Montagrin et al., 2018). We favor social space and use it here as a matter of preference, not rigor, since these terms may be used interchangeably.

The first observation of organized spatial positioning in *Drosophila* is credited to Sexton and Stalker, who noticed that groups of female *Drosophila paramelanica* touch one

another with their forelegs to maintain uniform spacing at high group density (Sexton and Stalker, 1961). This observation was rediscovered by Schneider et al. (2012) over 50 years later in *D. melanogaster*. Repeated video recordings of flies in a homogenous group revealed 'touching' behavior, which involves the foreleg of an 'interactor' touching the ariste, head, body, wing, or leg of an 'interactee.' Before touching, the interactor would typically approach the side of the interactee's body at acute angles, unlike in courtship when males tap the rear of a female's abdomen. This behavior can be classified using three social space parameters: (i) *distance* of the shortest line segment connecting the center of mass between the interacting flies; (ii) *angle* of the line segment connecting the centers of mass of both flies and the line segment protruding from the head of the interactor; (iii) the *time* fulfilled during these touch encounters (**Figure 1**). Schneider et al. (2012) defined a social interaction between multiple flies as distance $\leq$ 2 body lengths, angle $\leq$ 90 degrees, and time $\geq$ 1.5 s. Since this was repeatedly observed in a social context devoid of courtship behavior, these social space criteria arguably represent the most basic unit of social communication in flies. As flies house gustatory taste receptors within bristles on their legs (Vosshall, 2007), it is possible flies use touch, taste or both as a form of social communication, in addition to visual and olfactory sensory modalities (Vosshall and Stocker, 2007; Zhu, 2013). Additional studies have applied similar criteria for scoring social interactions, with some modification that involved relaxing the angle parameter (Bentzur et al., 2020) and restricting the distance parameter (Dawson et al., 2018; Liu et al., 2018).

Social space criteria defined by Schneider et al. (2012) were derived by observation and applied as a standard across different types of flies. This method did not consider differences in social space criteria that could occur between strains and species. This issue was addressed by the development of an algorithm that analyzes spatial positioning between flies and maps their typical social space in an unsupervised fashion (Schneider and Levine, 2014). More specifically, the algorithm analyzes the spatial positions of every fly in all tracked videos. Then background noise is eliminated by analyzing spatial positions of "virtual trials" which consist of fly tracks randomly sampled from separate videos. With that background subtraction, the algorithm identifies distance, angle and time parameters that are over-represented in videos of flies socially interacting compared to the non-social virtual trials. This can be interpreted as the typical spatial boundary between flies from the analyzed videos. Any fly crossing this boundary within the videos is considered socially interacting. For the remainder of this review, we will refer to social space criteria generated from this algorithm as "automated criteria" and all other criteria derived from human observation as "fixed criteria."

The social space algorithm was first applied to male and female *Canton-S* and *Oregon-R* strains of *Drosophila melanogaster*. The automated criteria that were computed differed from the previously published fixed criteria (distance $\leq$ 2 body lengths, angle $\leq$ 90 degrees, and time $\geq$ 1.5 s; Schneider et al., 2012). The distance parameters ranged between 1.75 and 2 body lengths, the angle parameters ranged between 115 and 160 degrees and the time parameters ranged between 0.4 and 0.6 s (Schneider and Levine, 2014). Using different methods of image analysis over time, Simon et al. (2012) and Jiang et al. (2020) demonstrated that the average nearest neighbor distance between flies studied in a group converges between 1.5 and 2 body lengths. Other researchers studying group dynamics in *Drosophila* have also applied a distance criterion between 1 and 3 body lengths based on their own observation (Pasquaretta et al., 2016a; Bentzur et al., 2020; Wice and Saltz, 2021). A recent comparative study conducted on 20 drosophilid species found that the average social distance of each species ranges between 1 and 3 body lengths. Additionally, the authors observed that the average leg length of each species relative to their body size positively correlated with social distance. This finding suggests that the variation in the social distance of flies can be explained by their morphology, and it further confirms that 2 body lengths is a reliable social space criterion to capture social encounters between individual flies in a group setting.

When using social space criteria to score the social behavior of flies in a group, it is important to consider how to minimize false-positive interactions. For instance, the automated criteria estimated by Schneider and Levine (2014) displayed an increase in the angle parameters and a decrease in the time parameters compared to the fixed criteria. A wider angle and a shorter time parameter would lead to an increase in the number of interactions, and indeed Schneider and Levine (2014) reported an increase of hundreds of social interactions with the automated criteria. Additionally, false-positive social interactions may occur when two flies, interacting over long periods of time, momentarily slip outside of the social space boundary. This may result in a lengthy interaction between two flies getting counted as several short interactions. Stricter social space criteria have been applied by other researchers, perhaps with the intention of minimizing false-positive interactions. One straightforward approach is reducing the distance parameter so that social interactions are only counted when flies are in close proximity. For example, Liu et al. (2018) recorded interactions exclusively when one fly's head approached and touched another fly's rear. Another strategy is the implementation of a 'gap length' parameter, which is a set time interval required to elapse before additional interactions between the same pair of flies are counted (Liu et al., 2018; Bentzur et al., 2020). Bentzur et al. (2020) reported that implementing a gap length of 4 s substantially reduced the number of consecutive interactions occurring between the same pairs of flies. Another alternative to filtering excessive interactions is counting subsequent social interactions between unique pairs of flies as done by Schneider et al. (2012). That is, interactions between A and B will not be counted two times in a row. When defining social space criteria, there is a trade-off between filtering false-positive and accepting the loss of true positive interactions and balancing this depends on the researcher. Finally, social space criteria should be redefined if different social contexts are being compared. Flies engaging in aggressive or sexual acts may posture their bodies differently than the touch events described previously and adjusting social space criteria to reflect this may become useful toward future

pursuits in the automated behavioral classification of *Drosophila* social interactions.

Social Networks

Within recent years, there has been increased interest applying SNA to study the sociality of *Drosophila* insects from computational, behavioral, neurobiological, and evolutionary perspectives (Schneider et al., 2012; Schneider and Levine, 2014; Pasquaretta et al., 2016a,b; Liu et al., 2018; Bentzur et al., 2020; Jezovit et al., 2020; Rooke et al., 2020; Alwash et al., 2021). All these studies consist of analyzing video footage tracked by machine vision software and applying social space criteria to generate social networks. Despite differences in the methodology of these experiments (see **Table 2**), remarkably similar experimental questions have been addressed (see **Table 3**). In this section, we review recent SNAs applied to *Drosophila.* We compile the various hypotheses tested in these studies and comment on the overlap found in the social network data. We also summarize the SNA methods in these studies and discuss the advantages of each for studying *Drosophila* social networks.

Video Acquisition and Tracking

First, the precision of social network data depends on reliable, error-free video tracking. The number of errors accumulated by video tracking is dependent on the level of contrast between the flies and the arena background, the length of the videos, and the number of flies (Robie et al., 2017). The most common tracking platform across the *Drosophila* social network literature is Ctrax, an open-source machine vision tracker (Branson et al., 2009). An inconvenient limitation of Ctrax is the requirement to tediously review the tracking data for errors that involve, for example, inconsistent identification of the same individual fly or changes in the size and orientation of the tracks. Each of these errors requires manual review and correction. In a recent experiment that repeatedly filmed 10 flies in an arena for 15 min, an automated error fixing script was applied to edit the tracking errors from Ctrax (Bentzur et al., 2020). An alternative called Flytracker, has been developed that claims to produce error-free tracking (Liu et al., 2018). Recently, Wice and Saltz (2021) cross-evaluated the performance of Flytracker with manual annotation of fly identities from 700 random frames and reported a strong correlation between automated tracking and manual tracking. While these alternatives may increase the speed of data collection, there is always the danger of harboring tracking errors that could lead to a loss of precision and integrity of the SNA. When considering a video tracking pipeline, the speed versus the precision should be weighed appropriately depending on the research objectives. For example, if social networks are generated from interactions defined by distance and angle parameters between flies, then it may be worth thoroughly reviewing and fixing tracking errors that swap identities, and that alter the size and orientation of the fly tracks, as done by multiple studies (Schneider et al., 2012; Jezovit et al., 2020; Rooke et al., 2020; Alwash et al., 2021). If the objective is to generate social networks from interactions defined exclusively by the distance between flies, errors in the orientation of the tracks, for example, can be tolerated and ignored.

Static and Iterative Network Generation

Three recent studies analyzed *Drosophila* social networks using the more conventional static network approach (Liu et al., 2018; Bentzur et al., 2020; Wice and Saltz, 2021). This method generates

TABLE 2 | A comparison of all published-to-date *Drosophila* social network studies with their network analysis methods summarized.

Publication	Social interaction criteria	Summary of network analysis	Group size	Length of video recordings	Tracking software	Post-tracking correction
Schneider et al., 2012	Time: 1.5 s. Distance: 2 body lengths. Angle: 90°	Unweighted, directed, iterative	12 flies	30 min	Ctrax	Yes (Fixerrors)
Pasquaretta et al., 2016a	Time: 0.5 s. Distance: 1 body length.	Weighted, directed, iterative.	12 flies	4 h	Ctrax	Yes (Fixerrors)
Liu et al., 2018	Touch only: head to tail contact for 0.5 s. Gap length between interactions: 0.5 s.	Weighted, directed, static	16 flies	1 h	Flytracker	No
Bentzur et al., 2020	Time: 2 s. Distance: 2 body lengths. Angle: <0°	Weighted, undirected, static	10 flies	15 min	Ctrax	Yes (FixTRAX)
Jezovit et al., 2020	Automated method (Schneider and Levine, 2014)	Unweighted, directed, iterative	12 flies	30 min	Ctrax	Yes (Fixerrors)
Rooke et al., 2020	Automated method (Schneider and Levine, 2014)	Unweighted, directed, iterative	6 flies, 12 flies, 24 flies	30 min	Ctrax	Yes (Fixerrors)
Alwash et al., 2021	Automated method (Schneider and Levine, 2014)	Unweighted, directed, iterative	12 flies	30 min	Ctrax	Yes (Fixerrors)
Wice and Saltz, 2021	Time: 0.6 s. Distance: <2.5 body length. Angle: <160°	Weighted, directed, static	20 flies	20 min	Flytracker	Yes*

**Authors cross-validated tracking by hand-annotating fly identities in a random sample of 700 frames.*

TABLE 3 | A summary of the research objectives and hypotheses tested in all published-to-date *Drosophila* social network studies.

Research objective	Publications
Quantification of the emerging properties of *Drosophila* social networks and group formation	Schneider et al., 2012; Pasquaretta et al., 2016a; Liu et al., 2018; Bentzur et al., 2020; Jezovit et al., 2020; Rooke et al., 2020; Alwash et al., 2021
The experimental effects of social isolation on social networks and group formation	Schneider et al., 2012*; Liu et al., 2018; Bentzur et al., 2020
The experimental effects of sensory deprivation on social networks and group formation	Schneider et al., 2012; Bentzur et al., 2020; Rooke et al., 2020
Analysis of social space	Schneider and Levine, 2014
Diffusion analysis - modeling spread of information flow between flies	Pasquaretta et al., 2016a,b
The experimental effects of density and group size on social networks	Rooke et al., 2020
Investigating the evolutionary factors of social networks and group formation	Jezovit et al., 2020; Wice and Saltz, 2021
Genetic underpinnings/heritability of social networks and group formation	Alwash et al., 2021; Wice and Saltz, 2021
Investigation of social networks from mixed groups	Pasquaretta et al., 2016a; Bentzur et al., 2020; Wice and Saltz, 2021

See **Figure 2 for re-analyzed data.*

TABLE 4 | Summary of the advantages and disadvantages involved in simplistic network analyses with fewer parameters (less information column) compared to more complex analyses that require more input but controls more confounds (more information column).

Factor	Less information	More information
Interaction definition	Fixed: • Assumes all individuals and social treatments interact in the same manner. • Can use published criteria.	Treatment-specific: • Requires criteria for all experimental treatments. • Ability to control for differences in interaction patterns when looking at group-level phenotypes.
Directionality of interaction	Undirected: • Assumes any interaction is bidirectional.	Directed: • Assumes interactions are directional.
Value of interaction	Unweighted: • Assumes many interactions between two individuals are as important as a single interaction. • Straightforward methods for network-permutations.	Weighted: • Keeps track of "strong" vs. "weak" interactions, be it time spent interacting or number of interactions. • Permutation methods often fail with small networks.
Network definition	Static: • The network is the accumulation of all interactions over the experimental period. • If structure changes over time, this can be hidden. • If un-saturated, comparisons between different network densities introduce confounds between density and organization of the network.	Iterative: • Can handle arbitrarily long experimental timeframes. • Requires a 'density' cut-off value.
Data normalization	Standardized *Z*-score: • Normalizes all measures to a standard scale. • Allows plotting various network measurements on the same axis. • Does not control for anything beyond measurement units.	Network permutation Z-score: • Usually done by generating randomized networks with a controlled network feature (e.g., degree distribution) and standardizing observed networks to null distribution of random networks. • Takes individual-level interaction propensities into account. • Provides unbiased measures of network organization.

a single network that represents the entire history of social interactions within a single video (visualized in **Figure 1**). The number of connections within these networks varies depending on the number of interactions observed. All three of these studies weighted the networks, offering additional information on the strength of connections. Four other *Drosophila* social network studies published to date utilized a dynamic iterative approach (Schneider et al., 2012; Jezovit et al., 2020; Rooke et al., 2020; Alwash et al., 2021). This method, published by Schneider et al. (2012), generates directed and unweighted iterations of networks in groups of flies. Unlike static networks, the iterative approach generates multiple networks from a single video at a controlled network density from a sliding boxcar filter (Kossinets and Watts, 2006; visualized in **Figure 1**). To summarize, one network iteration is built exclusively from a threshold number of unique interactions. When an additional unique interaction is observed, the oldest unique interaction is removed from the network and the newest interaction is added and this forms the second network iteration. This pattern continues and can produce hundreds or thousands of social network iterations in a single video, all offering snapshots of changing network structure over time. To score and compare the network measures of different types of fly groups, each iteration is standardized to thousands of random network permutations with equal in-degree and out-degree distributions. This normalization by degree distribution is then followed by averaging all iterations to summarize the network measure to a single data point. The result is an averaged *z*-score of all network iterations per video. This use of the *z*-score normalization attempts to evaluate properties of the group-wide behavioral interaction patterns independent of the observed individual interaction patterns (degree distribution). Overall this iterative method removes the confounds of network density and degree distribution when comparing networks across different treatment groups (Schneider et al., 2012).

The static and iterative methods each have their advantages and disadvantages (see **Table 4**). An advantage of the static network approach is that the results are intuitive, whereas the iterative approach is far more abstract to interpret. For example, in a static network, the betweenness centrality score (defined in **Table 1**) can be compared to each network node and the node with the highest score can be interpreted as being critical to the cohesion of the network. In the iterative approach, every node's score is averaged in each network iteration and all iterations are averaged. As a result, the iterative analysis sacrifices information about the individual fly in exchange for measuring the overall group. Another distinction between the two methods lies in

the network density. The iterative method controls the density of networks by capping the number of social interactions per network and analyzing iterations of density-controlled networks. It was found that iterative networks capped at a 25% network density for groups of 12 flies, which are networks consisting of 33 unique interactions, were more robust than other network densities (Schneider et al., 2012). The static approach simply allows all social interactions in a single video trial to fill out into a single network and therefore network density may vary between video trials. However, network density can be an informative behavioral measure of the animal group since denser networks indicate more social activity and this is not directly measured through the iterative approach. Instead, to gauge differences in network density in the iterative approach the researcher can compute the number of iterations, which is associated with higher social activity. Finally, the static and iterative approaches may be combined as seen in Pasquaretta et al. (2016a) where static networks were generated every 15 min from multiple hours of footage. This combines the simplicity of the static network methods with the advantage of measuring dynamic group activity over multiple time points.

Social Experience

While the methods of generating and analyzing *Drosophila* social networks differ, one question many of the studies address is how social experience affects the group dynamics of flies (Schneider et al., 2012; Liu et al., 2018; Bentzur et al., 2020). Schneider et al. (2012) examined the effects of 3-day social isolation by measuring the network properties of groups of flies that were all separately reared in isolation, compared to groups of flies reared in a socialized environment. There were no significant differences found in the average network measures between these treatments (Schneider et al., 2012). A limitation to this study was the use of the same fixed criteria (2 body lengths, 90 degrees, and 1.5 s) for the isolated flies and socialized flies. This did not consider potential differences in the social interaction patterns of isolated versus socialized flies. Therefore, to better understand how social experience influences the behavior of flies, we re-analyzed the Schneider et al. (2012) data using automated criteria (**Figure 2**). Indeed, we find that socially isolated flies tend to engage in longer social interactions than socialized flies (housed-together treatment). On the other hand, a treatment of socialized flies that were combined from separate housing groups (mixed-together treatment) tend to have a shorter interaction time compared to the housed-together treatment. We then generated social networks using the iterative approach and found that social isolation significantly affects the network structure. For example, global efficiency (defined in **Table 1**) is significantly higher in isolated flies, indicating that isolated flies have more redundant connections in their networks. Isolated flies also display a significantly lower betweenness centrality, indicating that there are fewer central individuals serving as a hub in the network. Across all measures, we observe greater variability in networks of isolated flies compared to the controls, particularly in assortativity and clustering coefficient (defined in **Table 1**). Lack of social experience in these groups of isolated flies may be contributing to these less predictable network measures. Other behavioral measures, such as the average interaction rate and percentage of interactions reciprocated, were also significantly lower in groups of isolated flies. The new analysis shown here underscores the importance of the automated criteria and makes the findings of Schneider et al. (2012) consistent with recent studies that have addressed these questions in other ways (Liu et al., 2018; Bentzur et al., 2020).

Liu et al. (2018) took a different approach to the same question. Rather than isolate virgin flies for 3 days, 9-day old flies were isolated for 6 days. Replicates of static, directed, and weighted social networks were generated from multiple video sources and then averaged. Liu et al. (2018) revealed groups of 16 flies that had been isolated tend to be more active, interact more often and produce networks with a higher clustering coefficient than groups of socialized flies. Additionally, a time course of 1-day to 6-day long isolation treatments showed that the average clustering coefficient is significantly greater than that of socialized flies at all time points. This suggests that a single day of isolation is sufficient to alter the clustering coefficient of flies, and this may be robust since we also report a higher clustering coefficient in isolated flies (**Figure 2**). In fact, Liu et al. (2018) also report a higher global efficiency in isolated flies, which also agrees with our re-analyzed data (**Figure 2**). This similarity illustrates that network measures are robust in flies of different ages since groups of 3-day old flies raised in isolation produce similar networks to 9-day old flies.

A more recent experiment by Bentzur et al. (2020) also found social isolation affects fly networks. Like Schneider et al. (2012), these authors collected flies as virgins and isolated or socialized them for 3 days before recording their behavior. In this study, the authors generated static networks, and they were analyzed two ways: (1) weighting nodes by the number of interactions to emphasize short and acute social patterns; (2) weighting nodes by the length of interactions to emphasize long-lasting social interaction patterns. The authors found that isolated flies displayed a lower average betweenness centrality than socialized flies in networks weighted by the number of interactions and the length of interactions, and our re-analyzed data further validates this finding (**Figure 2**). Bentzur et al. (2020) also found that, on average, isolated flies have a lower modularity score (defined in **Table 1**) in networks weighted both ways, indicating that isolated flies produce networks that are less compartmentalized. This can be attributed to their finding that social isolation leads to flies being more active as measured by increased velocity, and decreased instances of flies physically clustering and interacting over long periods. However, higher locomotor activity may lead to more frequent social interactions, resulting in a higher network density, and a higher average degree/strength. Indeed, this was reported in isolated flies (Liu et al., 2018; Bentzur et al., 2020) and may serve as a confound when assessing network structure. Alternatively, the aggregate clustering observed in socially experienced flies could reduce the number of social interactions, skewing the data to a lower network density. Interestingly, in networks weighted by the length of interactions, Bentzur et al. (2020) found no significant difference in the network density between isolated and experienced flies. Perhaps measuring networks weighted by length of interactions reduces the confounds that arise from differences in locomotor activity and frequency of social encounters because these networks

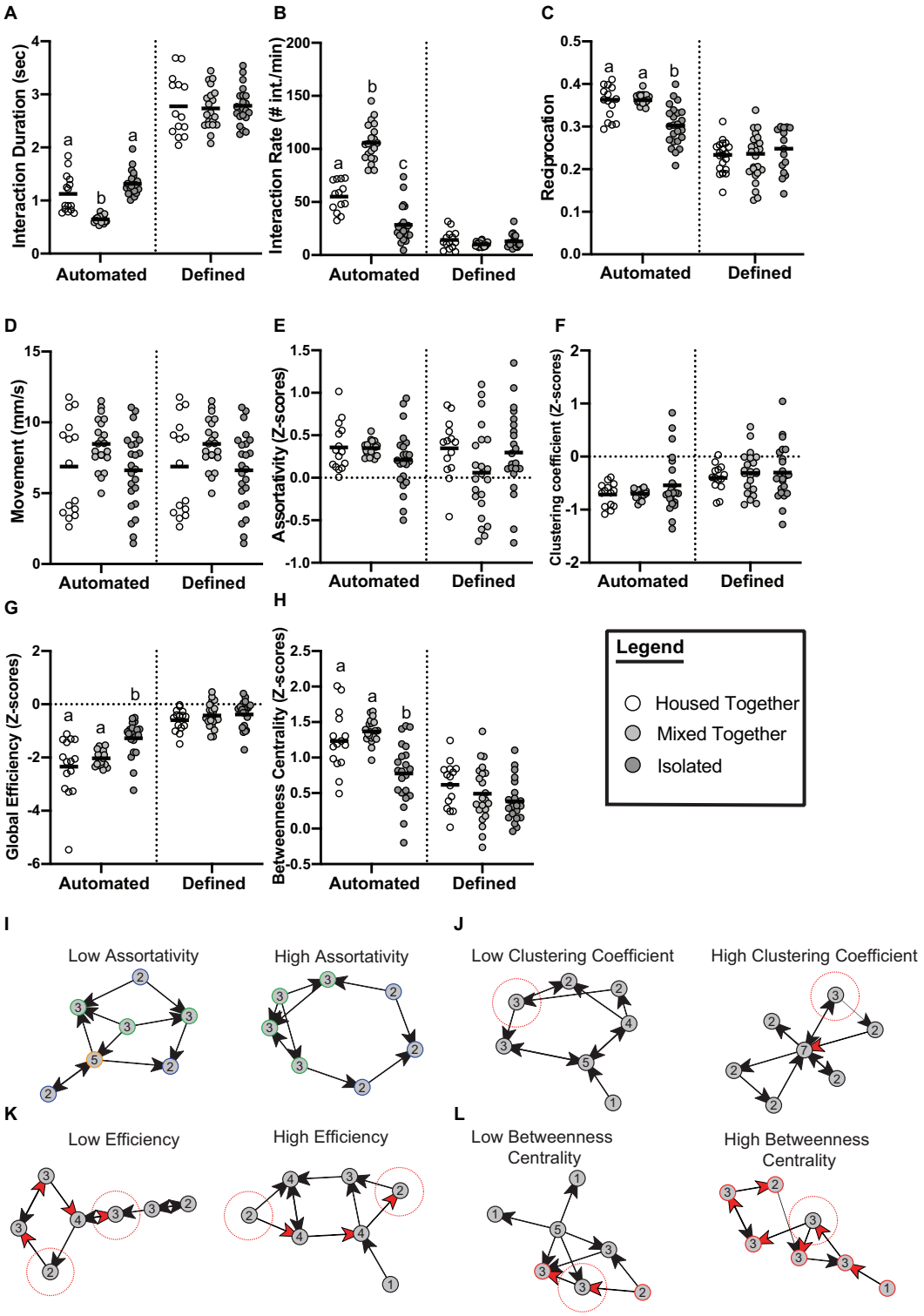

FIGURE 2 | Emerging properties of social networks after social isolation. Data from Schneider et al. (2012) re-analyzed with automated criteria compared to the original published data with fixed criteria reveals social experience significantly affects social interaction measures **(A–D)** and social network measures **(E–H)**. Flies were divided into three treatments: (i) Housed together (white) meaning all 12 flies in one video trial were raised together (n = 15 trials); (ii) Mixed together (light gray) meaning all 12 flies in one video trial were unfamiliar with each other from being raised with other flies (n = 22 trials); (iii) Isolated (dark gray) meaning all 12 flies in one video trial were completely socially isolated since eclosion (n = 24 trials). **(A)** Flies of the mixed group have significantly lower average interaction duration when analyzed using the automated criteria ($p \leq 0.0001$). **(B)** Flies of the isolated treatment have significantly lower rates of interaction when analyzed using the automated

(Continued)

FIGURE 2 | criteria ($p \leq 0.0001$). **(C)** Average proportion of interactions reciprocated were significantly lower in the isolated groups when analyzed using the automated criteria ($p \leq 0.0001$). **(D)** Movement did not significantly differ between the three treatments ($p = 0.0909$). **(E)** No significant differences between the three treatments were observed for assortativity when analyzed using the automated criteria ($p = 0.1027$). **(F)** No significant differences between the three treatments were observed for clustering coefficient when analyzed using the automated criteria ($p = 0.9540$). **(G)** Groups of isolated flies form networks with a significantly higher global efficiency compared to controls when using automated criteria ($p \leq 0.0001$). **(H)** Groups of isolated flies form networks with a significantly lower betweenness centrality compared to controls ($p \leq 0.0001$). Panels **(A–H)** were analyzed with one-way ANOVA with ranks to determine if statistical differences exist between the groups. Outliers were removed from all the datasets. Bars indicate mean. Letters indicate statistical significance. **(E–H)** Networks were generated from the following automated criteria: distance = 1.5 body lengths, angle = 115°, time = 0.55 s (housed-together); distance = 1.5 body lengths, angle = 110°, time = 0.5 s (mixed-together); distance = 1.5 body lengths, angle = 110°, time = 0.95 s (isolated). Measurements were standardized using *z*-scores as described by Schneider et al. (2012). Panels **(I–L)** defines and visualizes the network measurements analyzed [taken from Schneider et al. (2012)]. **(I)** Assortativity is the correlation between nodes of a similar degree (degree shown as number inside node). Low assortativity indicates nodes of a dissimilar degree tend to interact whereas high assortativity indicates more nodes of a similar degree tend to interact. **(J)** Clustering coefficient reflects the interconnectedness of the nodes in a given network. Networks with low clustering coefficient have a higher proportion of nodes (see focal node highlighted in red) with neighbors that are unlikely to interact. Networks with high clustering have a higher proportion of nodes (see focal node highlighted in red) whose neighbors are interconnected. **(K)** Global efficiency of a network is a measurement of the average shortest path length that information would flow through. Networks with a low efficiency score indicates less efficient information flow on average because the connections between nodes require more steps (visualized by 4 steps required for information to reach the two highlighted nodes through red arrows). Networks with high efficiency have less distances on average between nodes (visualized by 3 steps required for information to reach the two highlighted nodes through red arrows). **(L)** Betweenness centrality is a measure of how many shortest paths traverse a node, which can indicate the relative importance of a node for information flow. Networks with low betweenness centrality have fewer nodes that are critical for network cohesion. This is visualized by the node highlighted with the red dotted circle; this node can easily be bypassed. In the example network with high betweenness centrality, the node highlighted with the red dotted circle cannot be bypassed for information to travel through the network, and networks with high betweenness centrality have more central nodes like that.

favor connections between flies that spend longer periods of time socializing.

Despite the differences in methodology, three studies overlap in showing how social experience affects the group dynamics of flies. Two recent publications were the first to report these effects (Liu et al., 2018; Bentzur et al., 2020), and re-analyzing data from Schneider et al. (2012) further validates these two independent studies. Flies isolated for 3 days form social networks with a lower betweenness centrality (Bentzur et al., 2020) (**Figure 2H**). This results in less cohesive social networks with fewer central individuals holding the group together. Additionally, isolated flies form networks with a lower modularity (Bentzur et al., 2020), which indicates social isolation leads to less complex network structures. Taken together, these studies show that isolating flies hinders their ability to socialize within groups. This appears to contradict the finding that flies isolated for 1–6 days form networks with a higher clustering coefficient (Liu et al., 2018) (**Figure 2F**), indicating isolated flies on average may form cliquish groups. However, an automated classifier trained to detect instances of multiple flies physically aggregating found that isolated flies aggregate less than socialized flies (Bentzur et al., 2020). This illustrates the point that social networks capture patterns not necessarily intuitive to the human eye and future experiments would benefit by applying machine learning classifiers to measure additional qualities of social interactions. Measuring a wealth of behavioral classifiers, as done by Bentzur et al. (2020), would help validate and interpret the more abstract social network measures. Another recent experiment by Sun et al. (2020) studied the social attraction of free-walking flies by measuring their proximity to immobilized flies in arenas. With this assay, the authors found evidence that isolated flies exhibited a decrease in social attraction when compared to socialized flies. Finally, we find evidence that isolated flies are just as active as socialized flies and engage in fewer social interactions on average (**Figure 2**), which contradicts other studies (Liu et al., 2018; Bentzur et al., 2020). This highlights the benefit of automated criteria for generating social networks (**Table 4**). The behavior of the flies filmed may fluctuate based on experiments being completed at different times. The automated algorithm (Schneider and Levine, 2014) can take these behavioral fluctuations into account and estimate social space criteria reflective of the flies' behavior in the current experiment. Additionally, automated criteria can take behavioral differences between experimental treatments into account. For example, if socially isolated flies tend to interact differently than socially experienced flies, the automated criteria can correct for this and generate social networks that best represent the social environment being measured.

Effect of Density and Group Size on Social Networks

Each study that compared social networks of isolated and socialized flies examined groups of different sizes. A recent experiment by Rooke et al. (2020) demonstrated that group size affects features of social networks by comparing groups of 6, 12, and 24 flies across three different arena sizes. First, the authors found that the average locomotor activity of flies was similar across different group sizes and arena sizes, suggesting flies regulate their movement to compensate for decreased space. In terms of the social networks, the authors generated iterative, unweighted, and directed networks at controlled network density as published by Schneider et al. (2012). Rooke et al. (2020) found that groups of 6 and 12 flies form networks with a significantly lower average clustering coefficient than groups of 24 flies, and this was consistent across three different arena sizes. Additionally, groups of 12 and 24 flies form networks with a significantly higher average betweenness centrality than groups of 6 flies. This suggests that larger groups, on average, have more flies that are central and maintain greater cohesion across the group. Although the number of social interactions increases as the arena size and group size increase, properties of the social networks remain consistent across the same group size. Since the social networks were all generated at a controlled network density, and all flies were reared with equal social experience, differences in the network measures can be attributed to differences in group size. No matter how confined or dispersed a group of flies may be, the properties of the group shift only when the size of the group

changes. Perhaps individual flies may be sensitive to changes in group size based on visual feedback and through the perception of pheromone concentration and organize themselves in the group according to these signals.

Sensory Modalities and Group Formation

With *D. melanogaster* being one of the most popular organisms for behavioral genetic experiments, the wide availability of mutant strains and genetic tools to manipulate gene expression have been applied to social network experiments. To date, social networks have been generated for flies with disrupted visual, olfactory, gustatory, and acoustic modalities (see **Table 5**). Schneider et al. (2012) reported that the gustatory mutant $poxn^{\Delta XBs6}$ displayed an extreme reduction in the ability to form social networks (Schneider et al., 2012). More specifically, 40% of the videos filmed of these mutants did not harbor enough social interactions to form a single iterative network (Schneider et al., 2012). Recently, Jiang et al. (2020) also reported that *poxn* mutants, in addition to a range of other gustatory mutants, displayed an impaired ability to form physical social clusters. Together, this suggests chemosensory receptors are crucial for maintaining the sociality of flies.

TABLE 5 | Summary of various genes and sensory manipulations studied in *Drosophila* social network experiments.

Mutation/ gene	Role	Network findings	References
orco	Olfactory mutation.	Reduction in the ability to form networks.	Schneider et al., 2012
Iav^1	Hearing impaired mutation.	No effect on social network measures.	Schneider et al., 2012
$poxn^{\Delta XBs6}$	Gustatory mutation.	Reduction in the ability to form networks.	Schneider et al., 2012
w^{1118}	Mutation associated with neurological and visual defects and reduced life span.	Increased global efficiency.	Schneider et al., 2012
lush	Olfactory binding protein that is sensitive to male pheromones.	*lush*-inhibited fly networks increased clustering coefficient and betweenness centrality values in groups of 12 and 24 flies.	Rooke et al., 2020
foraging	Pleiotropic gene that influences several metabolic, physiological, behavioral (foraging) and developmental phenotypes.	The rover allele had higher global efficiency values while sitter allele had higher clustering coefficient and assortativity values.	Alwash et al., 2021
or65a	Olfactory receptor neurons that mediate chronic responses to male-specific pheromone cVA.	*Or65a*-inhibited fly networks had increased strength and decreased betweenness centrality values, along with reduced modularity.	Bentzur et al., 2020
or67d	Olfactory receptor neurons that mediate acute responses to male-specific pheromone cVA.	Inhibition of *or67d* neurons did not influence social networks.	Bentzur et al., 2020
cyp6a20	Associated with increased aggression.	Networks with a mixture of WT and *cyp6a20*-knockdown mutants leads to a reduction in betweenness centrality values.	Bentzur et al., 2020

Schneider et al. (2012) also demonstrated that hearing-impaired *inactive* mutants (iav^1) produced social networks that were not significantly different from wild-type flies. Surgical removal of ariste to ablate auditory perception in flies also had no effect on social clustering behaviors (Jiang et al., 2020). However, Jiang et al. (2020) reported that iav^1 mutants form more dispersed social clusters, unlike wild-type flies that are more tight-knit. This is also reflected in social space criteria for iav^1 mutants where the distance parameter was estimated to be larger than wild-type flies (Schneider and Levine, 2014). Although auditory mutants may socially interact and cluster less than wild-type flies, there is currently no evidence that manipulating auditory cues within a group of single-sex flies affects measures of social network structure (Schneider et al., 2012).

To disrupt vision, experiments have been conducted on flies in the dark. Schneider et al. (2012) reported that groups of flies filmed in the dark display a lower clustering coefficient and higher betweenness centrality, but these effects were not considered significant when accounting for multiple test correction (Schneider et al., 2012). Bentzur et al. (2020) found that groups of socially isolated flies behave more similarly in the light and dark compared to socially experienced flies. The authors reported that in networks of socially experienced flies, visual disruption leads to a significantly lower average betweenness centrality, opposite of what was reported by previous studies (Schneider et al., 2012). Despite disagreement in the social network data when subjecting flies to darkness, multiple studies report similarities in how flies aggregate and physically cluster. Using automated behavioral classification, Bentzur et al. (2020) reported that groups of flies in the dark aggregate less often and for shorter periods of time on average. Data by Jiang et al. (2020) also found that wild-type flies in the dark, along with *norpA33* visual mutants, cluster together less than wild-type flies. These two recent studies reinforce observations by Schneider et al. (2012) that darkness decreases the average interaction duration among groups of flies.

Arguably olfaction is the dominant sensory mechanism *Drosophila* depends on to locate foraging sites and conspecifics. Ablating olfaction is complex because *Drosophila* insects possess multiple olfactory receptors that are encoded by multiple genes. The olfactory mutant, *orco*, is known to have a severe loss of smell because it is deficient for a co-receptor that complexes with a variety of odorant receptors (Vosshall and Hansson, 2011). Social networks of *orco* mutants have been shown to have a significantly lower global efficiency than wild-type flies, with *orco* heterozygotes displaying an intermediate score (Schneider et al., 2012). This may indicate that the copy number of the *orco* gene leads to social interactions that, on average, result in a greater social distance between individuals in the network. In the same study, the *orco* mutants displayed a higher clustering coefficient and a higher assortativity compared to controls, although the differences were not statistically significant after multiple test correction (Schneider et al., 2012). Also, the *orco* mutant aggregates less with conspecifics compared to wild-type flies (Jiang et al., 2020). Overexpressing an *orco* transgene in the

olfactory system of these mutants led to the flies aggregating like wild-type flies (Jiang et al., 2020). This is similar to an observation of ant *orco* mutants that displayed a reduction in their ability to follow pheromone trails and cluster with other ants (Trible et al., 2017). This cross-species reduction in aggregation suggests that olfaction is crucial for the sociality of numerous insects, and it is no surprise that olfactory mutants produce social networks different from wild-type flies.

Behavioral Genetic Studies on Group Formation

In addition to studying the social behavior of fly mutants, the *Drosophila* model system offers genetic tools to manipulate the expression of genes in a tissue-specific manner through the GAL4-UAS system (Elliott and Brand, 2008). This system was applied to recent social network studies to examine the downstream behavioral effects of ablating specific olfactory sensing cells (Bentzur et al., 2020; Rooke et al., 2020). One experiment examined the social networks of flies where the olfactory receptor neurons Or65a and Or67d were inhibited by driving the expression of *kir2.1* in those cells. These olfactory receptors are known to be sensitive to cVA, a male-specific pheromone that mediates aggressive and copulatory behaviors in male flies (Bontonou and Wicker-Thomas, 2014). Interestingly, flies with inhibited Or67d neurons did not produce social networks drastically different from wild-type flies despite there being evidence that Or67d plays a role in social attraction (Bentzur et al., 2020; Sun et al., 2020). However, the inhibition of Or65a neurons leads to a significantly decreased average betweenness centrality (Bentzur et al., 2020). Another experiment focused on inhibiting the olfactory support cells that express the gene *lush*, which is expressed in trichoid sensillae of flies and aids in the binding of ligands to olfactory receptors (Rooke et al., 2020). By driving the expression of *kir2.1* in all *lush*-expressing cells, Rooke et al. (2020) found that *lush*-inhibited flies produce social networks different from controls in larger group sizes. More specifically, groups of 6 *lush*-inhibited flies formed social networks with an average clustering coefficient and betweenness centrality that resembles the wild-type controls. However, in groups of 12 and 24, the *lush*-inhibited flies formed social networks with a significantly higher betweenness centrality and clustering coefficient than wild-type controls (Rooke et al., 2020). Together, results of these studies indicate that different olfactory genes, expressed in different tissues, may play different roles in regulating group-wide social connections in flies.

Transgenic tools have also been used to manipulate the *foraging* (*for*) gene in a recent SNA study. This gene expresses natural polymorphisms in flies that influence behavioral phenotypes in the larval stage called rovers and sitters (Sokolowski, 1980). Alwash et al. (2021) demonstrated that networks of adult rovers and sitters form different social networks, suggesting this gene influences the behavior of adult flies. Sitter flies were shown to display a higher interaction duration and were more likely to reciprocate interactions, whereas rover flies were more active and displayed higher interaction rates. Compared to rovers, sitters formed networks with a higher assortativity and clustering coefficient, as well as a lower global efficiency suggesting there is less efficient information flow within these groups of flies. Alwash et al. (2021) also used separate transgenic lines, generated by Allen et al. (2017; see for details), that carry 1 copy, 2 copies and 4 copies of the *for* allele, respectively. By comparing social networks across these lines, it was found that *for* gene dosage affects the average assortativity, clustering coefficient and global efficiency measures. Additionally, the average interaction duration, the average rate of interactions, the proportion of interactions reciprocated and the activity of flies all changed across different dosages of *for*. The authors confirm that many of the social network differences observed between rovers and sitters are influenced by the *for* locus. These findings characterize the influence of a specific gene on social network dynamics in *Drosophila*, shedding light on the genetic underpinnings of sociality.

Multiple independent experiments that measured the social behavior of *Drosophila* mutants and transgenic flies with inhibited neurons revealed that sociality of flies is multisensory. In unisex groups of *D. melanogaster*, auditory sensory systems do not appear to play a role in social organization (**Table 5**). Visual, gustatory, and olfactory manipulations cause flies to behave differently than wild-type flies in several ways (Schneider et al., 2012; Bentzur et al., 2020; Jiang et al., 2020; Rooke et al., 2020). However, these studies investigating the sensory mechanisms behind collective behavior are limited to unisex groups. It is possible that mixed groups of male and female flies may generate social structures that depend on a wider range of sensory systems since, for example, auditory cues are critical for courtship in flies (von Schilcher, 1976). Future studies should consider manipulating the composition of the social groups when inhibiting genes of interest to widen our knowledge of *Drosophila* social structures, like how Rooke et al. (2020) studied flies with *lush* inhibition at a variety of group sizes. It remains difficult to define how differences in precise network measures of mutants translate to differences in social organization, especially since various social network experiments utilize different methods of generating and analyzing networks. However, experiments that focused on social attraction and aggregation of flies used similar mutants and transgenic tools and found overlapping results to social network studies (Bentzur et al., 2020; Jiang et al., 2020; Sun et al., 2020). For example, Sun et al. (2020) reported that a combination of both vision and olfaction are crucial for the social attraction behavior of flies. This suggests that social networks capture some aspect of group-level social organization that is genetically and neurologically controlled. Recent work has demonstrated that the *for* gene plays a role in the social organization of adult flies since different polymorphisms are associated with differences in social network structure and manipulating the *for* gene influences this structure (Alwash et al., 2021). Further experimentation in social attraction and aggregation of flies at the neuronal and genetic level can assist in unraveling how abstract social network measures translate to a real-world group structure.

Social Transmission

To date, one group analyzed *Drosophila* social networks to directly study information flow, like many social network studies on ant colonies (Blonder and Dornhaus, 2011;

Mersch et al., 2013; Stroeymeyt et al., 2018). Social communication within *Drosophila* groups can inform naïve flies about the presence of oviposition sites (Battesti et al., 2014) and the presence of predatory insects (Kacsoh et al., 2018). Pasquaretta et al. (2016a) applied SNA to examine how information spreads within a group of informed and naïve flies. This was done by video recording a 4-h training phase designed to inform focal flies of an oviposition site. Then social networks were generated within groups consisting of 8 informed flies and 4 uninformed flies (Pasquaretta et al., 2016a). Static, directed, and weighted social networks were generated every 15 min from 4 h of video footage. Afterward, every trained and untrained female fly was subjected to an oviposition site choice assay to determine if the mean and variance of social network measures predict whether uninformed flies follow or avoid the choices made by informed flies. Uninformed flies followed the correct choice when informed flies had less variable network distances from other individuals, as measured by weighted closeness centrality (defined in **Table 1**). Uninformed flies also followed when informed flies had a similar number of social contacts, as measured by eigenvector centrality (defined in **Table 1**) and when informed flies exchanged information to a similar extent, as measured by information centrality index (defined in **Table 1**). On the contrary, uninformed flies were less likely to follow the correct choice when they had a high betweenness centrality in the social network. Taken together, this suggests that when informed flies participate in most social interactions within the group, the uninformed flies are more likely to follow, and information is passed from the informed to the uninformed flies. In groups where uninformed flies were central to group cohesion (high betweenness centrality), the informed flies had less influence in transmitting the site preference. The authors also reported a remarkable finding where informed flies were more likely to avoid the media they were trained to prefer if they formed clusters, measured by a higher mean clustering coefficient. Properties of a social group are complex, and this highlights how individual foraging preferences can shift based on social associations within a group.

Diseases can also be transmitted via social interactions within a group. Utilizing the SNA approach in bumblebee colonies, for example, shed light on the relationship between interaction rate and parasitic transmission (Otterstatter and Thomson, 2007; Naug, 2008). So far, no studies to date have used SNA to explore how social interaction and network properties affect disease transmission in *Drosophila*. However, one study by Dawson et al. (2018) investigated how the social environment affects cancer progression in flies. In a homogenous group, cancerous flies were found to have higher interaction rate and duration than in heterogeneous groups consisting of cancerous and healthy flies. Additionally, Dawson et al. (2018) showed that tumor progression is slower when cancerous flies are kept in a homogenous group, and tumor progression is faster when cancerous flies are in isolation or within a group of healthy individuals. The use of the SNA approach can allow us to investigate the relationship between disease progression and social interactions even further by analyzing global network measures.

Evolution of Social Organization

Recently, a social network comparative study was conducted on 20 drosophilid species. Generating iterative, directed, and unweighted networks from groups of 12 male flies and groups of 12 female flies across all species, Jezovit et al. (2020) found no phylogenetic patterns for the species differences observed in assortativity, clustering coefficient, betweenness centrality, and global efficiency. This mirrors the results of a social network comparative analysis conducted on primates that also reported no evidence of phylogenetic signal in species-specific social networks (Pasquaretta et al., 2014). However, significant phylogenetic signal was found for the variation observed in social distance Jezovit et al. (2020). Social distance also correlated with the relative leg length of each species, suggesting morphological traits can influence behavioral evolution in flies. Next, the authors extracted averaged climate data from the geographic range of each drosophilid species and tested for correlations with each species' averaged social network score. The authors found that variation in the climate data predicted species differences in the social network measures better than the differences found in the flies' general behavioral characteristics such as average locomotor activity, average interaction duration, and average tendency to reciprocate interactions. Considering that each fly species descended from an inbred stock domesticated to the laboratory environment, it is surprising that factors of each species' environment predicted differences in their social network measures. From these findings, we hypothesize that group-level organization is a behavioral trait that adapted to the abiotic selective pressures of each species' habitat. For example, *Drosophila* species from tropical environments tend to have shorter cuticular hydrocarbons and rely more on visual sensory modalities than arid-adapted species (reviewed by Jezovit et al., 2017) and these ecological categories may also be relevant to species' social structures measured by SNA. Finally, Jezovit et al. (2020) collected two independent datasets of social networks for 5 species, separated by 2 years at the time of collection. Consistent trends in the relative species' differences were found for average assortativity, clustering coefficient, betweenness centrality, and global efficiency. This replication shows that species-specific social networks are robust and may represent phenotypes that emerge from physiological and behavioral mechanisms in individual flies.

Another recent comparative study by Wice and Saltz (2021) investigated the evolutionary relevance of social network measures across 20 different *D. melanogaster* strains. The authors generated static and directed social networks from mixed groups of flies using fixed criteria (see **Table 2**). These groups consisted of 10 males and 10 females, and each individual was genotypically distinct. The authors measured in-strength, out-strength, betweenness centrality, clustering coefficient, and eigenvector centrality for each fly within the group, and then compared the distribution of these measures for each genotype. This study stands out from other social network studies in that the authors were focused on measuring the characteristics of individual nodes and not the overall network structure. By comparing average network measures across numerous strains, the authors reported the broad

sense heritability measure of clustering coefficient, betweenness centrality, and eigenvector centrality. Interestingly, betweenness centrality displayed the highest broad sense heritability score where genotypic differences account for 16.6% of the variation in this network measure (Wice and Saltz, 2021). This corroborates a prediction made by Schneider et al. (2012) that betweenness centrality may be a heritable trait based on robust differences observed between two *D. melanogaster* strains. To study environmental effects on social network measures, the authors reared the flies in various environments differing in calorie concentration and in the ratio of protein to carbohydrate content. The authors found no effect of environmental variation on betweenness centrality, similar to what Jezovit et al. (2020) found when comparing social networks across multiple *Drosophila* species. There is also evidence that various drosophilid species maintain consistent group structures across separate experiments, reinforcing the idea that social networks are emergent properties built from some genetic foundation shared by the individuals in the group (Jezovit et al., 2020). This view is strengthened by emerging evidence of specific genes accounting for differences in social networks within *Drosophila* (Bentzur et al., 2020; Rooke et al., 2020; Alwash et al., 2021). If social networks measure some heritable aspect of social behavior, then we can begin to consider that these properties are phenotypes that diversified through evolutionary selection mechanisms.

FUTURE DIRECTIONS

Throughout this review, we have outlined experiments that all suggest *Drosophila* insects form organized and reproducible social networks when individuals aggregate. Despite *Drosophila* having long been considered solitary, a variety of organized collective behaviors have been uncovered in recent years. These collective behaviors provide a conceptual understanding of how social networks may function in fly groups. For example, flies in groups collectively escape from environmental threats (Ramdya et al., 2015) and enhance the survival of offspring through communal oviposition (Lihoreau et al., 2016). Oviposition site choice is influenced by social interaction with conspecifics. Battesti et al. (2012) demonstrated that when "teacher flies" are trained to deposit eggs on one of two food options, naïve "student" flies follow the same choice as the teachers after socially interacting. In addition, female flies arrest oviposition upon detection of predatory threats and can transmit this response to flies unaware of the threat (Kacsoh et al., 2015). Furthermore, flies in smaller group sizes exhibit a higher tendency to freeze their movement upon the detection of a predator (Ferreira and Moita, 2020), emphasizing the fitness benefits individuals gain from group formation. While it is unclear whether flies transmit information to one another, the above studies indicate that social interactions can lead to flies becoming informed of a stimulus, and 'information transfer' is a convenient term to describe this phenomenon. Applying SNA to these behavioral studies offers the opportunity to explore this concept of information transmission more precisely, and how other factors such as group size, density, and individual status contribute to the group-level output. So far one study applied SNA methods to study the oviposition site-choice phenomenon. The authors found that oviposition site choice influence from teacher flies are inhibited when student flies have stronger social ties in the group (Pasquaretta et al., 2016a). Interactions shared between flies appears to influence fitness-enhancing behaviors and this process can be visualized with networks.

Across animal social network studies, it is often reported that individuals maintain fixed positions in a social network over time (Brent et al., 2013; Krause et al., 2017; Blaszczyk, 2018; Stanley et al., 2018a; Canteloup et al., 2020). Other studies have reported a different view that individuals shift roles to maintain the stability of their social group and this flexibility maintains the group after individuals are lost due to predation and other stresses (Naug, 2008; Goldenberg et al., 2016; Firth et al., 2017; Formica et al., 2017). In flies there is evidence that the network position of individuals (degree) fluctuates, but the overall network structure of the group remains fixed over time (Schneider et al., 2012). A similar finding was reported in ants where an individual's degree offered no predictive power over their degree later in the experiment (Blonder and Dornhaus, 2011). When studying animal groups in a controlled laboratory environment, there is evidence suggesting that individuals may not maintain fixed positions within social groups. This serves as an example how studying social networks in flies can enrich the broader animal social network literature. Areas of debate in these fields could be settled through social network experimentation in flies where vast resources are available to manipulate the organism genetically and physiologically, and large datasets can be acquired in controlled conditions.

The broader animal social network literature would also benefit from more studies manipulating the social environment of animals in controlled ways. In this review we outlined studies that examined social networks with manipulated group size and density (Rooke et al., 2020) and social networks from mixed groups of individuals with various social experiences (Pasquaretta et al., 2016a; Bentzur et al., 2020; Wice and Saltz, 2021). Future studies in *Drosophila* social networks should consider studying even more complex, mixed social environments. For instance, Jezovit et al. (2020) found that male-only social networks differ from female-only social networks in some species. Would mixing the sexes provide an intermediate social network phenotype, or could some interaction effect be observed? Wice and Saltz (2021) demonstrated females tend to occupy different social network positions than males when both sexes are mixed into the same social groups, but the authors did not attempt to study social organization of the group as a whole. Future experiments could analyze various layers of mixed-sex networks by generating separate networks from numerous criteria. Social space criteria can be refined to measure courtship and mating interactions or aggressive interactions. How would the properties of courtship and aggression networks compare to the properties of the general social networks? Experiments on courtship networks exist in the broader animal social network literature (Ryder et al., 2008;

Oh and Badyaev, 2010; Formica et al., 2012; Fisher et al., 2016) and it would be worthwhile to determine if the *Drosophila* courtship networks overlap with these other studies.

Finally, *Drosophila* has a long history of serving as a model organism for the genetic basis of social behavior. Applying social network methods to screen well-studied mutants may aid in uncovering genetic mechanisms of sociality. For instance, a recent study found a potential role the *foraging* gene plays in the collective behavior of flies that can be measured using social networks (Alwash et al., 2021). Heritable factors in social network measures has also been reported in humans, rhesus macaques, and flies (Fowler et al., 2009; Brent et al., 2013; Wice and Saltz, 2021), reinforcing the idea that robust social network measures represent phenotypes of collective group structures. Although there is evidence that social network phenotypes do not map well onto phylogenetic trees (Jezovit et al., 2020), it does not rule out that these social behaviors have no underlying and conserved biological mechanisms. The circadian clock is one example of a conserved biological system that is pervasive across various organisms, yet circadian rhythms as a behavior vary across organisms from different habitats (Dunlap et al., 2004; Sehgal, 2015). Further experimental efforts using *Drosophila* and the vast genetic tools available within this system could uncover genetic and neurological mechanisms governing collective behavior. These findings may one day contribute toward identifying ancient mechanisms of sociality similar to how other pervasive mechanisms, like the circadian clock, have been uncovered in *Drosophila*.

AUTHOR CONTRIBUTIONS

JJ and NA wrote the first draft and planned the manuscript with JL. JJ and NA designed the figures. All authors edited the manuscript.

FUNDING

This study was supported by the Canadian Institutes of Health Research (CIHR), Natural Sciences and Engineering Research Council (NSERC), Canada Research Chair (CRC), and Canadian Institute for Advanced Research (CIFAR).

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