SOCIAL DECISION-MAKING IN ANIMALS

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PUBLISHED IN: Frontiers in Behavioral Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-907-6
DOI 10.3389/978-2-88963-907-6

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SOCIAL DECISION-MAKING IN ANIMALS

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Citation: Oliveira, R. F., Curley, J. P., Leadbeater, E., eds. (2020). Social Decision-Making in Animals. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-907-6

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frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 19 March 2018
doi: 10.3389/fnbeh.2018.00050

Specificity in Sociality: Mice and Prairie Voles Exhibit Different Patterns of Peer Affiliation

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Social behavior is often described as a unified concept, but highly social (group-living) species exhibit distinct social structures and may make different social decisions. Prairie voles (*Microtus ochrogaster*) are socially monogamous rodents that often reside in extended family groups, and exhibit robust preferences for *familiar* social partners (same- and opposite-sex) during extended choice tests, although short-term preferences are not known. Mice (*Mus musculus*) are gregarious and colonial, but in brief laboratory tests of social preference they typically prefer social novelty. This preference for novel vs. familiar peers may represent a species-specific difference in social decision-making between mice and prairie voles. However, the tests used to measure preferences in each species differ markedly in duration and degree of contact, such that the behaviors cannot be directly compared. We assessed whether social preferences for novelty or familiarity differed between mice and prairie voles of both sexes when assessed with matching protocols: the sociability/social preference test (SPT) typically used in mice (short, no direct contact), and the partner preference test (PPT) used in voles (long, direct contact). A subset of voles also underwent a PPT using barriers (long, no direct contact). In the short SPT, behavior did not differ between species. In the longer test, pronounced partner preferences emerged in prairie voles, but mice exhibited no social preferences and rarely huddled. No sex differences were evident in either test. Direct physical contact was required for partner preferences in huddling time in voles, but preference for the partner chamber was evident with or without contact. Both prairie voles and mice are social, but they exhibit important differences in the specificity and extent of their social behavior. While mice are often used to study social approach and other behaviors, voles are a more suitable species for the study of selective social relationships. Consideration of these differences will be important for studies examining the neural mechanisms supporting different kinds of peer social behavior.

Keywords: partner preference, social approach, prairie vole, mouse, social behavior, sociability, selective, affiliation

OPEN ACCESS

Edited by:
James P. Curley, University of Texas at Austin, United States

Reviewed by:
Karen Lisa Bales, University of California, Davis, United States
Alexa H. Veenema, Michigan State University, United States

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Received: *25 January 2018*
Accepted: *28 February 2018*
Published: *19 March 2018*

Citation:
Beery AK, Christensen JD, Lee NS and Blandino KL (2018) Specificity in Sociality: Mice and Prairie Voles Exhibit Different Patterns of Peer Affiliation. Front. Behav. Neurosci. 12:50. doi: 10.3389/fnbeh.2018.00050

INTRODUCTION

Social groups are a common feature of many species; life in such groups can be supported by affiliative interactions among group members, as well as by lack of anti-social behaviors such as aggression and territoriality. Not all social species prefer familiar social contacts and repeated interactions, however. In rodents, selective affiliation between adults is often

studied in voles: prairie voles are socially monogamous rodents that show opposite-sex and same-sex preferences for a familiar partner (i.e., partner preferences; Williams et al., 1992; DeVries et al., 1997), and meadow voles live in winter social groups and form enduring, selective partner preferences for adult peers (Beery et al., 2008, 2009; Ondrasek et al., 2015). In contrast, laboratory mice typically prefer novel individuals in brief tests of social interaction (Moy et al., 2004). Because the behavioral tests used in mice and voles differ markedly, it is unknown whether these differences arise from differences in tests or from species-specific differences in social behavior in mice and voles.

Once passed over in favor of larger model organisms, mice are now the most common laboratory mammal—by one estimate accounting for 46% of mammalian research subjects in physiology, up from 4% at the turn of the 20th century and just 6%–10% in the 1980s before the advent of transgenic mouse research (Beery and Zucker, 2011). Despite their popularity as laboratory research models, some social behaviors are not exhibited by mice and therefore cannot be studied in this species. For example, mice are promiscuous breeders, and studies of prairie voles, California mice and other monogamous species have led to many insights about the formation of selective social bonds for mates and how these vary across species (Carter et al., 1995; Donaldson and Young, 2008; Turner et al., 2010; Johnson and Young, 2015). Studying diverse species is also important to determine the variety of pathways supporting behaviors, as well as the generalizability or translatability of findings across species (Donaldson, 2010; Phelps et al., 2010; Taborsky et al., 2015).

Selective partner preferences may be another behavior mice do not display and cannot be used to study. Alternatively, differences in peer-directed social behavior between mice and voles may be an artifact of different testing circumstances. Social preferences in voles are most commonly assessed using the partner preference test (PPT), while social investigation and social interest in mice are most commonly assessed in social interaction with a single novel individual (e.g., File and Seth, 2003), social recognition/habituation tests (e.g., Choleris et al., 2003; Bielsky et al., 2004), or in the three-chambered social approach/preference tests (e.g., Yang et al., 2011).

The PPT was originally developed in the laboratory of Dr. C. Sue Carter (Williams et al., 1992) and assesses the extent of social contact and time in proximity to a partner relative to a stranger. The PPT has been used extensively to assess how different manipulations alter formation and maintenance of *preferences for a mate* in monogamous prairie voles and to a lesser degree in other monogamous species (Ahern et al., 2009; Kingsbury and Goodson, 2014; Carp et al., 2016). The PPT is also used to assess factors affecting social preferences for *same-sex peers* in meadow voles (Beery and Zucker, 2010; Anacker et al., 2016a,b), prairie voles (DeVries et al., 1997), and occasionally other rodents (e.g., Triana-Del Rio et al., 2011). One study has examined long-term social preferences of female mice during an 18 h three-chambered social choice test with stimulus mice housed behind wire mesh (Harrison et al., 2016).

The three-chambered sociability/social preference test (also called the Crawley sociability test) was devised in 2004 as a modification of the PPT and other social tests, specifically oriented toward measuring social approach. It has been widely used to assess both sociability and social preferences in mice (Moy et al., 2004, 2007; Nadler et al., 2004; Schwartzer et al., 2017), with similar tests used in rats (Smith et al., 2015, 2017). To assess sociability, mice are typically given a choice between a novel object (an empty wire pencil cup) and a social stimulus (a pencil cup covering a novel mouse). In order to assess social preference (herein referred to as the social preference test, SPT), mice are presented with one novel and one familiar social stimulus under the pencil cups. In this variant, males and females of multiple mouse strains (including oxytocin null mutants) preferred novel individuals (Moy et al., 2004; Crawley et al., 2007).

There are important differences between the SPT and PPT. Test durations are markedly different at 10 min and 3 h long, respectively. Prior PPT studies have shown that in prairie voles, preferences manifest by the end of the first hour and become significant by the second and third hour, with no enhancement from longer testing intervals (Williams et al., 1992). The PPT also allows for extensive physical contact compared to the SPT. Social stimulus animals in the PPT are tethered around the neck, allowing contact with the focal individual, as well as free movement throughout a portion of the chamber. Thus, social proximity in the PPT refers to huddling time, whereas proximity in the SPT indicates social investigation. Finally, familiar animals in the SPT are typically only briefly familiarized with each other; they are not individuals with which lasting relationships are likely to have formed, reducing the likelihood of detecting preferences based on such relationships. For these reasons, behavior may differ in important ways between these assessments, obscuring our understanding of species-specific differences in behavior.

MATERIALS AND METHODS

Animal Subjects

Prairie voles were bred locally and housed with a same-sex cage-mate from weaning. Voles are photoperiodic and were maintained on a 14:10 h light:dark cycle, consistent with summer conditions. Twelve prairie voles (six male and six female) were used as focal test subjects in the SPT, and 1 week later the PPT.

C57BL/6 and C57BL/10 mice were bred locally and were maintained on a 12:12 light cycle. Mice were weaned into groups of 2–4 and separated to pairs at least 1 week prior to testing. Sixteen mice were used as focal test subjects (eight male and eight female; half of each sex were C57BL/6 and half were C57BL/10).

Additional individuals of matched species, sub-strain and sex were used as social partners or strangers. Tests were conducted at 6.3 $\pm$ 0.5 months of age (mean $\pm$ SEM). All procedures adhered to recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Research Council, and were approved by the Institutional Animal Care and Use Committee at Smith College.

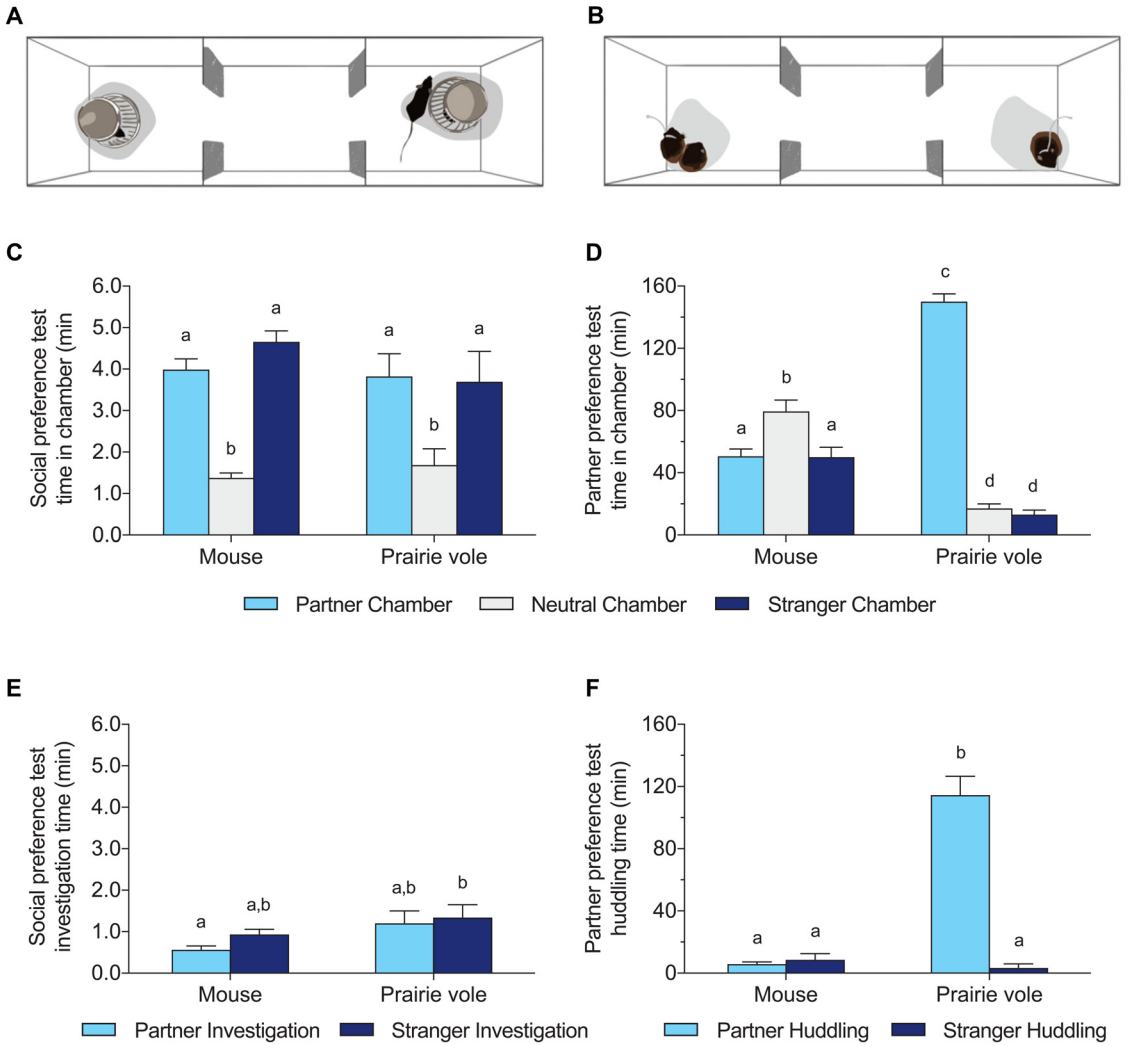

FIGURE 1 | Social behavior differences between mice and prairie voles across test types. **(A)** Schematic version of the 10 min social preference test (SPT), and **(B)** schematic of the 3 h partner preference test (PPT). Both tests were run with both species. **(C,D)** Time spent in the chamber occupied by the familiar partner, no subject, or a stranger in the SPT **(C)** and PPT **(D)**. **(E,F)** Time spent adjacent to the cup (investigation time) or the tethered subject (huddling time) across tests. Different letters above the bars indicate groups significantly different in *post hoc* tests corrected for multiple comparisons.

Social Preference Tests

The social preference version of the three-chambered social approach test was used to assess the inclination to seek social novelty, modeled on Yang et al. (2011). A linear apparatus (20 × 75 × 30 cm) was divided into three equal compartments. Stimulus animals were placed under wire pencil cups (Galaxy pencil holder, Spectrum Diversified) at each end of the apparatus, while the center chamber remained empty (**Figure 1A**). Two social stimuli were used: a familiar same-sex social partner (the cage-mate) and a novel individual of the same species, sex, and (if relevant) sub-strain as the focal individual and partner. Positions of the familiar and novel stimulus animals were alternated between tests. Familiar individuals were cage-mates of the focal subject and thus even more familiar than in the classic mouse test, as in novelty preference tests in rats (Smith et al., 2015, 2017), and more comparable to the familiar subjects in a PPT. Focal individuals were acclimated to the center of the apparatus for 5 min prior to test onset. Tests lasted 10 min and were video recorded for analysis.

Partner Preference Tests

PPTs were conducted as described previously (Ahern et al., 2009; Anacker et al., 2016a,b), using the same apparatuses as the SPT. Familiar and novel social stimulus animals were tethered at opposite ends of the apparatus (**Figure 1B**). Tethered animals were acclimated to the chamber for 5 min before placement of the focal animal in the center neutral chamber. Tethered subjects are typically calm after this duration. Tests lasted 180 min and were video recorded for analysis.

A subset of voles (n = 6 males) received a second, modified PPT conducted using pencil cups in place of tethers. These "cup PPTs" were used to distinguish the effects of test duration from the effects of access to full physical contact. Prior research in our lab has shown that voles tested in multiple PPTs exhibit equivalent huddling over time and across tests (Beery et al., 2009).

Data Analysis

Video recordings were scored using Intervole Timer v1.6 (Annaliese Beery) without knowledge of the partner and stranger positions. Comparisons across species were conducted via two-way analysis of variance (ANOVA) examining effects of species (mouse/vole) and stimulus (partner/stranger) on time adjacent to the stimulus. *Post hoc* tests (Tukey's HSD) were used to detect differences between all groups. Chamber times were analyzed in the same manner. Although all possible pairs are compared in this method, only 4/6 (adjacent) and 9/12 (chamber) pairings are useful to interpret (e.g., Mouse partner chamber vs. mouse center chamber is useful, but not mouse partner chamber vs. vole center chamber). Partner preference in each group was defined as significantly more time adjacent to the partner than the stranger. Preference score was defined as relative preference for the partner (time adjacent to the partner/time adjacent to the partner+stranger). Preference scores and activity within each test apparatus were compared using t-tests across species.

Sexes and strains were used in equal numbers across all conditions and thus pooled. We conducted sub-group analyses to explore effects of sex and strain on behavioral outcomes using t-tests between males and females of each species and between C57BL/6 and C57BL/10 mice for each outcome. No statistically significant sex or strain differences were found.

Statistical analyses were performed in JMP 8.0 and GraphPad Prism 7.0. Results were considered significant at $p < 0.05$, and all tests were conducted two-tailed.

RESULTS

Species Differences in Social Behavior in the Partner Preference Test

Mice and voles differed profoundly in their behavior in the 3 h PPT (**Figures 1B,D,F**). Prairie voles spent extensive time in physical contact with their partner (mean 114 ± 12 min), in contrast to mice (mean 5.9 ± 1.4 min). Two-way ANOVA of huddling time showed significant effects of species (mouse/vole), huddling target (partner/stranger), and their interaction (species: $F_{(1,52)} = 77.28$, $p < 0.0001$; huddling target: $F_{(1,52)} = 84.63$, $p < 0.0001$; interaction: $F_{(1,52)} = 93.59$, $p < 0.0001$). Prairie voles exhibited significant preferences for huddling with the partner over the stranger ($p < 0.0001$, **Figure 1F**), and huddled significantly more with their partners than did mice ($p < 0.0001$, Tukey's HSD). Mice did not exhibit significant preferences for either the partner or stranger, and spent little time huddling with either subject.

Two-way ANOVA of time spent in each chamber showed significant effects of chamber type (partner/neutral/stranger), and chamber interaction with species (chamber: $F_{(2,78)} = 83.78$, $p < 0.0001$; species: N.S.; interaction: $F_{(2,78)} = 123.4$, $p < 0.0001$). Mice spent significantly more time alone in the neutral chamber than in the partner chamber or the stranger chamber (each $p < 0.05$, Tukey's HSD). Prairie voles spent significantly more time in the partner chamber than did mice ($p < 0.0001$, Tukey's HSD), and more time in the chamber with their partner than either the neutral or stranger chamber (each $p < 0.0001$, Tukey's HSD).

Partner Preference Expression Requires Physical Contact

PPTs were compared to modified "cup PPTs" in six male voles to determine whether differences in behavior in the SPT and PPT are due to the difference in duration, difference in physical contact, or both. Access to physical contact strongly affected time in proximity to the partner (**Figure 2A**). Two-way ANOVA showed a significant effect of test type (PPT/cup PPT), stimulus subject (P/S), and interaction between these factors on time adjacent to a stimulus vole (stimulus subject: $F_{(1,20)} = 23.47$, $p < 0.0001$; test type $F_{(1,20)} = 9.61$, $p = 0.0056$; interaction: $F_{(1,20)} = 12.82$, $p = 0.002$). Partner preferences were only evident in the standard PPT, and partner huddling in the standard PPT was higher than all other outcomes (**Figure 2A**; multiple comparisons using Tukey's HSD between all groups, groups with different letters differed significantly).

In contrast, chamber times were equivalent across the two test types (**Figure 2B**). Two-way ANOVA with chamber (P/N/S) and test type (PPT/cup PPT), and their interaction as factors, yielded a significant effect of chamber ($F_{(2,30)} = 77.06$, $p < 0.0001$) but no effect of test type or interaction between test type and chamber time. *Post hoc* tests revealed that time spent in the partner chamber was significantly higher than other chamber times in both test types, and was indistinguishable across test types (Tukey's HSD between all groups).

No Species Differences in Social Behavior in the Social Preference Test

Behavior was more similar between mice and voles in the 10 min SPT with pencil cups. On average, mice spent less time near the partner than the stranger, while voles investigated both subjects roughly equally, but the difference in preference scores was not significant (0.36 ± 0.035 in mice vs. 0.51 ± 0.093 in voles; $t_{(26)} = 2.06$, $p = 0.12$). Focal animals of both species spent more time in some chambers of the SPT than others (**Figures 1A,C,E**), but there was no effect of species on the distribution of chamber times (two-way ANOVA: effect of chamber $F_{(2,78)} = 27.20$, $p < 0.0001$, no effect of species or interaction). Contact time with the wire cups enclosing stimulus animals differed between species (two-way ANOVA:

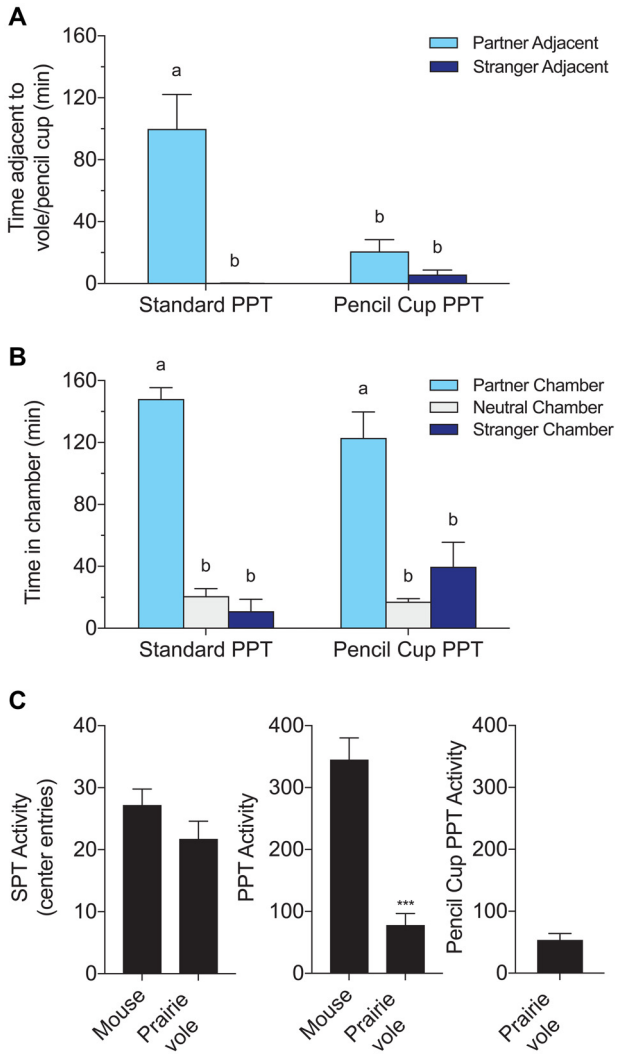

FIGURE 2 | Comparison of a standard PPT involving a tethered partner and stranger to a modified test using wire pencil cups over the stimulus voles. Both versions of the test lasted 3 h. **(A)** Time adjacent to the tethered stimulus subject or pencil cup containing the subject. **(B)** Time in each chamber. Chamber times were equivalent with and without direct contact, but partner preferences in time spent adjacent to the partner were only found in the test that allowed full contact. Letters indicate groups with significantly different means. **(C)** Species comparisons of activity, defined as the number of entries to the center chamber. Mice were significantly more active over the 3-h PPT (***$p < 0.0001$, *t*-test).

effect of species $F_{(1,52)} = 6.560$, $p = 0.013$) but there were no significant differences across species in groups matched on stimulus identity (e.g., partner adjacent in mice vs. partner adjacent in voles).

Activity

Mice were more active/exploratory than voles in the PPT, with 345 ± 35 vs. 78 ± 18 (mean ± SEM) crossings from a side chamber to the center chamber ($t_{(22)} = -6.75$, $p < 0.0001$, **Figure 2C**). Mice also crossed into the center chamber more often in the shorter SPT, but this difference was not significant (27 ± 3 vs. 22 ± 3 crossings). There was no difference in activity between voles tested in the regular PPT and the same voles tested in the cup PPT.

DISCUSSION

Mice and voles exhibited pronounced differences in social contact in the PPT, independent of sex and consistent with the existence of strongly selective social preferences for familiar peers in prairie voles (DeVries et al., 1997; Lee et al., unpublished data) but not mice. Voles spent the majority of the test huddling with their partner and over 90% of the test in occupied chambers. Mice did not exhibit significant partner preferences, spent 56% of the test in occupied chambers, and only 8% in social contact with another mouse. In 18-h modified PPTs in wild female mice using wire cage dividers, time in the social chambers was close to 50%, as in the present study, although huddling could not be measured (Harrison et al., 2016). Wild mice also demonstrated more positive behaviors like grooming and sniffing, and less negative social behaviors like fighting and chasing, over the course of 3 days of observed cohabitation (Harrison et al., 2016), and some nursing females form significant associations with other specific females (Weidt et al., 2014). Thus, mice can form relationships in specific circumstances, although they do not exhibit the extensive social huddling and social preference present in voles. Rats also appear to lack preferences for familiar individuals (Schweinfurth et al., 2017). These species-specific differences in social structure indicate the use of different animal models for different social behaviors. In particular, voles are more suitable for the study of selective social relationships.

In the present study, different testing scenarios led to important differences in conclusions about social behavior. Both the duration of the test and the ability to engage in social contact involved in the 3-h PPT shaped the outcomes observed. The major differences between mice and prairie voles in social preference and social contact time detected in long tests of social behavior were not detected in the shorter SPT. Differences between chamber times in the tests lacking physical contact (SPT and cup PPT) in the same voles particularly reinforce the importance of test duration for the expression of partner preferences. While the SPT has proven valuable for assessing sociability and investigation, it does not detect the formation of social relationships. Comparison of the modified "cup" PPTs to standard PPTs demonstrates that the physical contact permitted by the standard PPT was necessary for the expression of partner preferences in time adjacent to the stimulus animals. However, chamber times were similar across the cup and standard PPTs, suggesting that chamber times in this long test can illustrate social preference irrespective of contact. These results are similar to findings in opposite-sex tests of titi monkeys, who showed preferences for proximity to a social partner but not increased contact with an enclosing barrier (Carp et al., 2016).

In our study, the preference for social novelty in mice in the SPT was not significant. Novelty preference may be

more pronounced in juvenile mice tested at 6 weeks of age (e.g., Moy et al., 2004), or greater familiarity with the familiar mouse may reduce novelty preference.

As research on social behaviors and disorders grows, mice are increasingly being used to study the hormones, neurotransmitters, and genes involved in different social behaviors, the roles of life experience, and sources of individual variation (e.g., Ferguson et al., 2001; Choleris et al., 2003; Curley et al., 2009; Dölen et al., 2013). As the present study demonstrates, both test format and choice of species impact social outcomes in such studies; long tests in socially selective species such as prairie and meadow voles are important for the study of specific relationships between peers. The use of multiple species with distinct patterns of social behavior should thus provide greater insight into the substrates of peer relationships in diverse mammals including humans.

DATA AVAILABILITY

The dataset generated for this study is archived at the Open Science Framework website at http://osf.io/j29t5.

AUTHOR CONTRIBUTIONS

JDC, NSL and KLB conducted the study and contributed to the design. AKB conceived the study, coordinated the study, analyzed the data, prepared figures and wrote the manuscript. All authors critically revised the manuscript and gave approval for publication.

FUNDING

This work was supported by grants from the National Science Foundation (award 1257162) and the National Institute of Mental Health of the National Institutes of Health (award R15MH113085).

ACKNOWLEDGMENTS

We are grateful to Candy Gonzalez, Katherine (Bei) Heald, and Natalie Larson for assistance with behavioral testing, and to the staff of the Animal Quarters for care of our animals.

REFERENCES

Ahern, T. H., Modi, M. E., Burkett, J. P., and Young, L. J. (2009). Evaluation of two automated metrics for analyzing partner preference tests. *J. Neurosci. Methods* 182, 180–188. doi: 10.1016/j.jneumeth.2009.06.010

Anacker, A. M. J., Christensen, J. D., LaFlamme, E. M., Grunberg, D. M., and Beery, A. K. (2016a). Septal oxytocin administration impairs peer affiliation via V1a receptors in female meadow voles. *Psychoneuroendocrinology* 68, 156–162. doi: 10.1016/j.psyneuen.2016.02.025

Anacker, A. M. J., Reitz, K. M., Goodwin, N. L., and Beery, A. K. (2016b). Stress impairs new but not established relationships in seasonally social voles. *Horm. Behav.* 79, 52–57. doi: 10.1016/j.yhbeh.2016.01.004

Beery, A. K., Loo, T. J., and Zucker, I. (2008). Day length and estradiol affect same-sex affiliative behavior in the female meadow vole. *Horm. Behav.* 54, 153–159. doi: 10.1016/j.yhbeh.2008.02.007

Beery, A. K., Routman, D. M., and Zucker, I. (2009). Same-sex social behavior in meadow voles: multiple and rapid formation of attachments. *Physiol. Behav.* 97, 52–57. doi: 10.1016/j.physbeh.2009.01.020

Beery, A. K., and Zucker, I. (2010). Oxytocin and same-sex social behavior in female meadow voles. *Neuroscience* 169, 665–673. doi: 10.1016/j.neuroscience.2010.05.023

Beery, A. K., and Zucker, I. (2011). Sex bias in neuroscience and biomedical research. *Neurosci. Biobehav. Rev.* 35, 565–572. doi: 10.1016/j.neubiorev.2010.07.002

Bielsky, I. F., Hu, S.-B. B., Szegda, K. L., Westphal, H., and Young, L. J. (2004). Profound impairment in social recognition and reduction in anxiety-like behavior in vasopressin V1a receptor knockout mice. *Neuropsychopharmacology* 29, 483–493. doi: 10.1038/sj.npp.1300360

Carp, S. B., Rothwell, E. S., Bourdon, A., Freeman, S. M., Ferrer, E., and Bales, K. L. (2016). Development of a partner preference test that differentiates between established pair bonds and other relationships in socially monogamous titi monkeys (*Callicebus cupreus*). *Am. J. Primatol.* 78, 326–339. doi: 10.1002/ajp.22450

Carter, C. S., DeVries, A. C., and Getz, L. L. (1995). Physiological substrates of mammalian monogamy: the prairie vole model. *Neurosci. Biobehav. Rev.* 19, 303–314. doi: 10.1016/0149-7634(94)00070-h

Choleris, E., Gustafsson, J.-A. A., Korach, K. S., Muglia, L. J., Pfaff, D. W., and Ogawa, S. (2003). An estrogen-dependent four-gene micronet regulating social recognition: a study with oxytocin and estrogen receptor-α and -β knockout mice. *Proc. Natl. Acad. Sci. U S A* 100, 6192–6197. doi: 10.1073/pnas.0631699100

Crawley, J. N., Chen, T., Puri, A., Washburn, R., Sullivan, T. L., Hill, J. M., et al. (2007). Social approach behaviors in oxytocin knockout mice: comparison of two independent lines tested in different laboratory environments. *Neuropeptides* 41, 145–163. doi: 10.1016/j.npep.2007.02.002

Curley, J. P., Davidson, S., Bateson, P., and Champagne, F. A. (2009). Social enrichment during postnatal development induces transgenerational effects on emotional and reproductive behavior in mice. *Front. Behav. Neurosci.* 3:25. doi: 10.3389/neuro.08.025.2009

DeVries, A. C., Johnson, C. L., and Carter, C. S. (1997). Familiarity and gender influence social preferences in prairie voles (*Microtus ochrogaster*). *Can. J. Zool.* 75, 295–301. doi: 10.1139/z97-037

Dölen, G., Darvishzadeh, A., Huang, K. W., and Malenka, R. C. (2013). Social reward requires coordinated activity of nucleus accumbens oxytocin and serotonin. *Nature* 501, 179–184. doi: 10.1038/nature12518

Donaldson, Z. R. (2010). We're the same... but different: addressing academic divides in the study of brain and behavior. *Front. Behav. Neurosci.* 4:41. doi: 10.3389/fnbeh.2010.00041

Donaldson, Z. R., and Young, L. J. (2008). Oxytocin, vasopressin, and the neurogenetics of sociality. *Science* 322, 900–904. doi: 10.1126/science.1158668

Ferguson, J. N., Aldag, J. M., Insel, T. R., and Young, L. J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *J. Neurosci.* 21, 8278–8285.

File, S. E., and Seth, P. (2003). A review of 25 years of the social interaction test. *Eur. J. Pharmacol.* 463, 35–53. doi: 10.1016/s0014-2999(03)01273-1

Harrison, N., Lopes, P. C., and König, B. (2016). Oxytocin and social preference in female house mice (*Mus musculus domesticus*). *Ethology* 122, 571–581. doi: 10.1111/eth.12505

Johnson, Z. V., and Young, L. J. (2015). Neurobiological mechanisms of social attachment and pair bonding. *Curr. Opin. Behav. Sci.* 3, 38–44. doi: 10.1016/j.cobeha.2015.01.009

Kingsbury, M. A., and Goodson, J. L. (2014). Pair bond formation is impaired by VPAC receptor antagonism in the socially monogamous zebra finch. *Behav. Brain Res.* 272, 264–268. doi: 10.1016/j.bbr.2014.06.042

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., et al. (2004). Sociability and preference for social novelty in five inbred

strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav.* 3, 287–302. doi: 10.1111/j.1601-1848.2004.00076.x

Moy, S. S., Nadler, J. J., Young, N. B., Perez, A., Holloway, L. P., Barbaro, R. P., et al. (2007). Mouse behavioral tasks relevant to autism: phenotypes of 10 inbred strains. *Behav. Brain Res.* 176, 4–20. doi: 10.1016/j.bbr.2006.07.030

Nadler, J. J., Moy, S. S., Dold, G., Simmons, N., Perez, A., Young, N. B., et al. (2004). Automated apparatus for quantitation of social approach behaviors in mice. *Genes Brain Behav.* 3, 303–314. doi: 10.1111/j.1601-183x.2004.00071.x

Ondrasek, N. R., Wade, A., Burkhard, T., Hsu, K., Nguyen, T., Post, J., et al. (2015). Environmental modulation of same-sex affiliative behavior in female meadow voles (*Microtus pennsylvanicus*). *Physiol. Behav.* 140, 118–126. doi: 10.1016/j.physbeh.2014.12.021

Phelps, S. M., Campbell, P., Zheng, D.-J., and Ophir, A. G. (2010). Beating the boojum: comparative approaches to the neurobiology of social behavior. *Neuropharmacology* 58, 17–28. doi: 10.1016/j.neuropharm.2009.06.043

Schwartzer, J. J., Onore, C. E., Rose, D., and Ashwood, P. (2017). C57BL/6J bone marrow transplant increases sociability in BTBR T^{+} $Itpr3^{tf}$/J mice. *Brain Behav. Immun.* 59, 55–61. doi: 10.1016/j.bbi.2016.05.019

Schweinfurth, M. K., Neuenschwander, J., Engqvist, L., Schneeberger, K., Rentsch, A. K., Gygax, M., et al. (2017). Do female Norway rats form social bonds? *Behav. Ecol. Sociobiol.* 71:98. doi: 10.1007/s00265-017-2324-2

Smith, C. J. W., Mogavero, J. N., Tulimieri, M. T., and Veenema, A. H. (2017). Involvement of the oxytocin system in the nucleus accumbens in the regulation of juvenile social novelty-seeking behavior. *Horm. Behav.* 93, 94–98. doi: 10.1016/j.yhbeh.2017.05.005

Smith, C. J. W., Wilkins, K. B., Mogavero, J. N., and Veenema, A. H. (2015). Social novelty investigation in the juvenile rat: modulation by the μ-opioid system. *J. Neuroendocrinol.* 27, 752–764. doi: 10.1111/jne.12301

Taborsky, M., Hofmann, H. A., Beery, A. K., Blumstein, D. T., Hayes, L. D., Lacey, E. A., et al. (2015). Taxon matters: promoting integrative studies of social behavior: NESCent Working Group on Integrative Models of Vertebrate Sociality: Evolution, Mechanisms, and Emergent Properties. *Trends Neurosci.* 38, 189–191. doi: 10.1016/j.tins.2015.01.004

Triana-Del Rio, R., Montero-Domínguez, F., Cibrian-Llanderal, T., Tecamachaltzi-Silvaran, M. B., Garcia, L. I., Manzo, J., et al. (2011). Same-sex cohabitation under the effects of quinpirole induces a conditioned socio-sexual partner preference in males, but not in female rats. *Pharmacol. Biochem. Behav.* 99, 604–613. doi: 10.1016/j.pbb.2011.06.006

Turner, L. M., Young, A. R., Römpler, H., Schöneberg, T., Phelps, S. M., and Hoekstra, H. E. (2010). Monogamy evolves through multiple mechanisms: evidence from V1aR in deer mice. *Mol. Biol. Evol.* 27, 1269–1278. doi: 10.1093/molbev/msq013

Weidt, A., Lindholm, A. K., and König, B. (2014). Communal nursing in wild house mice is not a by-product of group living: females choose. *Naturwissenschaften* 101, 73–76. doi: 10.1007/s00114-013-1130-6

Williams, J. R., Catania, K. C., and Carter, C. S. (1992). Development of partner preferences in female prairie voles (*Microtus ochrogaster*): the role of social and sexual experience. *Horm. Behav.* 26, 339–349. doi: 10.1016/0018-506x(92)90004-f

Yang, M., Silverman, J. L., and Crawley, J. N. (2011). "Automated three-chambered social approach task for mice," in *Current Protocols in Neuroscience*, eds J. N. Crawley, C. R. Gerfen, M. A. Rogawski, D. R. Sibley, P. Skolnick and S. Wray (Hoboken, NJ: John Wiley & Sons, Inc.) Chapter 8:Unit 8.26.

Conflict of Interest Statement: 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: 06 November 2018
doi: 10.3389/fnbeh.2018.00263

Spontaneous Discriminative Response to the Biological Motion Displays Involving a Walking Conspecific in Mice

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Recent translational studies using mice have contributed toward elucidating the neural, genetic, and molecular basis of social communication deficits. Nevertheless, many components of visual processes underlying mice sociality remain unresolved, including perception of bodily-movement. Here, we aimed to reveal the visual sensitivity of mice to information on bodily motion using biological motion displays depicted by simple geometric dots. We introduced biological motions extracted from walking mice vs. corresponding meaningless scrambled motions, in which the spatial configurations of each path of dots were shuffled. The apparatus was a three-chambered box with an opening between the chambers, and each side chamber had a monitor. We measured the exploration time of mice within the apparatus during the test, with two types of displays being presented. Mice spent more time in the chamber with the scrambled motion displays, indicating that animals spontaneously discriminated stimuli, with the scrambled motion being relatively novel. Furthermore, mice might have detected socially familiar cues from the biological motion displays. Subsequent testing revealed that additional mice showed no bias to the static versions of the stimuli used in the Movie test. Thus, we confirmed that mice modulated their behavior by focusing on the motion information of the stimuli, rather than the spatial configurations of each dot. Our findings provide a new perspective on how visual processing contributes to underlying social behavior in mice, potentially facilitating future translational studies of social deficits with respect to genetic and neural bases.

Keywords: biological motion, social cognition, mice, comparative psychology, motion perception

OPEN ACCESS

Edited by:
James P. Curley,
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Reviewed by:
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Received: *15 January 2018*
Accepted: *17 October 2018*
Published: *06 November 2018*

Citation:
Atsumi T, Ide M and Wada M (2018) Spontaneous Discriminative Response to the Biological Motion Displays Involving a Walking Conspecific in Mice. Front. Behav. Neurosci. 12:263. doi: 10.3389/fnbeh.2018.00263

INTRODUCTION

Over the last three decades, studies have elucidated human social cognition based on visual cues produced by the eyes, face, and bodily-parts of other individuals, providing information about emotions and intentions (Happé et al., 2017). Animal studies develop our knowledge about the evolutionary and neural basis of cognition. Although rodents are one of the most accessible laboratory animals, knowledge about visual processing for social cues, including bodily-movement, remains limited.

The visual perception of motion related to social information is evolutionally fundamental (Troje, 2013). Researchers interested in the social significance of motion information have focused

on assessing how biological motion is perceived (BM; Johansson, 1973). BM displays are usually made by attaching light sources on an actor's body and recording their movements in a dark environment. Motion is isolated from other sources of information, like shape and color; however, the displays are readily identified as depicting the actor's bodily movement and various actions (Troje, 2013). Some studies have shown that perception of BM is strongly linked to social cognition in humans (Pavlova, 2012). For instance, people with autism spectrum disorders (ASD), characterized by social deficits, are less sensitive to BM (Blake et al., 2003; Klin et al., 2009; Koldewyn et al., 2010).

Some studies of non-human animals have reported the ability for individuals to discriminate BM from comparative displays (cats: Blake, 1993; bottlenose dolphins: Herman et al., 1990). Recent studies, however, have distinguished between the ability to discriminate stimuli acquired through simple training and other discriminative responses (Pavlova, 2012). Some species cannot generalize their learning to novel BM displays (baboons; Parron et al., 2007; rats: MacKinnon et al., 2010; pigeons: Dittrich et al., 1998; Yamamoto et al., 2015; rhesus macaques: Vangeneugden et al., 2010). The discrimination training employed in these studies might induce a behavioral strategy in which the animals use local movements of dots as a discriminative cue.

In some cases, it seems reasonable to focus such natural behavioral repertoires on the perception of social signals. Studies of newly hatched domestic chicks employed imprinting techniques and showed their innate sensitivity to BM stimuli made from a video clip of a walking hen (Regolin et al., 2000; Vallortigara et al., 2005; Vallortigara and Regolin, 2006). A study of marmosets showed that they gaze longer at BM displays than other control stimuli, including static, inverted, scrambled, and rotated versions of the BM (Brown et al., 2010). Another study, focusing on the gazing behavior of dogs, examined the effect of oxytocin, which is a neuropeptide hormone implicated in reproductive and social behaviors (Kovács et al., 2016). The authors found that oxytocin enhanced differences in the gaze distribution of dogs between BM and control stimuli. A study of medaka fish showed that the BM stimuli induced shoaling behavior (Nakayasu and Watanabe, 2014). Further evidence of the social significance of BM was provided by a study with neural recordings of monkeys (Oram and Perrett, 1994). When viewing the BM display, the authors found that the anterior superior temporal polysensory (STP) area was activated, a region responsible for processing various socially relevant visual cues (Allison et al., 2000).

Previous studies suggested that mice might perceive visual cues, such as painful facial expressions, postures, or gestures (Langford et al., 2006, 2010), and whole-body actions in social contexts (Watanabe et al., 2016). Watanabe et al. (2016) demonstrated that videos of mouse social behavior are visually attractive to conspecifics, with animals staying longer in places with particular video clips. Two studies on rodents examined their acquired discrimination of BM displays by other species, such as a walking hen and human (MacKinnon et al., 2010; Foley et al., 2012). However, whether BM displays induce untrained behaviors remain untested. It remains unclear whether the visual preferences of mice are elicited by socially relevant motion information other than visual properties, such as shape and color.

This study aimed to elucidate the processing of motion information by mice using bodily actions as the core component of social cognition. Spontaneous discrimination of BM displays should be strictly linked to adaptive behavior. We examined whether mice differentiate BM displays of walking mice from control stimuli without any training (Movie test). The possibility that animals respond to the local motion of each geometric particle was examined by introducing scrambled motions as control stimuli originating from BMs, by altering their global appearance. In the subsequent tests, static versions of the BM displays were presented to examine how motion information contributes to behavioral modulation (Static image test).

MATERIALS AND METHODS

Subjects

Twenty-four male mice (*Mus musculus*) of C57BL/6N were purchased from Charles River Laboratories, Japan. Twelve mice were assigned to the movie test, and the remaining individuals were used in the static image test. The mice used in our study were 6∼9 weeks old. The mice were housed in group cages, with four individuals per cage. The mice were provided with food and water *ad libitum*. The housing room was maintained at 23°C on a 12 h light/dark cycle. Before the study, the mice were not exposed to any of the experimental material in this study.

All animal procedures were performed in accordance with the institutional guideline for laboratory animals and approved by the animal care committee of Research Institute of National Rehabilitation Center for Persons with Disabilities (#28-AE1).

Apparatus

We focused on whether the subjects showed spontaneous discriminative behaviors to the visual stimuli. We then developed an experimental set up based on a three-chambered sociability test apparatus for rodents (Moy et al., 2004). This is a standardized testing protocol used to examine the behavioral phenotype regarding the social communication capacity in a strain. In this protocol, the time spent in the chamber with a familiar or an unfamiliar conspecific of the subject is measured. Naïve and wild-type mice generally tend to stay longer in the chamber with any conspecific or with novel individuals of conspecific (Moy et al., 2004). Mice modulate such place preferences in the presence of real organisms and with the appearance of video recorded conspecifics (Watanabe et al., 2016). If mice are able to perceive BM, they would be expected to spend a different amount of time in the chamber with BM compared the chamber with the control stimuli, without any learning.

The testing apparatus was a rectangular, three-chambered box fabricated by O'Hara & Co., Japan. Each chamber was 20 cm L × 40 cm W × 22 cm H. Dividing walls were made from clear plexiglass, with small rectangular openings (5 cm W × 3 cm H), allowing access into each chamber. The chambers of the apparatus were cleaned using fresh paper chips with 70% ethanol

before each trial. To present visual stimuli, a small LCD monitor (5 inch HDMI LCD (B), 800 × 480 resolution, cocopar) covered by a customized plastic case (5 cm L × 11 cm W × 15 cm H) was mounted on the wall on the opposite side of each door in both the left and right chambers. The top of the apparatus was an opening, above which a web camera (HD Webcam C615, Logicool) and a handy video camera (Everio GZ-E565, JVC) mounted above for the online measurements of trajectories of mice and for offline coding, respectively.

Stimuli

The sensitivity of mice to BM stimuli was tested using a binary choice between a pair of point-light animations. The point-light animations were composed of 6∼8 light points and were displayed at a speed of 24 frames/s. We created the stimuli employed in the current study using movie clips of real mice (**Figure 1**, top). We collected movie clips in which the adult male mice of C57BL/6N strain walk across the video camera's recording area. Scenes of mice walking were extracted from the sources, and involved at least one stroke of moving legs of the actor (14∼20 frames). The end frame of the scene was manually defined by the experimenter. One stroke was defined as the period of time from the movement of the limbs of the mouse away from the floor in the first frame until the return of the limb to the same location. Each scene was looped during presentation by joining the last frame of the scene to the first frame. Three scenes by three different actors were obtained. We then created three stimulus sets for the Movie test involving both BM and control movie clips (Set 1∼3), by using Adobe Flash CS6. White colored point-lights were placed at key points on the body area (tip of nose, root of an ear, hands of fore- and hind-limbs, root of the tail, and the midpoint of the tail) of the actor mouse in a scene, and dots occluded by a body part were not plotted on that particular frame. Then, the view of the original scene was replaced with a uniform dark background, with only the dots remaining. These modified movie clips from each scene were used as BM movies (**Figure 1**, left). To create control stimuli, the locations of each light-point at the beginning of the movie clip were shuffled, retaining entire motion path of each dot across the frames (scrambled motion: SM; **Figure 1**, right). For Static image test, the static stimuli were derived from the movie stimuli used in the Movie test. We extracted the first frames of each movie clip and introduced them as stimuli, and used three static pictures from the BM and SM clips, respectively. Presentation number and order of each stimulus was systematically controlled across the mice during experiments (see Procedure).

These stimuli were displayed using a self-made script in Matlab 2015a (Mathworks) with Psychtoolbox-3 (Brainard, 1997; Pelli, 1997) on MacBook Pro (Apple). Each stimulus was fitted within a 6.5 cm W × 4.5 cm H area on the monitor. The size of each dot on the monitors was 0.1 cm diameter. Examples of BM and SM movie clips are presented in the supplementary online material (**Videos S1**, **S2**, respectively).

Procedure

The test mouse was first introduced to the middle chamber. At the beginning of the habituation phase, the mouse was allowed to explore the entire test box for 5 min (**Figure 2**, left). The subject was returned to the center of the chamber at the end of this phase. Then, the doorways to the two side chambers were obstructed by opaque plastic occlusions for approximately 1 min (**Figure 2**, center). Then, the handy camera was turned

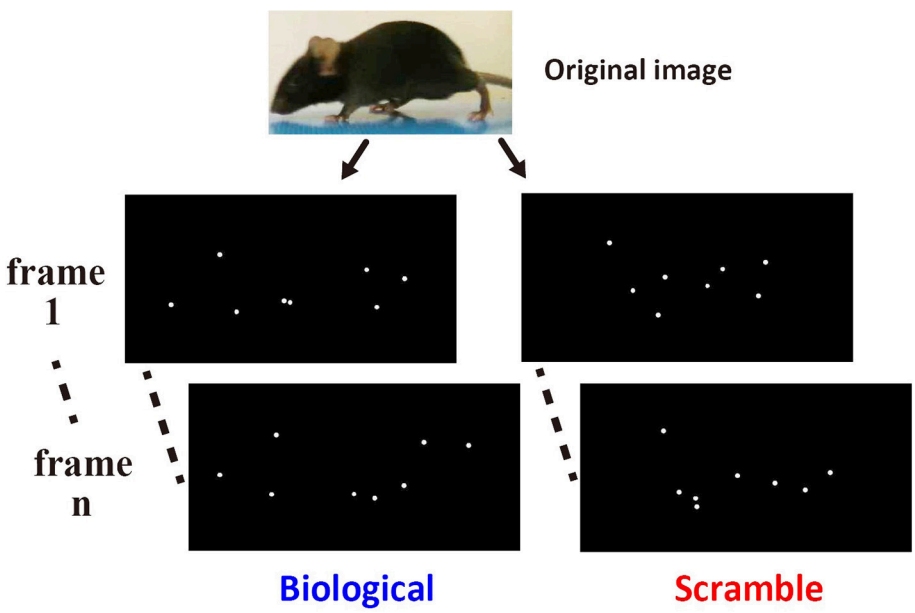


FIGURE 1 | Example of the stimuli used in the Movie test. The left images show two non-successive frames from a point-light animation sequence depicting normal biological motion extracted from an original video clip (top). The right images show the corresponding frames from an animation containing the same dots undergoing the same local motions, only with their initial start locations shuffled.

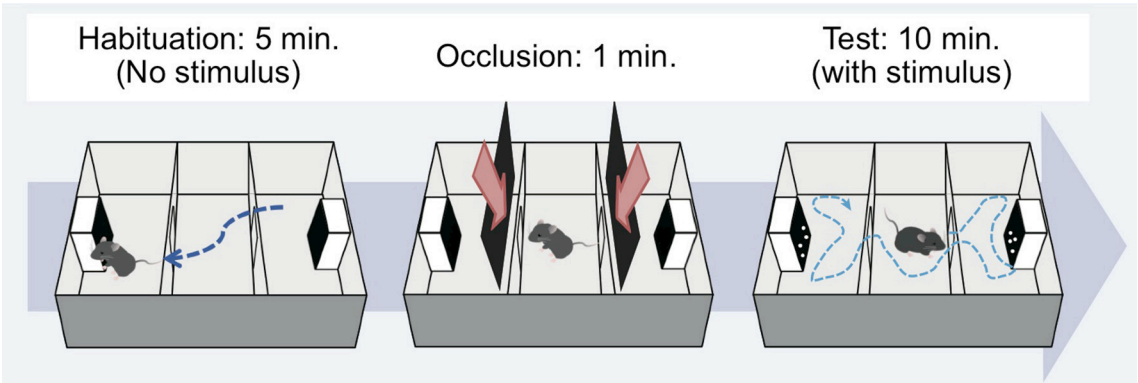


FIGURE 2 | Apparatus and procedure. Mice were tested by using a three-chambered apparatus (Moy et al., 2004). Habituation phase (**left**): the subject moved around freely for 5 min to acclimate to the apparatus without any stimulus on the monitors. Occlusion phase (**middle**): the subject was placed in the central compartment for 1 min to prepare for subsequent testing. Test phase (**right**): the doors to the side compartments were opened and the mouse again freely moved for 10 min. Mouse movement during the experiments was recorded by web camera, and the time spent in each compartment was calculated using tracking software.

on. Soon after that, a tracking software (ANY-maze version 4.75, Muromachi Kikai) and the presentation of the stimuli on each monitor were started, and the occlusions were immediately removed. One monitor presented the test stimulus, while the other showed the control stimulus, simultaneously. The subject was allowed to explore the entire test box for a 10 min session (**Figure 2**, right). During the 10 min period, one stimulus set of BM and SM was constantly presented. The amount of time spent in the left and right chambers during the period was estimated by the online tracking software and a human blind coder offline. An entry was defined as the center of the body area in one chamber. Each mouse experienced one trial of this testing per day, with a total of six trials throughout the experiment (over 6 days).

The location of the stimulus type (left vs. right side chamber) was systematically alternated between trials. The first chamber in which a BM movie was presented was counterbalanced across the subjects. Three stimulus sets were presented twice during the 6 days, and the chambers displaying each stimulus were alternated across days. Hence, the mouse was presented each movie clip once in each side chamber. We assumed that this manipulation would induce sufficient exploratory behavior by mice across days, ensuring no chance of acquiring any association between a specific stimulus and a stimulus type-dependent behavioral modulation (Simon et al., 1994; Rubinstein et al., 1997). The combination of the movie clips and the chambers where the stimuli were presented differed across days for each subject, and the order was counterbalanced across mice.

The procedure used in the Static image test was identical to that in the Movie test, except that mice were only tested for 2 days. The number of times that the static versions of each stimulus were presented was identical to those presented in the first 2 days of the Movie test.

RESULTS

Movie Test

Figure 3A shows one representative result derived using the tracking software. Over 10 min, the mice did not remain in a specific chamber, but moved in and out of the three chambers. We calculated the average time spent in each chamber with BM and SM stimuli across the days for each mouse (over 6 days, **Figure 3B**). We found that mice spent longer in the chamber with SM movies [paired t-test, $t_{(11)} = -5.44$, $p = 0.0002$, effect size: Cohen's $d = 2.72$, 95% confidence interval: CI = [−78.11, −33.11]]. This time difference between the stimulus conditions indicates that visual stimuli, even those depicted by simple moving dots, modulate mouse behavior in novel experimental apparatus. We confirmed the time-course effect to elucidate whether the subjects became habituated to the stimuli over the course of the experiment. The proportion of time spent in the BM chamber for the entire time spent in both chambers was calculated (6 days, **Figure 3C**). One-way repeated measure ANOVA revealed no main effect of days [$F_{(3.64,40.07)} = 1.58$, $p = 0.20$, effect size: $\eta_p^2 = 0.13$], with no indication of long-term habituation over time. The place preference in the chambers with SM stimuli was, therefore, maintained throughout the 6 days. In addition, the difference between conditions was already apparent in the first 2 days, in which each mouse experienced the presentation of each stimulus condition in both the left and right chambers [$t_{(11)} = -4.42$, $p = 0.001$, $d = 2.29$, CI = [−111.86, −37.54], **Figure 4A**]. Subsequent analysis for short-term habituation within a session revealed that the proportion of time spent in chambers with BM was stable for all minutes during the first 2 days [no main effect of time in each session: $F_{(4.77,567.74)} = 0.77$, $p = 0.57$, $\eta_p^2 = 0.006$]. A paired t test showed a significant difference between two types of stimuli in the first minute of the 2 days [$t_{(11)} = -3.20$, $p = 0.009$, $d = 1.65$, CI = [−22.90, −4.23]]. The time differences between conditions that appeared in the early periods suggest that the behavioral bias of mice was not formed by any type of learning, rather their innate sensitivity to the stimuli.

Analyses for durations of residential behaviors demonstrated that the movie clips modulated the time mice spent in each chamber. This bias relied on the spontaneous responses of the animals and was strongly maintained throughout the experiment. Despite these clear differences, the occupancy time in the

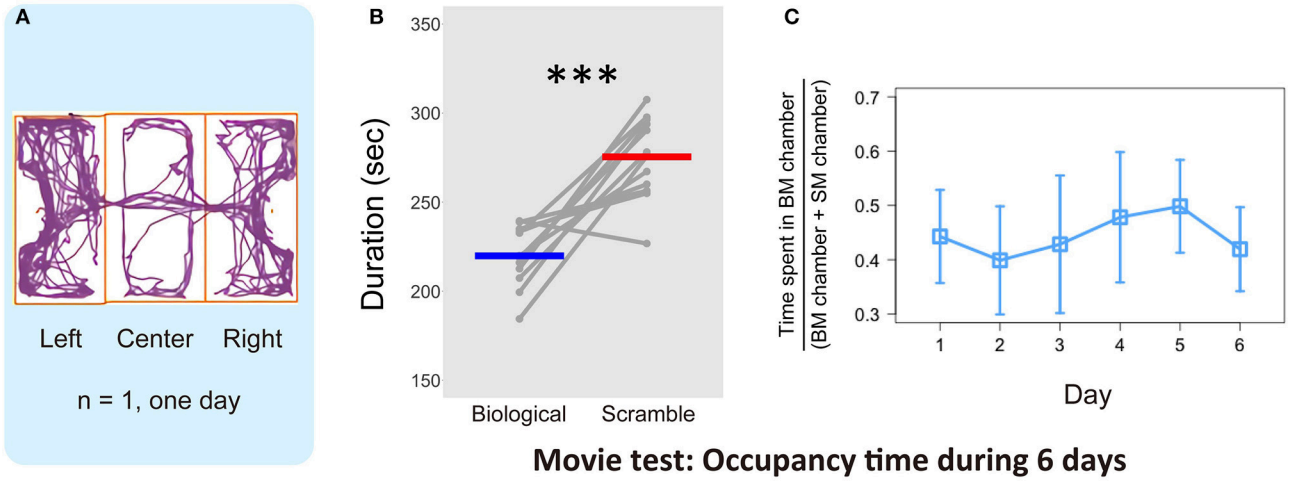


FIGURE 3 | Results of the time spent in the chambers with each stimulus in the Movie test. **(A)** One representative example of mouse locomotion for 10 min in 1 day estimated by automated video tracking software. Purple line indicates the trajectory, and orange rectangles represent each chamber area. **(B)** Average time spent in each chamber over 6 days and individual plot of each mouse ($N = 12$). Gray dots and lines indicate the individual data, and horizontal bars colored blue and red show the average time in each condition, respectively. The difference of the average time between Biological and Scramble movies was significant (***$p < 0.001$). **(C)** Proportion of time spent in BM chamber across the 6 days ($N = 12$). Error bars represent the ±95% confidence interval of the mean.

chamber provided insufficient information about whether the mice preferred to view the stimuli. **Figure 4B** shows a heat map that was estimated from the average occupancy time across the subjects. An example of the motion trajectory of a mouse (**Figure 3A**) and the heat map indicated that the subjects spent time in front of the monitors and in other areas, such as walls and corners. As a result, we questioned whether the effect of bias toward SM displays was true. Thus, we performed an additional analysis to estimate the occupancy time in front of each monitor. The visual acuity of mice is considered to be 0.5∼0.55 cycles per degree (cpd) (Prusky et al., 2000; Prusky and Douglas, 2004). We used stimuli formed of 0.1 cm diameter dots. We ensured that each dot could be clearly viewed by mice from the midpoint of the chamber (5 cm away from the monitor, 0.88 cpd). We assumed that the distance was suited for the broad visual field of mice to view the global appearance of each stimulus (180° for monocular vision, and 40° for binocular vision; Dräger, 1978). The region of interest (within an ∼5 cm diameter of the screens) and the occupancy time of these regions were estimated based on the frame-by-frame positions of mice coded by the software. We used the trajectories from the first 2 days and the average occupancy times across days for each subject in the statistical analysis. **Figure 4C** shows the time spent in front of the monitors with each condition. The occupancy time in front of the SM movie clip was longer than that in front of the BM clip [$t_{(11)} = -2.33$, $p = 0.04$, $d = -0.62$, CI = [−16.01, −0.44]]. Even in the area closer to the stimuli, mice remained longer in front of SM than BM displays.

The possibility that our animals avoided BM displays rather than preferred to approach SM stimuli remained. If the animals tended to keep a distance from BM clips, the time spent in front of the monitors with BMs would be relatively shorter than the time spent in front of the SM monitors. We analyzed the proportion of time spent in front of the monitors during the entire time in each chamber. There was, however, no difference between the conditions [$t_{(11)} = -0.24$, $p = 0.82$, $d = -0.05$, CI = [−2.68, 2.16]; **Figure 4D**]. Thus, the longer residency time in the SM chambers was associated with a visual preference for the control stimuli, rather than avoiding the BM chamber.

The location of mice was biased to the chamber with the SM display, but whether the animals paid attention to either display could not be determined. Previous studies reported attention-based behavioral repertoires toward biological motion stimuli in animals. Our tracking software was limited to detecting mice attentional behaviors only; thus, further analysis with a human blind-coder was required. First, the normal coding of the time spent in each chamber during the first 2 days performed by the human coder and the computer software were highly correlated, indicating significant reliability of human coding [$r = 0.99$, $t_{(46)} = 43.09$, $p < 2.2 \times 10^{-16}$, CI = [0.98, 0.99]]. Next, the time that the mouse spent within 1 cm diameter of the center of the LCD display's bottom, and paid attention to the monitor, was measured by the human coder. We measured the attentional behaviors including approaches into the region of interest and non-visual modalities, such as sniffing, touching, and nose poking. There was no difference between the time invested in attentional behaviors in front of the monitors during the entire time in each chamber [$t_{(11)} = -0.38$, $p = 0.71$, $d = -0.15$, CI = [−5.13, 3.63]; **Figure 5A**]. We also found no difference in the proportion of the time during the entire time in each chamber [$t_{(11)} = 0.87$, $p = 0.40$, $d = 0.36$, CI = [−1.32, 3.05]; **Figure 5B**]

Finally, we intended to determine the source of the modulation of mice residency time. An earlier study has already reported an orienting behavior toward non-socially relevant stimulus such as scrambled bot movements, and its visual novelty seemed to drive the behavior (Kovács et al., 2016). If the visual

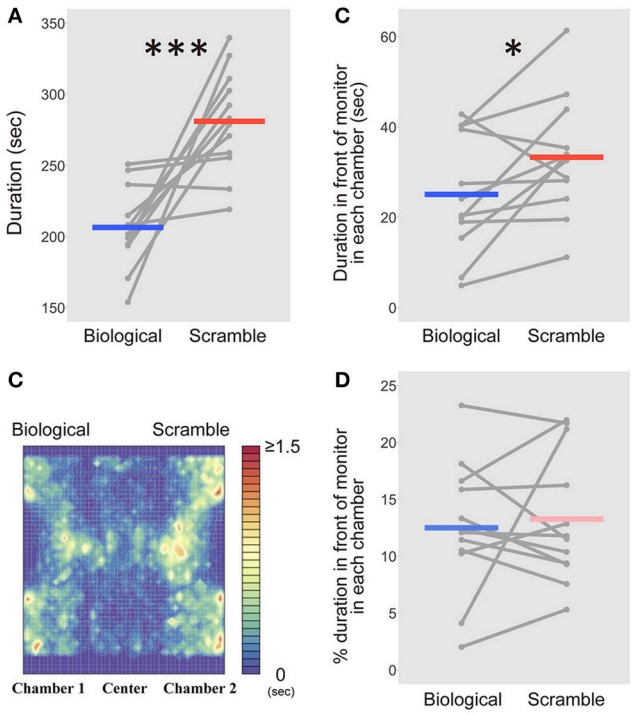


FIGURE 4 | Results of the time spent in the chambers with each movie stimulus condition in the Movie test over 2 days ($N = 12$). **(A)** The difference between Biological and Scramble movies was significant (***$p < 0.001$). **(B)** A heat map of activity in the three-chambered apparatus during the test phases was estimated by averaging the occupancy times across the days for each mouse. **(C)** The time spent in front of the monitors. The difference between Biological and Scramble movies was significant (*$p < 0.05$). **(D)** Percentage of duration spent in front of the monitors during the entire time in each chamber. Gray dots and lines indicate the individual data, and horizontal colored bars show the averaged time in each condition, respectively.

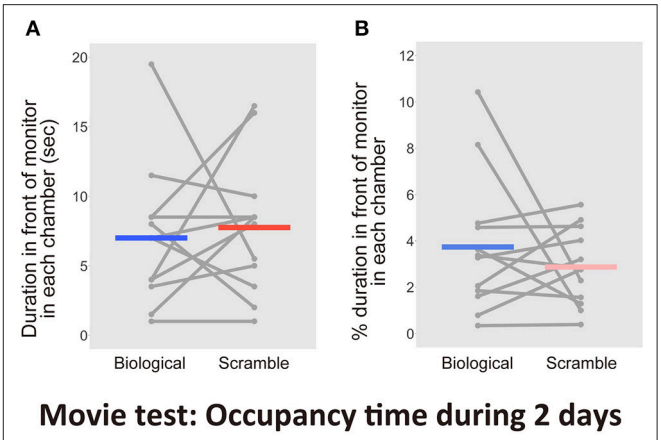


FIGURE 5 | Results of the time spent in front of each monitor including attentional behaviors in the Movie test over 2days ($N = 12$). **(A)** The time spent in front of the monitors. **(B)** Percentage of duration spent in front of the monitors during the entire time in each chamber. Gray dots and lines indicate the individual data, and horizontal colored bars show the averaged time in each condition, respectively.

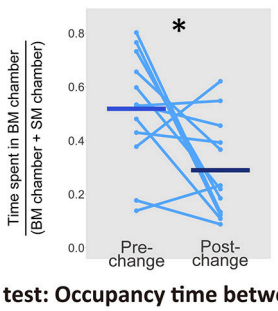
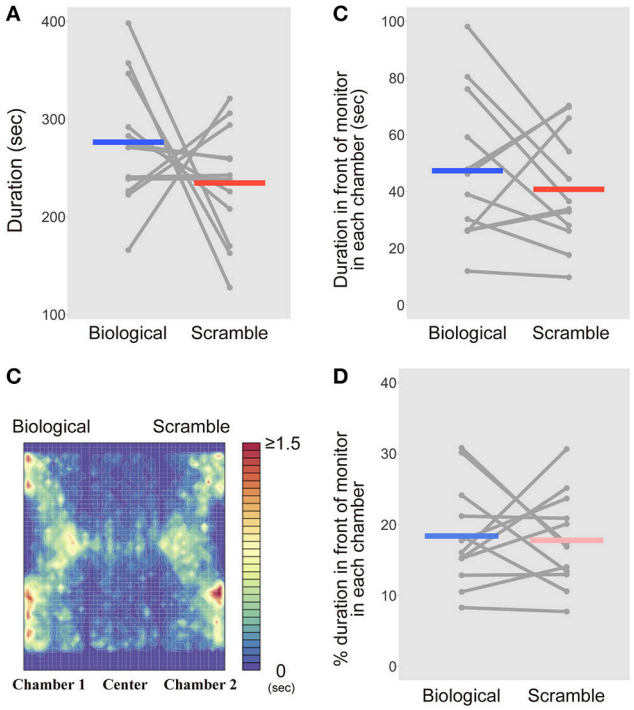


FIGURE 6 | Proportion of time spent in front of monitors with BM chamber between pre- and post-change of stimulus sets in the Movie test ($N = 12$). Light blue dots and lines indicates the individual data, and horizontal bars show the averaged time. The difference between 2 days was significant (*$p < 0.05$).

novelty of the display induced the orienting behavior of our mice, relatively greater preference for SM displays must be seen after movie clips were changed to new ones. We, therefore, calculated the ratio of time spent in front of displays (within about 5 cm diameter of the screens) with BM movies to total sum of the times spent in front of monitors with the 2 conditions for each subject. We then examined difference between the averaged times of the first pre- and post-change for stimulus set (**Figure 6**). The proportion of time spent with BM movies significantly decreased after the stimulus set change [a paired t-test, $t_{(11)} = 2.66$, $p = 0.022$, $d = 1.15$, CI = [0.04, 0.42]]. This indicates that the novelty of each set of stimuli influenced the residency time of the mice, and summing up, this novelty effect would be more obvious in SM condition.

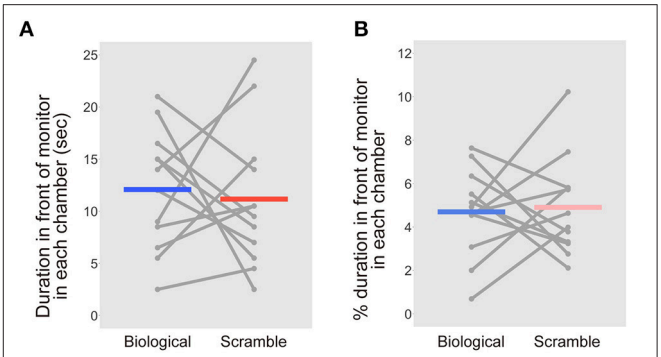
Static Image Test

The results of the Movie test demonstrated that the SM stimuli visually attracted mice, as they remained nearby for longer. Thus, the mice detected bodily-motion information of conspecifics from the BM displays, with the relative novelty of the SM displays potentially eliciting greater interest. However, it was still not known whether the motion information in the stimuli worked, or whether the simple spatial coordination of point-lights contributed in some way. The SM displays were obtained by rearranging the initial locations of each dot in the BM stimuli. If the special arrangements were relevant, static stimuli might also extract the same behavioral biases. Thus, we analyzed the responses of mice to static stimuli extracted from the movie clips employed in the previous test. We examined whether the discriminative behavior of mice relied on the motion information of the stimuli. We conducted the Static image test for 2 days, because the significant bias to SM stimuli was observed during the early period in the Movie test. **Figure 7A** shows the average time spent in each chamber with the static version of the BM and SM across the days for each mouse. We found no difference between stimulus condition [paired t-test, $t_{(11)} = 1.19$, $p = 0.26$,



Static image test: Occupancy time during 2 days

FIGURE 7 | Results of the tests with the static picture stimuli in Static image test over 2 days ($N = 12$). **(A)** Bars indicate the time spent in the chambers with each type of static stimuli extracted from Biological and Scramble movies of mice. **(B)** A heat map of activity in the three-chambered apparatus during the test phases was estimated by averaging occupancy times across mice. **(C)** Time spent in front of the monitors estimated by the software. **(D)** Percentage of duration spent in front of the monitors during the entire time in each chamber. Gray dots and lines indicate the individual data, and horizontal colored bars show the averaged time in each condition, respectively.



Static image test: Occupancy time during 2 days

FIGURE 8 | Results of the time spent in front of each monitor, including attentional behaviors, in the Static image test over 2 days ($N = 12$). **(A)** Time spent in front of the monitors. **(B)** Percentage of duration spent in front of the monitors during the entire time in each chamber. Gray dots and lines indicate the individual data, and horizontal colored bars show the averaged time in each condition.

$d = 0.67$, CI $= [-35.76, 119.23]$]. **Figure 7B** presents the activity within the apparatus estimated from occupancy times. By analyzing the occupancy time in front of the images within 5 cm, we found no difference between the two conditions [$t_{(11)} = 0.83$, $p = 0.43$, $d = 0.28$, CI $= [-10.94, 24.11]$, **Figure 7C**]. We analyzed the proportion of time spent in front of the monitors to the entire time in each chamber. There was also no difference between the two conditions [$t_{(11)} = -0.30$, $p = 0.77$, $d = -0.11$, CI $= [-8.90, 6.79]$; **Figure 7D**]. Thus, the behavioral bias obtained in the Movie test relied on the motion information of the stimuli. This result indicated that mice preferred the SM movie clips because of their integrated information, rather than local cues, such as the spatial configurations of dots.

Finally, we aimed to validate the time spent occupying the area within 1 cm of the monitors, which included the time spent for attentional behaviors. The entire time spent in each chamber during the first 2 days recorded by the human coder and the computer were highly correlated [$r = 0.998$, $t_{(46)} = 102.87$, $p < 2.2 \times 10^{-16}$, CI $= [0.996, 0.999]$]. The significant reliability of the human coding was again confirmed. The human coder then evaluated the amount of attention mice paid to the monitor. As a result, we found no difference between the time spent for attentional behaviors in front of the monitors [$t_{(11)} = 0.34$, $p = 0.74$, $d = 0.15$, CI $= [-4.997, 6.830]$; **Figure 8A**]. There was no difference between the proportion of residency time including attentional behaviors in front of the monitors in each chamber throughout the entire period [$t_{(11)} = -0.23$, $p = 0.83$, $d = -0.10$, CI $= [-2.24, 1.82]$, **Figure 8B**].

DISCUSSION

The current study examined whether mice spontaneously discriminate BMs depicted by multiple point-light dots from a control stimulus. The Movie test revealed the animals have longer residency time in chambers with SM displays, in which the initial positions of the dots were shuffled but the original pendulum movement was preserved. This bias disappeared when we presented the static version of these stimuli. The results of the two consecutive tests suggested that mice are able to differentiate between the two types of stimuli by focusing on the entire appearance of the movies, rather than local features. Therefore, our results suggest that mice obtain bodily motion information, which is considered as a crucial source of social cognition (Blake and Shiffrar, 2007) from just the movie stimuli of point-light displays.

Previous reports argued that rodents discriminate BM displays from other stimuli; however, it is not known whether the animals perceived moving dots in an integrated way to perform tasks (MacKinnon et al., 2010; Foley et al., 2012). Non-human species often fail to group each motion of dots as a single stimulus (Dittrich et al., 1998; Yamamoto et al., 2015). Consequently, how local features contribute to the performance of the animal should be examined with care. The current study is the first to report that discriminative behavior in rodents is not elicited by the individual motion of dots nor their spatial configurations.

Discrimination between two types of stimuli was observed during exploratory behavior in Movie tests. Our data showed that exploration over three chambers was common among the mice. Hence, the mice had sufficient opportunity to view our stimuli during exploratory behaviors. This tendency was common across days, but the mice did not show any habituation for movie type-driven in both long-term and short-term periods. The behavior was not the result of avoiding BM displays. The bias toward SM movie clips seemed stable over 6 days period because of relatively greater visual interest in the stimuli than BM stimuli.

There could be some other possible reasons why we observed the bias toward SM, i.e., the bias was not a result of the on-display stimuli. One possibility is that an idiosyncratic movie clip attracted the mice and led to apparent longer residency time with SM. We thus, individually measured the residency times with each movie clip over 6 days (Supplementary Data). Comparisons between 2 stimulus types in each movie sets showed a trend in which the residency time with SM was longer than the other (**Supplementary Figure 1**). This suggests that the bias to SM displays was not the effect of a particular movie stimulus. Another concern is the possibility that our finding was simply obtained by chance. If a residency time in another area within the chambers, in which the monitors were not visible, such as corners, is more suited to explain the present result, it would be difficult to conclude that the visual perception really contributed. We checked the residential behavior of mice at corners of both sides of the chamber during the first 2 days. However, the bias toward SM was not due to the time spent at corners (see more details in Supplementary Data and **Supplementary Figure 2**).

Three-chambered test paradigms have been used in many translational studies on social deficits in mice, such as autism-spectrum disorders (ASD). Mice typically show preferences for socially novel conspecifics over familiar ones (Moy et al., 2004). The high reproducibility of these behavioral traits in mice, along with their preference for social novelty, has been repeatedly confirmed. This paradigm is broadly accepted by studies assessing the sociability of mice (Silverman et al., 2010). Considering the results of the current study, biological motion information extracted from the same species was more familiar to the subjects than scrambled motion information. Thus, the biased orienting behavior might be explained by a general preference for novel stimuli in mice. In actuality, an analysis focusing on the effect of changing the set of stimuli revealed that a visually novel set of movie clips modulated the residency time of the mice. Novelty preferences were also detected when examining BM perception in dogs (Kovács et al., 2016). Dogs received an intranasal administration of oxytocin, which is a neuropeptide hormone that is closely related to social cognition. The authors showed that the dogs exhibited longer fixation to scrambled motion displays than BM stimuli. They suggested that the ability of dogs to perceive BM more easily might enhance their visual attention to unfamiliar scrambled motions. The point-light displays employed in the current study were novel to the mice. Thus, our data support the concept that socially novel signals elicit visual preferences in mice. Further study is necessary to examine whether the familiarity-dependent orienting behavior in mice is innate, or whether it is formed by daily observations of other individuals.

Our results also indicated that the behavior of mice was not elicited by learning the point-light displays, because the bias appeared during the early period of the experiments. Spontaneous discrimination of biological motion stimuli suggests that individuals within a species use social information to communicate with other individuals. Biological motion stimuli often elicit orienting behaviors toward stimuli by humans or non-human animals. Such behaviors include approaching (Regolin et al., 2000; Vallortigara et al., 2005; Vallortigara and Regolin, 2006; Miura and Matsushima, 2016), shoaling (Nakayasu and Watanabe, 2014), and eye gazing (Simion et al., 2008; Kéri and Benedek, 2009; Klin et al., 2009). Sensitivity to body-movement contributes to non-verbal communications (Pavlova, 2012), which optimize adaptive behavior for processing signals produced by conspecifics. Our results confirm that mice also exhibit spontaneous discrimination of BM by conspecifics, supporting the concept that communication among animals depends on the appearances of bodily-movement. In the present study, our visual stimuli seemed insufficient to elicit any bias in attentional behaviors regarding non-visual modality (e.g., sniffing), but were sufficient for inducing approaches toward SM. This phenomenon might reflect modality-specific preferences to visual motion stimuli in mice.

The analysis of the reduced region of interest revealed that the occupancy time was significantly longer for a viewing distance within 5 cm (the midpoint of the left or right chamber) from the screen with SM. In the present study, it was still unclear whether the mice could distinguish each dot in our stimuli because of their poor visual acuity. Each dot presented on the monitor was 0.1 cm in diameter, which corresponded to 0.88 cpd when the mouse observed the stimuli at a distance of 5 cm from the monitor. While the visual acuity of mice is approximately 0.5 cpd (Prusky et al., 2000; Prusky and Douglas, 2004), it remains a possibility that the dots on the screen might be viewed as blurry by the animals. Each stimulus (consisting of 6∼8 dots) was spread over nearly the entirety of the screen width of 6.5 cm, which was subtended at an angle of 66° on the mouse retina. Previous studies employing a similar visual angle of screen width reported neural activities of mice with a fixed view point, by presenting visual stimuli of lower spatial frequencies than that of the visual acuity of the mice (Niell and Stryker, 2008, 2010). In our study, the mice could freely move inside the apparatus and change their viewpoints and needed only to infer the global form, rather than explicitly distinguishing the individual dots. We therefore argue that our stimuli could elicit a behavioral bias toward unfamiliar, non-biological motion displays.

The results of our study do not enable us to determine whether the mice modulated their behaviors depending on physical and ecological contexts of BM. Our BM video clips presented walking from a fixed viewpoint, such as on a walker on treadmill. Although this type of movie allowed us to retain the whole-body appearance of the point-light walker, it might be physically unnatural for the mice. Many previous studies have shown that non-human animals could

perceive BM displays of a fixed viewpoint (chicks: Regolin et al., 2000; Vallortigara et al., 2005; Vallortigara and Regolin, 2006, dogs: Kovács et al., 2016, primates: Oram and Perrett, 1994; Brown et al., 2010). These indicate that we could assume BM perception by non-human animals based on a treadmill-like walking action. Previous studies of rodents that reported the capacity of their discriminative learning revealed that the animals could differentiate these types of displays from the controls (MacKinnon et al., 2010; Foley et al., 2012).

Another concern is the possibility that our SM displays exhibited other actions of mice. It was difficult to regard the overall appearance of the SM displays as representing some other meaningful bodily actions, because we randomly rearranged the positions of each dot keeping the course of the motion trajectory. Thus, there was limited possibility that actions resembling those more commonly attractive than walking were randomly generated in these control movie clips. To elucidate whether our control stimuli were potentially meaningful, the relative attractiveness of various biological motions must be tested. To the best of our knowledge, it remains unknown whether non-human animals modulate their responses toward various types of BM depending on the bodily actions involved; addressing this point is a hurdle in the understanding of the context-dependent role of motion. To take this study forward, future work should explore whether context-dependent motion stimuli, such as physically and ecologically natural action repertoires of the mouse, induce different behavioral reactions in the animals.

What regions of the brain are involved in BM perception in mice? To date, various human studies have elucidated the key regions of the brain for perception. The posterior superior temporal sulcus (pSTS) was reported as the representative brain area and is thought to play an important role in social perception (Allison et al., 2000; Grossman et al., 2000; Grossman and Blake, 2001, 2002; Peuskens et al., 2005; Saygin, 2007). Some part of the fusiform gyrus and extrastriate cortex, which specifically respond to human bodily shapes, also seem to be responsible (Jokisch et al., 2005; Peelen et al., 2006). Furthermore, the premotor area responds to self-generated bodily movement, with observed bodily-motion produced by other individuals being related to the brain region (Saygin, 2007). It is difficult to identify most of the human brain regions that correspond to those in mice at present. However, some possible brain regions in mice might have similar perception to humans. A previous study reported mice and rats could detect the coherent motion of multiple point-light dots (Douglas et al., 2006). This global motion perception, like BM perception, depends on the spatiotemporal integration of moving dots in the visual system. Another study showed that viewing coherently moving dots induces the activation of both the frontal cortex and visual cortex in mice, whereas incoherent motion only activates the visual cortex (Han et al., 2017). The detection of unified motion involved in BM might be associated with this region. The mouse premotor area (M2) might share some homologous functions with humans for motor execution. M2 is thought to be crucial for goal-directed actions underlying motor planning (Gremel and Costa, 2013). Recent studies have elucidated the mice brain circuits with respect to social and emotional domains co-working with behavioral responses induced by visual cues from other organisms. The anterior cingulate cortex (ACC) and amygdala have been identified with the representative responsible brain regions to code affective or noxious signals from observed individuals (Jeon et al., 2010; Burkett et al., 2016). These brain activities also accompany the observation of itching behavior (Yu et al., 2017). Studies using mice have suggested that the brain circuit responsible for BM perception is found in the pathway from visual cortex through to the frontal cortex, M2, ACC, and amygdala. To examine this hypothesized circuit for the BM perception of mice, socially and emotionally valuable stimulus categories should be employed. A simple geometric moving visual stimulus simulating a potential predator induces the emotional behaviors of mice, such as freezing and flight responses (De Franceschi et al., 2016). Therefore, viewing the BM display made from potential predators might also activate the brain, including the ACC and amygdala. We should verify that the BM perception of mice reflects the visual system, which detects structural information from observed motion in the frontal cortex, and then engages it to motor planning in M2, and emotional information processing in the ACC and amygdala. Future studies targeting the brain circuits might reveal the convergence of perception of bodily motion derived from different brain structures acquired during the evolution of different species.

Our data suggested the innate sensitivity of mice to bodily motion information; however, this result must be carefully considered by examining whether we can extend this finding to social cognition. In humans, researchers have shown the linkages between BM detection and social skills by analyzing patients with social deficits (Blake and Shiffrar, 2007). A previous study demonstrated the impaired BM discrimination of rats with social deficits, such as an autism model (Foley et al., 2012). The researchers employed a discrimination learning procedure, not providing additional evidence of the impaired sensitivity toward BM. In other words, additional experiments are required to determine whether the behavioral bias toward SM displays is impaired in mice with social deficits, such as the ASD model.

Recent translational studies using mice have demonstrated the neural and genetic basis of social deficits, such as ASD (as reviewed by Provenzano et al., 2012). Mice are still useful for studies of its visual domain, because transgenic and knockout models of this disorder can be generated easily (Pinto and Enroth-Cugell, 2000). Studies using ASD model mice might be able to support the relationship between their responses to BM and other social behaviors. Aberrant sociality in mice is basically assessed by focusing orienting behaviors toward other individuals. Although a number of genetic mutations and interferences during early neurodevelopment induce ASD-like abnormal behaviors, it is not well known whether these alternations are associated with domain-general sociability or modality-dependent social behaviors. The results of this study provide a new approach toward elucidating the neural and genetic basis of the entire

social behavior of mice from the complex background cognitive capacity.

CONCLUSION

The mice modulated their orienting behavior depending on the BM displays showing a conspecific walking as depicted by simple geometric dots. There, however, remains a possibility that our control stimuli were not the best to test the perception of BM in mice. In this study, we examined their responses toward displays of walking-actions and of walking-actions with the initial dot positions rearranged. Future study should test the action-dependent attractiveness of BMs. To tackle the current topic is expected to improve our knowledge of socially relevant visual processing in the mammalian brain and might contribute to therapeutic screening for social communication deficits derived from altered visual recognition of bodily motion.

AUTHOR CONTRIBUTIONS

TA, MI, and MW conceived and designed the experiments. TA, MI, and MW performed the experiments. TA analyzed the data and wrote the manuscript. All authors approved the final version and agreed to be accountable for all aspects of the work.

FUNDING

This work was supported by JSPS Kakenhi (JP26700012, 17H01757, JP23300151, JP18J01787, and JP18K18705).

ACKNOWLEDGMENTS

We thank Taemi Nawa for looking after the animals and providing technical assistance and Dr. Mrinmoy Chakrabarty for providing valuable and insightful suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00263/full#supplementary-material

Video S1 | An example of Biological motion movie clip.

Video S2 | An example of Scrambled motion movie clip.

REFERENCES

Allison, T., Puce, A., and McCarthy, G. (2000). Social perception from visual cues: role of the STS region. *Trends Cogn. Sci.* 4, 267–278. doi: 10.1016/S1364-6613(00)01501-1

Blake, R. (1993). Cats perceive biological motion. *Psychol. Sci.* 4, 54–57. doi: 10.1111/j.1467-9280.1993.tb00557.x

Blake, R., and Shiffrar, M. (2007). Perception of human motion. *Annu. Rev. Psychol.* 58, 47–73. doi: 10.1146/annurev.psych.57.102904.190152

Blake, R., Turner, L. M., Smoski, M. J., Pozdol, S. L., and Stone, W. L. (2003). Visual recognition of biological motion is impaired in children with autism. *Psychol. Sci.* 14, 151–157. doi: 10.1111/1467-9280.01434

Brainard, D. (1997). The psychophysics toolbox. *Spat. Vis.* 10, 433–436. doi: 10.1163/156856897X00357

Brown, J., Kaplan, G., Rogers, L. J., and Vallortigara, G. (2010). Perception of biological motion in common marmosets (*Callithrix jacchus*): by females only. *Anim. Cogn.* 13, 555–564. doi: 10.1007/s10071-009-0306-0

Burkett, J. P., Andari, E.,Johnson, Z. V., Curry, D. C., de Waal, F. B. M., and Young, L. J. (2016). Oxytocin-dependent consolation behavior in rodents. *Science* 351, 375–378. doi: 10.1126/science.aac4785

De Franceschi, G., Vivattanasarn, T., Saleem, A. B., and Solomon, S. G. (2016). Vision guides selection of freeze or flight defense strategies in mice. *Curr. Biol.* 26, 2150–2154. doi: 10.1016/j.cub.2016.06.006

Dittrich, W., Lea, S., Barrett, J., and Gurr, P. (1998). Categorization of natural movements by pigeons: visual concept discrimination and biological motion. *J. Exp. Anal. Behav.* 70, 281–299. doi: 10.1901/jeab.1998.70-281

Douglas, R. M., Neve, A., Quittenbaum, J. P., Alam, N. M., and Prusky, G. T. (2006). Perception of visual motion coherence by rats and mice. *Vis. Res.* 46, 2842–2847. doi: 10.1016/j.visres.2006.02.025

Dräger, U. C. (1978). Observations on monocular deprivation in mice. *J. Neurophysiol.* 41, 28–42. doi: 10.1152/jn.1978.41.1.28

Foley, A. G., Gannon, S., Rombach-Mullan, N., Prendergast, A., Barry, C., Cassidy, A. W., et al. (2012). Class I histone deacetylase inhibition ameliorates social cognition and cell adhesion molecule plasticity deficits in a rodent model of autism spectrum disorder. *Neuropharmacology* 63, 750–760. doi: 10.1016/j.neuropharm.2012.05.042

Gremel, C. M., and Costa, R. M. (2013). Premotor cortex is critical for goal-directed actions. *Front. Comput. Neurosci.* 7:110. doi: 10.3389/fncom.2013.00110

Grossman, E., Donnelly, M., Price, R., Pickens, D., Morgan, V., Neighbor, G., et al. (2000). Brain areas involved in perception of biological motion. *J. Cogn. Neurosci.* 12, 711–720. doi: 10.1162/089892900562417

Grossman, E. D., and Blake, R. (2001). Brain activity evoked by inverted and imagined biological motion. *Vis Res.* 41, 1475–1482. doi: 10.1016/S0042-6989(00)00317-5

Grossman, E. D., and Blake, R. (2002). Brain areas active during visual perception of biological motion. *Neuron* 35, 1167–1175. doi: 10.1016/S0896-6273(02)00897-8

Han, H. B., Hwang, E., Lee, S., Kim, M. S., and Choi, J. H. (2017). Gamma-band activities in mouse frontal and visual cortex induced by coherent dot motion. *Sci. Rep.* 7:43780. doi: 10.1038/srep43780

Happé, F., Cook, J. L., and Bird, G. (2017). The structure of social cognition: In(ter)dependence of sociocognitive processes. *Annu. Rev. Psychol.* 68, 243–267. doi: 10.1146/annurev-psych-010416-044046

Herman, L. M., Morrel-Samuels, P., and Pack, A. A. (1990). Bottlenose dolphin and human recognition of veridical and degraded video displays of an artificial gestural language. *J. Exp. Psychol. Gen.* 119, 215–230. doi: 10.1037/0096-3445.119.2.215

Jeon, D., Kim, S., Chetana, M., Jo, D., Ruley, H. E., Lin, S., et al. (2010). Observational fear learning involves affective pain system and Cav1.2 Ca2+ channels in ACC. *Nat. Neurosci.* 13, 482–488. doi: 10.1038/nn.2504

Johansson, G. (1973). Visual perception of biological motion and a model for its analysis. *Atten. Percept. Psycho.* 14, 201–211. doi: 10.3758/BF03212378

Jokisch, D., Daum, I., Suchan, B., and Troje, N. F. (2005). Structural encoding and recognition of biological motion: evidence from event-related potentials and source analysis. *Behav. Brain Res.* 157, 195–204. doi: 10.1016/j.bbr.2004.06.025

Kéri, S., and Benedek, G. (2009). Oxytocin enhances the perception of biological motion in humans. *Cogn. Affect. Behav. Neurosci.* 9, 237–241. doi: 10.3758/CABN.9.3.237

Klin, A., Lin, D. J., Gorrindo, P., Ramsay, G., and Jones, W. (2009). Two-year-olds with autism orient to non-social contingencies rather than biological motion. *Nature* 459, 257–261. doi: 10.1038/nature07868

Koldewyn, K., Whitney, D., and Rivera, S. M. (2010). The psychophysics of visual motion and global form processing in autism. *Brain* 133, 599–610. doi: 10.1093/brain/awp272

Kovács, K., Kis, A., Kanizsár, O., Hernádi, A., Gácsi, M., and Topál, J. (2016). The effect of oxytocin on biological motion perception in dogs (*Canis familiaris*). *Anim. Cogn*. 19, 1–10. doi: 10.1007/s10071-015-0951-4

Langford, D. J., Bailey, A. L., Chanda, M. L., Clarke, S. E., Drummond, T. E., Echols, S., et al. (2010). Coding of facial expressions of pain in the laboratory mouse. *Nat. Methods* 7, 447–449. doi: 10.1038/nmeth.1455

Langford, D. J., Crager, S. E., Shehzad, Z., Smith, S. B., Sotocinal, S. G., Levenstadt, J. S., et al. (2006). Social modulation of pain as evidence for empathy in mice. *Science* 312, 1967–1970. doi: 10.1126/science.1128322

MacKinnon, L. M., Troje, N. F., and Dringenberg, H. C. (2010). Do rats (*Rattus norvegicus*) perceive biological motion? *Exp. Brain Res*. 205, 571–576. doi: 10.1007/s00221-010-2378-0

Miura, M., and Matsushima, T. (2016). Biological motion facilitates filial imprinting. *Anim. Behav*. 116, 171–180. doi: 10.1016/j.anbehav.2016.03.025

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., et al. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes Brain Behav*. 3, 287–302. doi: 10.1111/j.1601-1848.2004.00076.x

Nakayasu, T., and Watanabe, E. (2014). Biological motion stimuli are attractive to medaka fish. *Anim. Cogn.* 17, 559–575. doi: 10.1007/s10071-013-0687-y

Niell, C. M., and Stryker, M. P. (2008). Highly selective receptive fields in mouse visual cortex. *J. Neurosci*. 28, 7520–7536. doi: 10.1523/JNEUROSCI.0623-08.2008

Niell, C. M., and Stryker, M. P. (2010). Modulation of visual responses by behavioral state in mouse visual cortex. *Neuron* 65, 472–479. doi: 10.1016/j.neuron.2010.01.033

Oram, M. W., and Perrett, D. I. (1994). Responses of anterior superior temporal polysensory (STPa) neurons to "biological motion" stimuli. *J. Cogn. Neurosci*. 6, 99–116.

Parron, C., Deruelle, C., and Fagot, J. (2007). Processing of biological motion point-light displays by baboons (*Papio papio*). *J. Exp. Psychol. Anim. B* 33, 381–391. doi: 10.1037/0097-7403.33.4.381

Pavlova, M. A. (2012). Biological motion processing as a hallmark of social cognition. *Cereb. Cortex* 22, 981–995. doi: 10.1093/cercor/bhr156

Peelen, M. V., Wiggett, A. J., and Downing, P. E. (2006). Patterns of fMRI activity dissociate overlapping functional brain areas that respond to biological motion. *Neuron* 49, 815–822. doi: 10.1016/j.neuron.2006.02.004

Pelli, D. (1997). The videotoolbox software for visual psychophysics: transforming numbers into movies. *Spat. Vis.* 10, 437–442. doi: 10.1163/156856897X00366

Peuskens, H., Vanrie, J., Verfaillie, K., and Orban, G. A. (2005). Specificity of regions processing biological motion. *Eur. J. Neurosci*. 21, 2864–2875. doi: 10.1111/j.1460-9568.2005.04106.x

Pinto, L. H., and Enroth-Cugell, C. (2000). Tests of the mouse visual system. *Mamm. Genome* 11, 531–536. doi: 10.1007/s003350010102

Provenzano, G., Zunino, G., Genovesi, S., Sgadó, P., and Bozzi, Y. (2012). Mutant mouse models of autism spectrum disorders. *Dis. Markers* 33, 225–239. doi: 10.1155/2012/916082

Prusky, G. T., and Douglas, R. M. (2004). Characterization of mouse cortical spatial vision. *Vis. Res*. 44, 3411–3418. doi: 10.1016/j.visres.2004.09.001

Prusky, G. T., West, P. W., and Douglas, R. M. (2000). Behavioral assessment of visual acuity in mice and rats. *Vis. Res*. 40, 2201–2209. doi: 10.1016/S0042-6989(00)00081-X

Regolin, L., Tommasi, L., and Vallortigara, G. (2000). Visual perception of biological motion in newly hatched chicks as revealed by an imprinting procedure. *Anim. Cogn* 3, 53–60. doi: 10.1007/s100710050050

Rubinstein, M., Phillips, T. J., Bunzow, J. R., Falzone, T. L., Dziewczapolski, G., Zhang, G., et al. (1997). Mice lacking dopamine D4 receptors are supersensitive to ethanol, cocaine, and methamphetamine. *Cell* 90, 991–1001. doi: 10.1016/S0092-8674(00)80365-7

Saygin, A. P. (2007). Superior temporal and premotor brain areas necessary for biological motion perception. *Brain* 130, 2452–2461. doi: 10.1093/brain/awm162

Silverman, J. L., Yang, M., Lord, C., and Crawley, J. N. (2010). Behavioural phenotyping assays for mouse models of autism. *Nat. Rev. Neurosci*. 11, 490–502. doi: 10.1038/nrn2851

Simion, F., Regolin, L., and Bulf, H. (2008). A predisposition for biological motion in the newborn baby. *Proc. Natl Acad. Sci. USA*. 105, 809–813. doi: 10.1073/pnas.0707021105

Simon, P., Dupuis, R., and Costentin, J. (1994). Thigmotaxis as an index of anxiety in mice. *Influence of dopaminergic transmissions. Behav. Brain Res*. 61, 59–64. doi: 10.1016/0166-4328(94)90008-6

Troje, N. F. (2013). "What is biological motion? Definition, stimuli, and paradigms," in: *Social Perception* , eds M. D. Rutherford and V. A. Kuhlmeier (Cambridge: The MIT Press), 13–36.

Vallortigara, G., and Regolin, L. (2006). Gravity bias in the interpretation of biological motion by inexperienced chicks. *Curr. Biol*. 16, R279–R280. doi: 10.1016/j.cub.2006.03.052

Vallortigara, G., Regolin, L., and Marconato, F. (2005). Visually inexperienced chicks exhibit spontaneous preference for biological motion patterns. *PLoS Biol*. 3:e208. doi: 10.1371/journal.pbio.0030208

Vangeneugden, J., Vancleef, K., Jaeggli, T., Vangool, L., and Vogels, R. (2010). Discrimination of locomotion direction in impoverished displays of walkers by macaque monkeys. *J. Vis.* 1022, 1–19. doi: 10.1167/10.4.22

Watanabe, S., Shinozuka, K., and Kikusui, T. (2016). Preference for and discrimination of videos of conspecific social behavior in mice. *Anim. Cogn.* 19, 523–531. doi: 10.1007/s10071-016-0953-x

Yamamoto, E., Goto, K., and Watanabe, S. (2015). Pigeons (*Columba livia*) fail to connect dots in learning biological motion. *Anim. Cogn.* 18, 1187–1191. doi: 10.1007/s10071-015-0880-2

Yu, Y. Q., Barry, D. M., Hao, Y., Liu,. X. T., and Chen, Z. F. (2017). Molecular and neural basis of contagious itch behavior in mice. *Science* 355, 1072–1076. doi: 10.1126/science.aak9748

Conflict of Interest Statement: 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: 20 November 2018
doi: 10.3389/fnbeh.2018.00272

Oxytocin Manipulation Alters Neural Activity in Response to Social Stimuli in Eusocial Naked Mole-Rats

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The social decision-making network (SDMN) is a conserved neural circuit that modulates a range of social behaviors via context-specific patterns of activation that may be controlled in part by oxytocinergic signaling. We have previously characterized oxytocin's (OT) influence on prosociality in the naked mole-rat, a eusocial mammalian species, and its altered neural distribution between animals of differing social status. Here, we asked two questions: (1) do patterns of activation in the SDMN vary by social context and (2) is functional connectivity of the SDMN altered by OT manipulation? Adult subordinate naked mole-rats were exposed to one of three types of stimuli (three behavioral paradigms: familiar adult conspecific, unfamiliar adult conspecific, or familiar pups) while manipulating OT (three manipulations: saline, OT, or OT antagonist). Immediate early gene c-Fos activity was quantified using immunohistochemistry across SDMN regions. Network analyses indicated that the SDMN is conserved in naked mole-rats and functions in a context-dependent manner. Specific brain regions were recruited with each behavioral paradigm suggesting a role for the nucleus accumbens in social valence and sociosexual interaction, the prefrontal cortex in assessing/establishing social dominance, and the hippocampus in pup recognition. Furthermore, while OT manipulation was generally disruptive to coordinated neural activity, the specific effects were context-dependent supporting the hypothesis that oxytocinergic signaling promotes context appropriate social behaviors by modulating co-ordinated activity of the SDMN.

Keywords: social decision-making network, oxytocin, naked mole-rat, eusociality, immediate early gene, social behavior

OPEN ACCESS

Edited by:
James P. Curley, The University of Texas at Austin, United States

Reviewed by:
Kevin Donald Broad, University College London, United Kingdom
David A. Freeman, The University of Memphis, United States

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Received: *12 July 2018*
Accepted: *23 October 2018*
Published: *20 November 2018*

Citation:
Faykoo-Martinez M, Mooney SJ and Holmes MM (2018) Oxytocin Manipulation Alters Neural Activity in Response to Social Stimuli in Eusocial Naked Mole-Rats. Front. Behav. Neurosci. 12:272. doi: 10.3389/fnbeh.2018.00272

Abbreviations: ACC, anterior cingulate cortex; AH, anterior hypothalamus; AntPVN, anterior paraventricular nucleus; AON, anterior olfactory nucleus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; Caudate, caudate putamen; CeA, central amygdala; CG, cingulate cortex; dDG, dorsal dentate gyrus; IL, infralimbic cortex; LS, lateral septum; MeA, medial amygdala; MOB, main olfactory bulb; MS, medial septum; NAcc, nucleus accumbens; PG, periaqueductal gray; PIC, piriform cortex; PO, pre-optic area; PostPVN, posterior paraventricular nucleus; PrL, pre-limbic cortex; SON, supraoptic nucleus; Tu, olfactory tubercle; vDG, ventral dentate gyrus; VMH, ventromedial hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area.

INTRODUCTION

The social decision-making network (SDMN) is a highly conserved interconnected group of brain regions controlling behaviors related to sex, social dominance, parenting, and affiliation across vertebrates (O'Connell and Hofmann, 2011, 2012). Originally, the network was described by linking the social behavior network (adjacent tegmentum, AH, BNST, MeA, LS, PG, PO, and VMH) and the mesolimbic reward circuit (BLA, BNST, caudate, hippocampus, LS, NAcc, VP, and VTA) (Newman, 1999; O'Connell and Hofmann, 2011). A case can also be made for affiliated nodes such as the olfactory regions (AON, MOB, and Tu) and the medial prefrontal cortex (ACC, CG, IL, and PrL) due to their role in social cognition (Brennan and Kendrick, 2006; Tobin et al., 2010; Lee and Harris, 2013; Nakajima et al., 2014; Bicks et al., 2015; Toor et al., 2015; Oettl et al., 2016; Williamson et al., 2018). Collectively, these regions interact to coordinate incoming social information with context appropriate social responses. Indeed, context dependent behavioral plasticity is likely attributed to changes in coordinated neural activity between nodes of the network, rather than to differential activity of individual brain regions *per se* (Goodson and Kabelik, 2009; Teles et al., 2015; Johnson et al., 2016).

Function of the SDMN is influenced by oxytocin (OT). OT is a neuropeptide implicated in both social (e.g., maternal care, affiliation, and stress) and sexual (e.g., arousal, ejaculation, and motivation) behaviors across vertebrate species (O'Connell and Hofmann, 2012; Anacker and Beery, 2013). For example, manipulation of OT signaling alters multiple social behaviors including prosociality/aggression, social recognition, short-term social memory, alloparenting, and pup care (Ferguson et al., 2000, 2001; Francis et al., 2000; Champagne et al., 2001; Consiglio et al., 2005; Olazábal and Young, 2006a,b; Choleris et al., 2007; Beery et al., 2008; Reddon et al., 2014; Chang et al., 2015). Furthermore, variability in distribution patterns of OT receptors in SDMN regions suggests that oxytocinergic signaling contributes to species-specific adaptations in social behavior (reviewed in Anacker and Beery, 2013). Finally, both central and site-specific (NAcc) manipulation of OT receptor signaling disrupts coordinated activity among SDMN regions (Johnson et al., 2016, 2017). Thus, OT is a key mechanism for sculpting social behavior within and between species, prospectively through facilitating context-specific changes in coordinated activity between nodes of the SDMN.

The naked mole-rat (*Heterocephalus glaber*) exhibits the most extreme form of sociality known in mammals: eusociality. These small, approximately mouse-sized, rodents live in large colonies of up to ∼300 animals with reproduction restricted to one breeding female, the queen, and one to three males (Brett, 1991b; Lacey and Sherman, 1991). The rest of the colony consists of non-breeding subordinates of varying age, which are highly social toward members of their own colony. In contrast, naked mole-rats can be very xenophobic and highly aggressive toward intruding members of another colony (Lacey and Sherman, 1991). Colony members engage in diverse behaviors such as foraging and food-sharing, cooperative care of pups, vocal communication, communal huddling, and colony maintenance and defense (Withers and Jarvis, 1980; Jarvis, 1981; Brett, 1991a; Pepper et al., 1991). There is individual variability in performance of these behaviors, resulting in stable yet plastic task specialization (Jarvis, 1981; Lacey and Sherman, 1991; Mooney et al., 2015b). Subordinates have been further split into two subcastes: workers and soldiers, responsible for colony maintenance and colony defense, respectively (Jarvis, 1981; Lacey and Sherman, 1991; Mooney et al., 2015b).

The oxytocinergic system contributes to the remarkable sociality found in naked mole-rats. Subordinates of both sexes have more OT neurons than breeders in the PVN (Mooney and Holmes, 2013) and peripheral administration of OT to subordinates increases prosocial behaviors in-colony (huddling) and during unfamiliar conspecific interaction tests (proximity, investigation) (Mooney et al., 2014). Among subordinates, workers have higher levels of c-Fos/OT immunoreactive neurons than soldiers in the PVN, accompanied with lower levels of aggression, further suggesting OT promotes prosocial behavior in workers (Hathaway et al., 2016). Naked mole-rats also express OT receptors in the SDMN. They have more OT receptors in the CeA, MeA, BNST, and NAcc in comparison to solitary cape mole-rats, suggesting that OT action in these regions is associated with colonial living (Kalamatianos et al., 2010). Furthermore, OT receptor density varies within naked mole-rats with breeding males showing higher binding than breeding females in the NAcc and males overall showing higher binding than females in the MeA (Kalamatianos et al., 2010; Mooney et al., 2015a).

We hypothesize that OT signaling mediates coordinated neural activity within nodes of the SDMN to promote context-specific social behavior in naked mole-rats. To test this, we had two experimental goals: (1) to determine if coordinated neural activity in the SDMN varies according to social context and (2) to examine if activity of SDMN regions is altered by central OT manipulation. To achieve these goals, we exposed subordinate naked mole-rats of both sexes to three unique social stimuli: a familiar adult conspecific, an unfamiliar adult conspecific, or 1-week-old pups from their home colony while treating with either saline, OT, or an OT antagonist (OTA). Following social exposure, we used immunohistochemistry to stain for the immediate early gene c-Fos to assess activation of brain regions in the SDMN. We predicted that coordinated c-Fos expression within the SDMN regions would differ by social context, and that manipulation of central OT receptor signaling would disrupt these patterns of connectivity.

MATERIALS AND METHODS

Animals and Housing

A total of 75 adult subordinate naked mole-rats were used (32 females and 43 males). Animals were considered adults if they were both over 20 g in weight and over 1-year old. Animals weighed between 22 and 60 g were housed on a 12:12 light/dark cycle at 28–30°C and given *ad libitum* access to sweet potato and a wet 19% protein mash (Harlan Laboratories, Inc.). Animals lived in colonies comprised of large (45.75 cm L × 24 cm W × 15.25 cm H) and small (30 cm L × 18 cm

W × 13 cm H) polycarbonate cages connected by plastic tubes (25 cm L × 5 cm D). Animals were collected from one of nine colonies ranging in size from 19 to 49 individuals. All procedures adhered to federal and institutional guidelines and were approved by the University Animal Care Committee. A summary of the experimental workflow is presented in **Figure 1**.

Intracerebroventricular Cannulation

Experimental animals were implanted with intracerebroventricular cannulae targeting the lateral ventricle as previously described (Mooney and Holmes, 2015). Briefly, mole-rats were deeply anesthetized using isoflurane (induction: 3%, delivered at a rate of 1 L/min inhalation; maintenance: 2%, delivered at a rate of 1 L/min) and the surgical site was cleaned and sterilized with 70% EtOH and then 10% iodine solution (Betadine; repeated twice). Animals were positioned in a stereotaxic instrument (Benchmark^{TM}, MyNeurolab.com, St. Louis, MO, United States) and a 1.5 cm incision was made on the top of the head. The skin and muscle were shifted to reveal the skull, which was then cleaned with sterile saline and dried. A small hole was drilled in the skull 0.9 mm lateral and 1 mm anterior to bregma on the right side of the animal and a 22-gauge stainless-steel guide cannula with a 2 mm pedestal (Plastics One, Roanoke, VA, United States) was lowered to 3 mm below the top of the skull. Cyanoacrylate gel was applied to the base of the pedestal to secure the guide cannula to the top of the skull. A dummy cannula was inserted in the guide cannula to prevent exposure, infection, or occlusion. The muscle was then laid back on the skull around the cannula and the skin was sutured together over the pedestal of the guide cannula. A small dab of cyanoacrylate gel was placed on the outside of the dummy cannula at the juncture where the pedestal of the guide cannula and the cap of the dummy cannula meet. Ten minutes prior to the completion of surgery, animals were injected with ketoprofen (Anafen®, Merial; 5 mg/kg BW). This injection was also administered once a day for 3 days post-surgery. Animals were placed on top of a heating pad in a cage with clean bedding for 8 h for recovery before being returned to their home colonies.

Social Behavior Paradigms

Familiar Conspecific Interaction Tests

Twenty-one experimental animals (FAM; 9 females and 12 males) were removed from their colony and individually placed in a clear polycarbonate cage (L 43 × W 22 × H 21 cm) lined with corncob bedding. After a 20-min habituation period, an adult conspecific from the experimental animal's home colony (same-sex pairing, N = 12, opposite-sex pairing, N = 10; sex matching randomly assigned) was placed in the cage in the corner farthest away from each experimental animal's current position. Each animal's behavior was recorded for 20 min with a Sony Handycam®. All animals were then returned to their home colony. Twenty-four hours after the baseline test, animals were again removed from their home colony and placed in a clear polycarbonate cage for 10 min before receiving intracerebroventricular infusions of either OT, an OTA, or saline. Seven animals received 0.25 μg of OT (OT acetate salt hydrate or α-hypophamine, No. O6379; Sigma), seven animals received 1 ng of the specific OTA (d(CH2)51,Tyr(Me)2,Thr4,Orn8,des-Gly-NH29)-Vasotocin, No. H-2908 BACHEM), and seven animals received 1 μl of the sterile saline. These doses were chosen as they produce behavioral effects in similarly sized mammals (Ferguson et al., 2000; Shahrokh et al., 2010; Samuelsen and Meredith, 2011). For infusion, animals were lightly anesthetized with isoflurane (2% delivered at a rate of 1 L/min) and the dummy cannula was replaced with an internal infusion cannula that sat 0.1 mm below the base of the guide cannula. One microliter of the drug or saline was infused via 500-μl Bas gas-tight syringes (MD-0050; Bio Analytical Systems) connected to the internal cannula with PE50 tubing. Infusions were automated at a rate of 1 μl/min with a Harvard infusion pump (Harvard Apparatus Inc. 22, Natick, MA, United States). Animals were then removed from anesthesia and recovered in ∼2–3 min. After 10 additional minutes, a novel stimulus animal was introduced following the same procedure as for the baseline test. Target behaviors for the baseline test and the manipulation test (**Table 1**) were scored by an observer blind to the experimental condition using Observer XT software (Noldus).

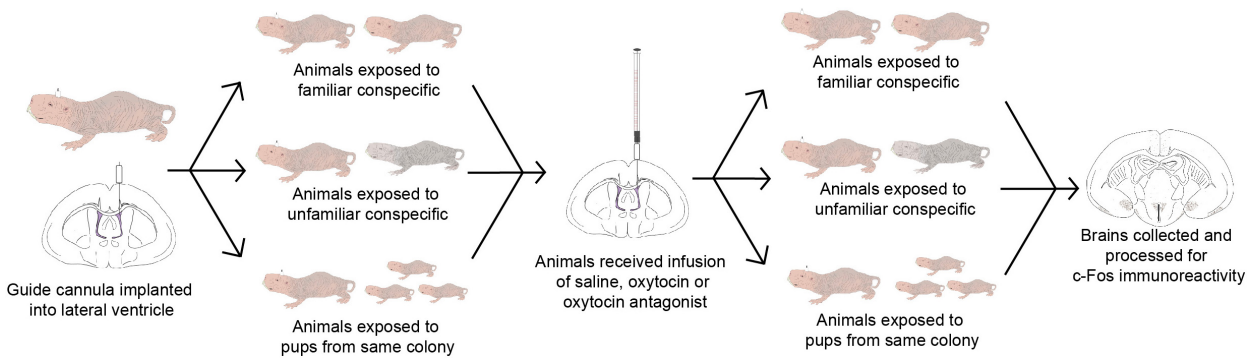

FIGURE 1 | Timeline of the experimental workflow. Animals were implanted with a guide cannula and given 7 days for recovery. On the 8th day, animals were exposed to either a familiar conspecific (n = 21), an unfamiliar conspecific (n = 30), or 3 pups from the same colony (n = 21). On the 9th day, animals were given an infusion of saline, OT, or OTA, and again exposed to either a familiar conspecific (n = 7 per OT manipulation), unfamiliar conspecific (n = 10 per OT manipulation), or 3 pups from the same colony (n = 7 per OT manipulation). Brains were collected 90 min following exposure to stimuli and processed for c-Fos immunoreactivity.

TABLE 1 | Behaviors of interest.

Behavior	Description
Aggression	Duration of physical attack on stimulus and stand-off with stimulus with teeth barred
Anogenital investigation	Duration of direct sniffing of the stimulus animal in anogenital region (sexual behavior)
Investigation (flank/face)	Duration of direct sniffing of the stimulus animal (aggressive)
Pup carrying	Duration of time with pup between the experimental animal's incisors
Pup investigation	Duration of time with the experimental animal's snout directed toward pup but without pup in the animal's incisors

Aggression, anogenital investigation, and investigation (flank/face) were scored in animals interacting with a familiar or unfamiliar conspecific. Pup carrying and pup investigation were scored in animals interacting with pups.

Unfamiliar Conspecific Interaction Tests

Thirty experimental animals (UNFAM; 15 females and 15 males) were used in this paradigm. All procedures and manipulations were identical to the familiar conspecific paradigm described above with the exception of the stimulus animal. In this case, an adult conspecific from an *unfamiliar* colony was used as the stimulus (same-sex pairing, $N = 12$, opposite-sex pairing, $N = 10$; sex matching randomly assigned). Ten animals received OT, 10 animals received OTA, and 10 animals received sterile saline.

Pup Interaction Tests

Twenty-four experimental animals (PUP; 8 females and 16 males) were used in this paradigm. Again, procedures and manipulations were identical to the FAM paradigm with the exception of the social stimulus. In this case, three pups (∼1 week old) from the experimental animal's home colony were placed in the cage in the corner farthest away from each animal's current position. Eight animals received OT, nine animals received OTA, and seven animals received sterile saline.

Tissue Collection and c-Fos Immunohistochemistry

One hundred minutes after the start of each interaction test (FAM, UNFAM, PUP) in which OT activity was manipulated, animals were overdosed with avertin (400 mg/kg) and rapidly decapitated. Brains were extracted and post-fixed in 4% paraformaldehyde for 4 h before being transferred to sucrose [30% in phosphate-buffered saline (PBS)] and stored for at least 24 h at 4°C. Brains were sliced coronally at 30 μm into four series on a freezing microtome. One series was stained for c-Fos immunoreactivity. Tissue was washed for 15 min (3 × 5 min) in PBS, followed by a 90-min incubation in blocking solution at room temperature [4% NGS, 0.3% TritonX, 3% H_2O_2 (3%) in PBS]. Tissue was then rinsed in PBS for 15 min (3 × 5 min) and incubated in c-Fos primary antibody [1:1200 rabbit anti-c-Fos polyclonal antibody (Santa Cruz Biotechnology) in PBS with 4% NGS, 0.3% TritonX] at 4°C for approximately 24 h. Tissue was rinsed in PBS for 15 min (3 × 5 min) and then incubated for 90 min at room temperature in a secondary antibody solution [1:200 goat anti-rabbit (Vector Laboratories) in PBS with 0.3% TritonX and 2% NGS]. Tissue was rinsed again in PBS for 15 min (3 × 5 min) and then incubated at room temperature for 90 min in avidin–biotin complex (ABC Elite, Vector Laboratories). Sections were washed again for 15 min (3 × 5 min) in PBS, and c-Fos was visualized using nickel-enhanced 3-3′-diaminobenzidine (DAB) for 3 min [2% DAB (1.25%), 0.2% H_2O_2 (3%), 0.24% NiCl (8%) in PBS]. Tissue was then mounted onto slides coated in pig gelatin, dehydrated, and coverslipped with Permount (Fisher Scientific). Experimental groups were yoked across staining cohorts.

c-Fos Quantification

The number of c-Fos immunoreactive cells was counted using either OpenCFU (Beta version 3.9.0) or ImageJ (Rasband, 2012; Geissmann, 2013). For a given brain region, counts for all animals were performed using the same program. As tissue tearing and damage was more likely on the side of the brain ipsilateral to the cannula implantation, counts were done unilaterally on the side contralateral to the implantation. On OpenCFU, this was done automatically with a threshold setting of 5 and a radius setting of 5. Counts were verified by an observer blind to the experimental condition for all sections. Areas counted using OpenCFU were the AH, BLA, BNST, CeA, LS, MeA, MS, NAcc, PG, PO, PVN, SON, and VMH. Using ImageJ, images were converted to 8-bit, a threshold of 180–190 applied, and particles larger than 10 pixels^2 counted. Areas counted using ImageJ were the ACC, AON, CA1, CA2, CA3, caudate, CG, dDG, IL, MOB, PIC, PrL, Tu, vDG, VP, and VTA. For each region of interest, a photomicrograph was taken on three consecutive slices of tissue using a microscope mounted camera at 200× magnification. The CA1, CA2, CA3, dDG, and vDG were taken at 400× magnification to prevent counting other surrounding regions. Photomicrographs contained the same area for all animals, corresponding to the given region; all c-Fos labeled cells in the photomicrograph were counted. The exceptions to this were the BNST, which only had pictures taken from two consecutive slices because only two sections reliably contained the BNST in each series, and the PAG and VTA, which only had one slice counted because more caudal sections were not collected during slicing.

Placement Confirmation

Before euthanizing animals, one saline-treated animal from each behavioral paradigm was also infused with 1 μl of india ink (10% v/v) in order to confirm that fluid was diffusing throughout the brain. For all animals, tissue was examined to ensure that the cannula clearly penetrated the lateral ventricle. Because we could not definitively confirm penetration of the ventricles in two FAM animals (one receiving saline and one receiving OTA), two UNFAM animals (both receiving OT), and three PUP animals (one receiving saline, one receiving OT, and one receiving OTA), these animals were excluded from analyses. Final sample sizes are reported in the section "Social behavior paradigms."

Statistical Analyses

All statistical analyses were performed on raw counts of c-Fos immunoreactive cells. First, a linear mixed-effects model was run

using the nlme and lmerTest packages in R (R Development Core Team, 2011; Kuznetsova et al., 2016; Pinheiro et al., 2016). c-Fos immunoreactivity was the response variable and behavioral paradigm, OT manipulation, brain region, and sex were predictor variables, using animal ID and immunohistochemistry batch as random effects. The linear mixed-effects model was used to determine if any predictor variables could be collapsed for the rest of the analyses; due to non-significant effects, sex was dropped as a variable for subsequent analyses. We then performed brain region-specific linear mixed-effects models with c-Fos as the response variable, behavioral paradigm, and OT manipulation as predictor variables, and batch as a random effect, again using the nlme and lmerTest packages in R. A Bonferroni correction was used to adjust for multiple testing (30 tests = p-value set at 0.0017). Main effects reaching this criterion ($p < 0.0017$) were followed with Tukey's HSD *post hoc* tests.

We next explored coordinated activity between brain regions by examining correlations in c-Fos expression across all brain regions measured for each behavioral paradigm-by-OT manipulation group. The Hmisc package in R was used to perform pair-wise Pearson correlations for all brain regions, followed by visualization using the corrplot package (Wei and Simko, 2016; Harrell, 2017). No thresholding was applied, but significant correlations are marked by white asterisks on the plots. To further explore the effect of behavioral paradigm and OT manipulation on how c-Fos is expressed between regions, networks were produced for each behavioral paradigm-by-OT manipulation group. For the networks, correlation p-values were corrected using the Benjamini–Hochberg procedure at a 5% false-discovery rate. These correlations were then extracted and plotted using Cytoscape (Shannon et al., 2003). Cytoscape's built-in Network Analyzer tool was used to plot node size according to degree (how many edges correspond to a given node) and edges according to correlation strength. On the networks, nodes are clustered and color-coded based on anatomy and literature-based functions into the following: olfactory (AON, MOB, PIC, Tu), mPFC (ACC, CG, IL, PrL), social behavior network (AH, BNST, LS, MS, PG, PO, VMH), amygdala (BLA, CeA, MeA), reward (caudate, NAcc, VP, VTA), OT production (AntPVN, PostPVN, SON), and hippocampal (CA1, CA2, CA3, dDG, vDG).

Finally, to examine if OT manipulation affected behavior, we tested two linear mixed-effects models as described above. First, to test whether injection manipulation itself affected behavior (because animals were anesthetized for ICV injections), we modeled difference in behavior duration between baseline and test day as the response variable, treatment and sex as independent variables, and animal ID as the random effect variable. Second, to test whether behavior on the test day was altered by OT manipulation, we modeled duration of behavior on test day as the response variable, treatment and sex as independent variables, and animal ID as the random effect variable. Both models were repeated for each behavior tested per behavioral paradigm and corrected for multiple testing using the Bonferroni method (FAM/UNFAM: three comparisons = p-value < 0.017; PUP: eight comparisons = p-value < 0.0062).

Then, to determine if c-Fos expression is related to behavior, we clustered brain regions using factor analysis to reduce analyses performed: all saline-treated animals across paradigms were included using the dimension reduction function in SPSS (IBM Corp, 2016). Principal axis factoring with a Promax rotation was used for the unsupervised clustering of brain regions; four clusters (as listed in the section "Results") were produced. Next, for each paradigm (collapsed across OT manipulation), a Pearson correlation between the summed c-Fos counts for a given cluster and given behavior on test day was performed. For FAM and UNFAM animals, duration of anogenital investigation, face/flank investigation, and aggression was scored. For PUP animals, duration of pup carrying and pup interaction was scored. P-values were corrected for multiple testing using the Bonferroni method (FAM/UNFAM: 12 comparisons = p-value < 0.0042; PUP: 8 comparisons = p-value < 0.0062).

RESULTS

Brain Region-Specific Analyses Reveal OT Manipulation and Paradigm Main Effects

The LME including all brain regions revealed a significant main effect of behavioral paradigm [$F(2,57) = 4.8065$, $p = 0.0118$], OT manipulation [$F(2,57) = 6.3197$, $p = 0.0033$], and brain region [$F(29,1608) = 108.4381$, $p < 0.0001$], but not sex [$F(1,57) = 1.0054$, $p = 0.3203$]. Significant interactions were detected for behavioral paradigm-by-brain region [$F(58,1608) = 4.7031$, $p < 0.0001$] and OT manipulation-by-brain region [$F(58,1608) = 2.1290$, $p < 0.0001$], but not for sex-by-brain region [$F(29,1608) = 0.3893$, $p = 0.9986$].

For brain region-specific analyses, only results that were statistically significant after correcting for multiple testing (Bonferroni method) are reported here ($p < 0.0017$). All effects with $p < 0.05$ are shown in **Table 2** while raw data are available in the **Supplementary Table 1**. A main effect of behavioral paradigm [$F(2,61) = 7.61$, $p = 0.0011$; **Figure 2A**] revealed altered c-Fos expression in the MOB, with PUP animals having higher expression of c-Fos relative to UNFAM animals ($p = 0.002$). This same pattern was seen in the Tu [$F(2,61 = 10.9$, $p < 0.0001$; **Figure 2B**], with a significant difference between PUP and UNFAM animals ($p = 0.00012$) and a trend between FAM and UNFAM animals ($p = 0.059$). In the IL, a main effect of behavioral paradigm [$F(2,61) = 7.3$, $p = 0.0015$; **Figure 2C**] revealed reduced c-Fos in UNFAM animals relative to both FAM and PUP animals whereas in the VP [$F(2,63)$, $p = 0.0002$; **Figure 2D**], c-Fos was higher in FAM animals relative to both UNFAM and PUP groups. Main effects of OT manipulation revealed higher c-Fos expression in OTA-treated animals relative to both saline- and OT-treated animals in the PIC [$F(2,63) = 6.9$, $p = 0.00167$; **Figure 3A**], ACC [$F(2,61) = 7.4$, $p = 0.0014$; **Figure 3B**], and PrL [$F(2,61) = 8.3$, $p = 0.000862$; **Figure 3C**].

TABLE 2 | Brain region-specific linear mixed effect model results listed by brain region with significance value (Sig) and direction of effect (Dir).

	Region	Main effect of behavioral paradigm	Main effect of OT manipulation	Behavioral paradigm-by-OT manipulation interaction
AON	Sig.	$F(2,61) = 3.55$, $p = 0.0346$		
	Dir.	PUP > UNFAM*		
MOB	Sig.	$F(2,61) = 7.61$, $p = 0.0011$		
	Dir.	PUP > UNFAM*		
Tu	Sig.	$F(2,61) = 10.95$, $p = 0.0001$	$F(2,61) = 5.86$, $p = 0.005$ OTA > Saline	
	Dir.	FAM/PUP* > UNFAM	OTA > OT*	
PIC	Sig.		$F(2,63) = 6.94$, $p = 0.002$	
	Dir.		OTA > Saline/OT*	
ACC	Sig.		$F(2,63) = 7.37$, $p = 0.001$ OTA > Saline*	
	Dir.			
IL	Sig.	$F(2,61) = 7.29$, $p = 0.0015$	$F(2,61) = 5/64$, $p = 0.006$	
	Dir.	FAM/PUP > UNFAM*	OTA > OT*/Saline	
PrL	Sig.		$F(2,61) = 8.32$, $p = 0.0006$	$F(2,61) = 2.63$, $p = 0.043$ PUP Saline/FAM OTA/PUP OTA/UNFAM OTA > UNFAM Saline*
	Dir.		OTA > Saline/OT*	
CG	Sig.			$F(4,58) = 4.38$, $p = 0.004$ FAM OT > UNFAM Saline FAM OT*/UNFAM OTA > PUP OTA
	Dir.			
PO	Sig.	$F(2,63) = 4.72$, $p = 0.012$		
	Dir.	FAM > UNFAM*/PUP		
AH	Sig.	$F(2,63) = 3.87$, $p = 0.026$		
	Dir.	FAM > UNFAM*/PUP		
VMH	Sig.	$F(2,63) = 6.41$, $p = 0.003$	$F(2,63) = 3.44$, $p = 0.038$	
	Dir.	FAM > UNFAM*/PUP	OTA > OT*/Saline	
PG	Sig.	$F(2,63) = 4.01$, $p = 0.023$	$F(2,63) = 4.70$, $p = 0.012$	
	Dir.	FAM > UNFAM*	OTA > OT*/Saline	
BNST	Sig.	$F(2,63) = 3.81$, $p = 0.027$		
	Dir.	FAM > UNFAM*		
LS				
MS				
AntPVN				
PostPVN				
SON	Sig.	$F(2,63) = 6.67$, $p = 0.002$	$F(2,61) = 6.80$, $p = 0.002$ OTA > Saline*	$F(4,63) = 3.83$, $p = 0.008$ FAM OT/FAM OTA/PUP Saline/PUP OT/Saline/PUP OTA/UNFAM OTA > UNFAM Saline* UNFAM OTA > UNFAM OT
	Dir.	FAM > UNFAM*/PUP*	OT > Saline	
MeA	Sig.		$F(2,61) = 4.83$, $p = 0.011$	
	Dir.		OTA > Saline/OT*	
BLA	Sig.		$F(2,63) = 3.55$, $p = 0.035$ OTA > OT*	
	Dir.			
CEA	Sig.	$F(2,63) = 3.15$, $p = 0.050$	$F(2,63) = 3.53$, $p = 0.035$	
	Dir.	FAM > UNFAM*/PUP	OTA > Saline*/OT	
Caudate				
VP	Sig.	$F(2,63) = 9.57$, $p = 0.0002$		
	Dir.	FAM > UNFAM/PUP*		
NAcc	Sig.		$F(2,63) = 3.60$, $p = 0.033$ OTA > OT/Saline	
	Dir.			
VTA				
CA1				
CA2				
CA3				
dDG				
vDG	Sig.	$F(2,57) = 3.42$, $p = 0.039$	$F(2,57) = 4.34$, $p = 0.018$ OTA > OT*	$F(4,57) = 3.67$, $p = 0.010$ PUP Saline > PUP OT*/UNFAM OT FAM OT*/FAM OTA*/UNFAM OTA > PUP OT FAM OTA > UNFAM OT*
	Dir.	FAM > UNFAM*		

All main effects with $p < 0.05$ are shown; however, only those with $p < 0.0017$ are considered significant following Bonferroni multiple testing correction. Significant post hoc effects ($p < 0.05$) are indicated with an asterisk (*). AON, anterior olfactory nucleus; MOB, main olfactory bulb; Tu, olfactory tubercle; PIC, piriform cortex; ACC, anterior cingulate cortex; IL, infralimbic cortex; PrL, pre-limbic cortex; CG, cingulate cortex; PO, pre-optic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PG, periaqueductal gray; BNST, bed nucleus of the stria terminalis; LS, lateral septum; MS, medial septum; AntPVN, anterior paraventricular nucleus; PostPVN, posterior paraventricular nucleus; SON, supraoptic nucleus; MeA, medial amygdala; BLA, basolateral amygdala; CeA, central amygdala; Caudate, caudate putamen; VP, ventral pallidum; NAcc, nucleus accumbens; VTA, ventral tegmental area; CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; dDG, dorsal dentate gyrus; vDG, ventral dentate gyrus; UNFAM, animals exposed to unfamiliar conspecific; FAM, animals exposed to familiar conspecific; PUP, animals exposed to pups; OT, oxytocin; OTA, oxytocin antagonist.

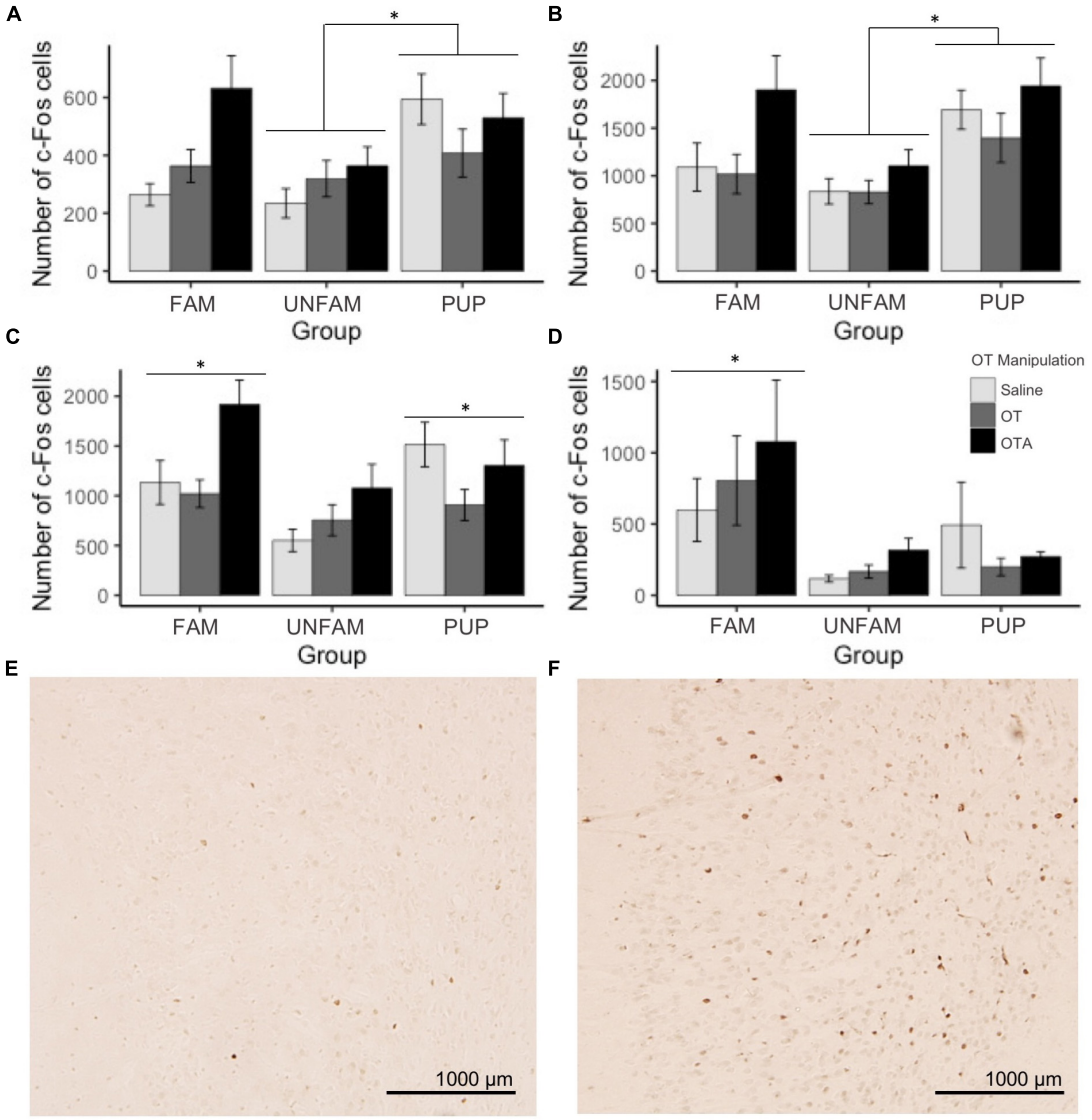

FIGURE 2 | Number of c-Fos cells ± SEM in brain regions where expression significantly varied by behavioral paradigm. UNFAM animals had a significantly lower number of c-Fos cells in the **(A)** MOB (main effect: $p = 0.0011$) relative to PUP ($p = 0.002$) but not FAM ($p = 0.42$), **(B)** Tu (main effect: $p < 0.0001$) relative to PUP ($p = 0.00012$) and FAM ($p = 0.059$), and **(C)** IL (main effect: $p = 0.0015$) relative to FAM ($p = 0.002$) and PUP ($p = 0.018$). **(D)** FAM animals had a significantly higher number of c-Fos cells in the VP (main effect: $p = 0002$) relative to UNFAM ($p = 0.0003$) and PUP ($p = 0.006$). Photomicrographs showing the reduced c-Fos immunoreactivity in the IL of an UNFAM animal **(E)** compared to the IL of a **(F)** FAM animal. IL, infralimbic cortex; Tu, olfactory tubercle; VP, ventral pallidum; UNFAM, animals exposed to an unfamiliar conspecific; FAM, animals exposed to a familiar conspecific.

Correlation Matrix Plots and Network Visualization Reveal the Disruption of SDMN Pathways by OT Manipulation

The correlation matrices were performed within each behavioral paradigm-by-OT manipulation group (**Figure 4**). Regions were clustered and color-coded based on literature and known functional connections in order to better visualize relationships between related regions. The plots demonstrate that manipulation of OT signaling, either by OT or OTA treatment, alters correlated activity among SDMN regions. In saline-treated FAM animals (**Figure 4A**), significant correlations are largely restricted to core social behavior network and reward-related regions. While OT (**Figure 4B**) had modest effects on c-Fos expression patterns, OTA (**Figure 4C**) caused a striking increase in positive correlations between almost all brain regions. In saline-treated UNFAM animals (**Figure 4D**), the SDMN-related regions are active and positively correlated. Treatment with OT (**Figure 4E**) or OTA (**Figure 4F**) disrupts these positive correlations. The saline-treated PUP animals (**Figure 4G**) are similar to UNFAM animals in that brain regions are significantly, positively correlated to one another and this pattern is disrupted by both OT (**Figure 4H**) and OTA (**Figure 4I**).

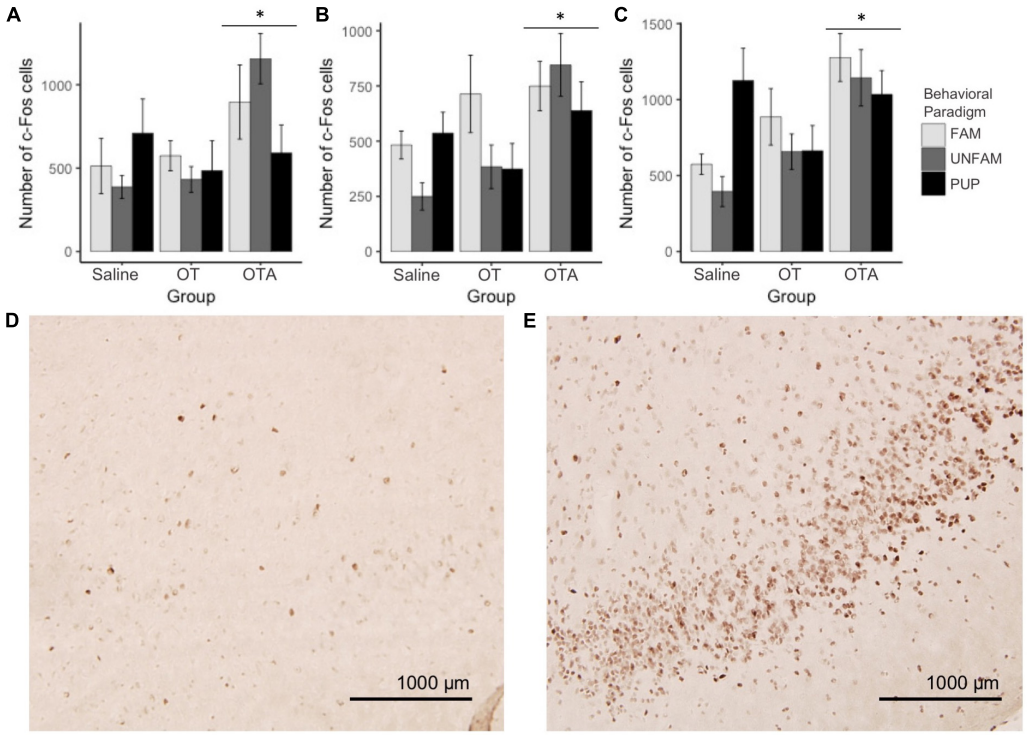

FIGURE 3 | Number of c-Fos cells ± SEM in brain regions where expression significantly varied by OT manipulation. OTA-treated animals had a significantly higher number of c-Fos cells in the **(A)** PIC (p = 0.00167), **(B)** ACC (p = 0.0014), and **(C)** PrL (p = 0.000862). Photomicrographs showing the reduced c-Fos immunoreactivity in the PIC of a **(D)** saline-treated animal compared to the PIC of an **(E)** OTA-treated animal. PIC, piriform cortex; ACC, anterior cingulate cortex; PrL, pre-limbic cortex.

The networks, like the correlation matrices, are plotted as behavioral paradigm-by-OT manipulation groups; however, unlike the correlation plots only relationships that were significant after applying Benjamini–Hochberg thresholding were included in the networks (5% false discovery rate). The networks confirm that OTA treatment alters correlated activity across the SDMN for all paradigms: by increasing connections in the FAM paradigm and disrupting connections in the UNFAM and PUP paradigms. OT had similar effects as OTA in the UNFAM and PUP paradigms. In the network for FAM saline-treated animals (**Figure 5A**), the NAcc, PIC, amygdalar regions, and some parts of the social behavior network are key nodes with highly correlated co-expression. OTA (**Figure 5C**) results in most all regions except the prefrontal cortex regions and SON highly co-expressing, while OT (**Figure 5B**) results in a more random expression pattern. In the network for UNFAM saline-treated animals (**Figure 5D**), the SBN, prefrontal cortex, amygdala, NAcc, SON, MOB, and dDG are important nodes. Almost all edges are removed by OT (**Figure 5E**) treatment while OTA (**Figure 5F**) activates hippocampal regions but results in few correlations between other regions. Finally, in the network for PUP saline-treated (**Figure 5G**), there is high co-expression in most of the SBN, amygdala, reward-related regions, PIC, AntPVN, and dDG. OT (**Figure 5H**) and OTA (**Figure 5I**) treatment eliminated most of these edges.

Factor Analysis Reveals Clusters of Related Regions Reflecting Known Patterns

There were no differences in behavior between baseline and test day, indicating that ICV injection did not in and of itself alter behavior. OT manipulation did not significantly affect behavior on test day. A summary of these results can be found in **Supplementary Tables 1**, **2**. Factor analysis revealed four clusters of brain regions (**Table 3**). Cluster 1 contains most classic SDMN regions (AH, BNST, LS, MeA, MS, PG, PIC, PO, VMH), cluster 2 is prefrontal cortex and OT production-related regions (ACC, AntPVN, CG, IL, PostPVN, PrL, SON), cluster 3 is hippocampal (CA1, CA2, CA3, dDG, MOB, vDG), and cluster 4 is olfactory (AON, Tu). Correlations between the summed counts of c-Fos within each cluster and behaviors revealed no significant relationships between any particular behavior and brain region cluster.

DISCUSSION

In this study, we investigated the effects of OT manipulation on coordinated activation of brain regions composing and related to the SDMN. By quantifying c-Fos immunoreactive cells following three different social paradigms, we demonstrated

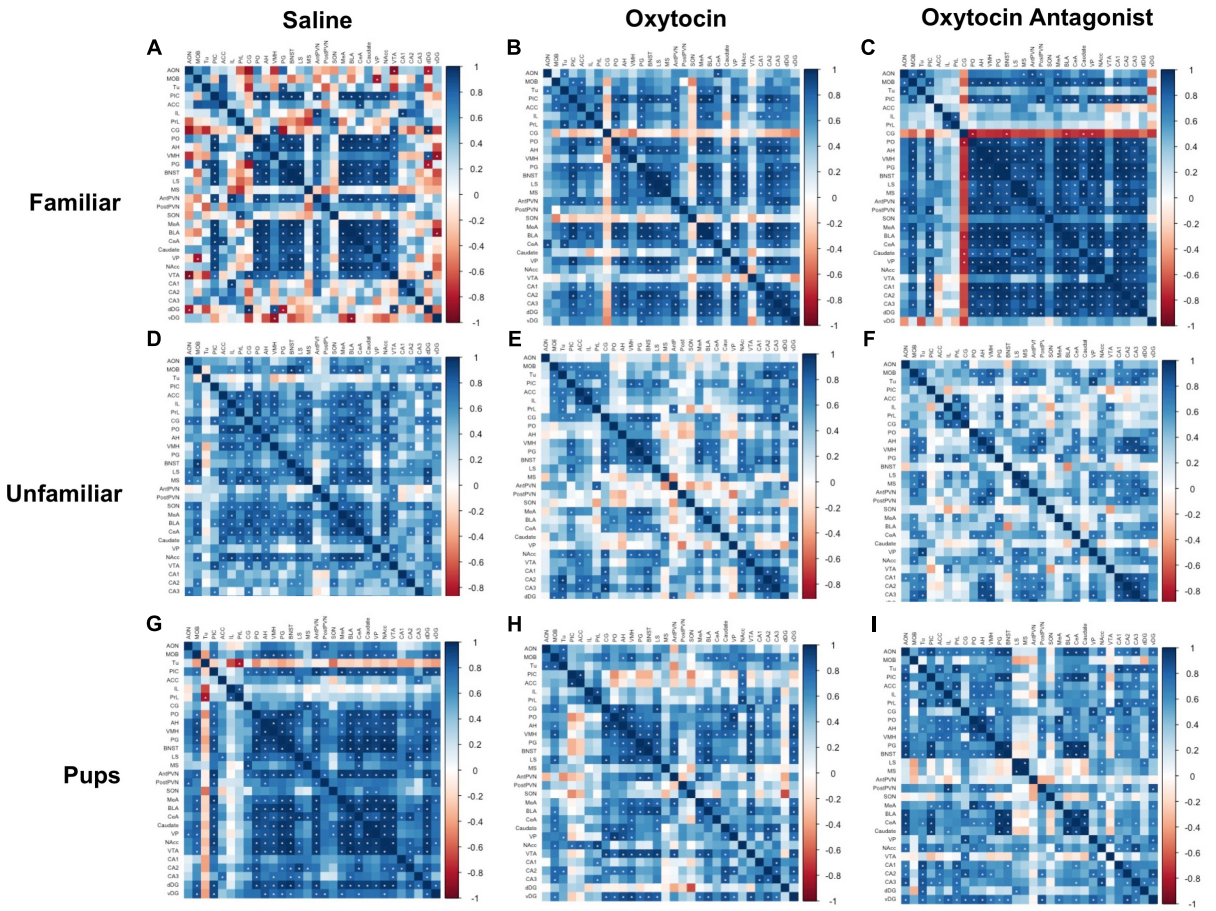

FIGURE 4 | Correlation matrices separated by behavioral paradigm and OT manipulation. Pair-wise Pearson correlations were calculated for c-Fos cell counts and all correlations plotted. Statistically significant correlations are marked by a white asterisk. **(A)** Saline-treated FAM, **(B)** OT-treated FAM, **(C)** OTA-treated FAM, **(D)** saline-treated UNFAM, **(E)** OT-treated UNFAM, **(F)** OTA-treated UNFAM, **(G)** saline-treated PUP, **(H)** OT-treated PUP, and **(I)** OTA-treated PUP. AON, anterior olfactory nucleus; MOB, main olfactory bulb; Tu, olfactory tubercle; PIC, piriform cortex; ACC, anterior cingulate cortex; PrL, pre-limbic cortex; IL, infralimbic cortex; CG, cingulate cortex; PO, pre-optic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PG, periaqueductal gray; BNST, bed nucleus of the stria terminalis; LS, lateral septum; MS, medial septum; AntPVN, anterior paraventricular nucleus; PostPVN, posterior paraventricular nucleus; SON, supraoptic nucleus; MeA, medial amygdala; BLA, basolateral amygdala; CeA, central amygdala; Caudate, caudate putamen; VP, ventral pallidum; NAcc, nucleus accumbens; VTA, ventral tegmental area; CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; dDG, dorsal dentate gyrus; vDG = ventral dentate gyrus.

that the SDMN is conserved in the highly social naked mole-rat and that it functions in a context-dependent manner. Furthermore, we report that OT manipulation, both via OT and OTA, is generally disruptive to coordinated neural activity, though specific effects are context-dependent. Finally, these data suggest that while structures such as the medial prefrontal cortex, hippocampus, and olfactory regions are important for processing social stimuli, they are not direct members of the SDMN.

By examining neural activity in SDMN regions following different behavioral paradigms, we have demonstrated that, perhaps not surprisingly, this network is functionally conserved in naked mole-rats. This is consistent with previous neurochemical analyses in SDMN regions in this species (Rosen et al., 2007, 2008; O'Connell and Hofmann, 2012; Holmes et al., 2013; Mooney et al., 2015a; Beery et al., 2016). Factor analysis shows the examined brain regions from 4 clusters (**Table 3**), the first of which includes all the SDMN brain regions together except the hippocampus. The other three clusters comprise anatomically and functionally related regions (i.e., medial prefrontal cortex, hippocampus, and olfactory regions, respectively). This pattern of clustering corroborates the existence of a SDMN in the naked mole-rat, and further suggests the other three clusters are perhaps related, but not directly a part of the SDMN. Interestingly, the SDMN showed correlated c-Fos immunoreactivity in the FAM saline-treated group. Given the colony living of naked mole-rats, their baseline experience is consistent interaction with multiple familiar conspecifics. When Newman proposed the social behavior network, one limitation was that without social stimuli, there is no baseline activity for the associated regions (Newman, 1999; O'Connell and Hofmann, 2011). In the case of eusocial naked mole-rats, it is likely the SDMN is always active and integral for navigating colony life.

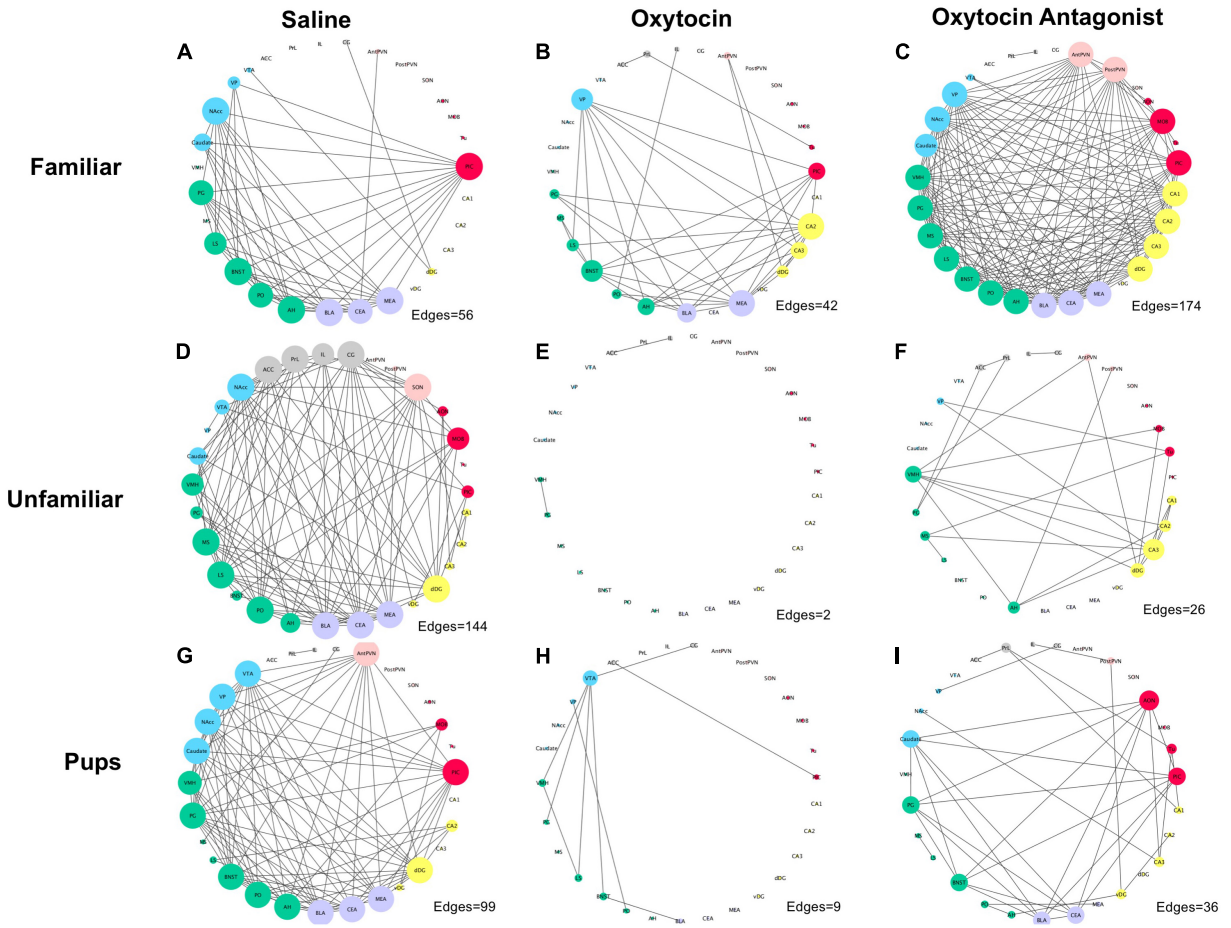

FIGURE 5 | Correlation networks separated by behavioral paradigm and OT manipulation. Pair-wise Pearson correlations were calculated for c-Fos cell counts with multiple-testing corrected for using the Benjamini–Hochberg method with a false discovery rate of 5%. Brain regions are color-coded based on anatomically and functionally similar groups (blue, reward; green, social behavior network; purple, amygdala; yellow, hippocampus; red, olfactory; pink, OT production cluster; and gray, medial prefrontal cortex). Node size is a measure of degree (how many edges are connected to a given node). **(A)** Saline-treated FAM, **(B)** OT-treated FAM, **(C)** OTA-treated FAM, **(D)** saline-treated UNFAM, **(E)** OT-treated UNFAM, **(F)** OTA-treated UNFAM, **(G)** saline-treated PUP, **(H)** OT-treated PUP, and **(I)** OTA-treated PUP. AON, anterior olfactory nucleus; MOB, main olfactory bulb; Tu, olfactory tubercle; PIC, piriform cortex; ACC, anterior cingulate cortex; PrL, pre-limbic cortex; IL, infralimbic cortex; CG, cingulate cortex; PO, pre-optic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PG, periaqueductal gray; BNST, bed nucleus of the stria terminalis; LS, lateral septum; MS, medial septum; AntPVN, anterior paraventricular nucleus; PostPVN, posterior paraventricular nucleus; SON, supraoptic nucleus; MeA, medial amygdala; BLA, basolateral amygdala; CeA, central amygdala; Caudate, caudate putamen; VP, ventral pallidum; NAcc, nucleus accumbens; VTA, ventral tegmental area; CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; dDG, dorsal dentate gyrus; vDG, ventral dentate gyrus.

The SDMN is thought to modulate behaviors necessary for navigating varied social environments (O'Connell and Hofmann, 2011). The behavioral paradigm-specific patterns of neural activity that we report support the hypothesis that coordinated activation of brain regions within the SDMN is context-dependent. Examination of the correlation matrices (**Figure 4**) reveals that while saline-treated animals in all three behavioral paradigms show coordinated activation of SDMN regions, the network incorporates the medial prefrontal cortex regions in UNFAM animals and hippocampal regions in PUP animals. The medial prefrontal cortex has been implicated in social approach, social ascent, facilitating aggressive behavior and in establishing social rank in mice, hence activation could be associated with need for assessing status and establishing hierarchy between the two unfamiliar animals (Wang et al., 2011; Takahashi et al., 2014; Lee et al., 2016; Williamson et al., 2018). It is interesting to speculate that the coordinated activation of hippocampal regions in PUP animals reflects learning of new colony members and is consistent with the increase in cell proliferation observed following pup exposure in prairie voles and fatherhood in male California mice, both species that exhibit biparental care (Ruscio et al., 2008; Hyer et al., 2016). Alternatively, this could be related to spatial memory required for returning pups to their nest as seen in female rats (Kinsley et al., 1999). In addition to paradigm-specific patterns of correlated activity, we also report paradigm-specific effects on c-Fos expression in individual brain regions. For example, the VP had significantly higher c-Fos immunoreactivity in FAM

TABLE 3 | Factor analysis results following application of principal axis factoring to saline-treated animals (collapsed across task), identifying four clusters of brain regions.

Cluster	Brain regions
Cluster 1	PIC, PO, AH, VMH, PG, BNST, LS, MS, MeA, BLA, CeA, Caudate, VP, NAcc, VTA
Cluster 2	ACC, PrL, IL, CG, AntPVN, PostPVN, SON
Cluster 3	MOB, CA1, CA2, CA3, dDG, vDG
Cluster 4	AON, Tu

PIC, piriform cortex; PO, pre-optic area; AH, anterior hypothalamus; VMH, ventromedial hypothalamus; PG, periaqueductal gray; BNST, bed nucleus of the stria terminalis; LS, lateral septum; MS, medial septum; MeA, medial amygdala; BLA, basolateral amygdala; CeA, central amygdala; Caudate, caudate putamen; VP, ventral pallidum; NAcc, nucleus accumbens; VTA, ventral tegmental area; ACC, anterior cingulate cortex; PrL, pre-limbic cortex; IL, infralimbic cortex; CG, cingulate cortex; AntPVN, anterior paraventricular nucleus; PostPVN, posterior paraventricular nucleus; SON, supraoptic nucleus; MOB, main olfactory bulb; CA1, cornu ammonis 1; CA2, cornu ammonis 2; CA3, cornu ammonis 3; dDG, dorsal dentate gyrus; vDG, Ventral dentate gyrus; AON, anterior olfactory nucleus; Tu, olfactory tubercle.

animals (**Figure 2C**). This region is involved in formation of social attachment in socially monogamous prairie voles (Lim and Young, 2004; Barrett et al., 2013; Zheng et al., 2013). In UNFAM animals, who are meeting a novel animal and presumably assessing relative status, there was significantly lower activation of the MOB, Tu, and IL (**Figures 2A–C**). The olfactory system is essential to pair bonding in prairie voles (Curtis et al., 2001), and, while naked mole-rat subordinates are reproductively inactive, they are highly affiliative with colony members. Naked mole-rats show preference for familiar olfactory cues over unfamiliar ones (Toor et al., 2015), likely contributing to in-colony social recognition and bonding. Interestingly, the IL (in addition to the PrL) is also activated in a shifting social environment in which social ascent is possible, as in the case of removing an alpha male in a mouse hierarchy (Williamson et al., 2018). Administration of a histone deacetylase inhibitor to the IL increases stress behaviors in a Syrian hamster model of social defeat (McCann et al., 2017), further implicating the IL in social interactions involving dominance.

The data reported here also support the hypothesis that OT mediates coordinated neural activity within the SDMN to promote context-specific social behavior. OTA treatment altered coordinated c-Fos expression in all tasks but while it increased connectivity in FAM animals, it reduced connectivity in UNFAM and PUP animals (**Figures 4**, **5**). Brain region-specific analyses revealed that in all behavioral paradigms, OTA significantly increased c-Fos expression in the PIC, ACC, and PrL (**Figure 3**), suggesting these regions are particularly sensitive to perturbations in OT signaling. While examination of OT receptor distribution in naked mole-rats did not evaluate anterior regions like the ACC and PrL, subordinate NMRs have negligible OT receptor binding in the PIC (Kalamatianos et al., 2010). Thus, OTA could be acting elsewhere to indirectly influence activity in these regions. Similar to OTA, we also found evidence that OT treatment alters connectivity in the SDMN with reduced coordinated activity in animals from the UNFAM and PUP paradigms (**Figure 5**). This is not necessarily surprising as exogenous OT is unlikely to reflect endogenous levels and, furthermore, our central delivery ensures OT would act at multiple targets, interfering with "normal" OT signaling. Importantly, we cannot infer whether more or fewer connections are optimal as we have yet to directly relate coordinated activity to specific behaviors. Rather, we can conclude that central OT manipulation affects connectivity in the SDMN in various social contexts and future studies will identify how specific regions are involved.

Indeed, the NAcc is a likely hub modulating the role of OT between other SDMN regions. OT acts in the NAcc to modulate sociosexual interactions and mating in prairie voles (Johnson et al., 2017): administration of an OT receptor antagonist directly to the NAcc changes connectivity between NAcc and other SDMN regions. The NAcc is also involved in reproductive behavior and social dominance in naked mole-rats. For example, OT receptor binding is higher in breeding male NAcc compared to breeding females (Mooney et al., 2015a) and expression of genes involved in social suppression of reproduction (*Kiss1*, *Npvf*, *Gpr147*, *Tac3r*) varies by sex and status (Faykoo-Martinez et al., 2018). The current data demonstrate that the NAcc is a key node in all three behavioral paradigms (**Figure 5**). In the FAM network for saline-treated animals, NAcc coordinated activity is enhanced by OTA but knocked out by OT treatment. In the UNFAM network for saline-treated animals, the NAcc is the key active node out of the mesolimbic reward circuit and is knocked out by both OT and OTA. Finally, in the PUP network for saline-treated animals, all mesolimbic reward circuit regions are key nodes. This includes the NAcc, which is then knocked out to varying extents by OT and OTA treatment. Given its role in assigning valence to social stimuli, it will be important to evaluate OT effects directly in the NAcc to better understand how this region alters coordinated neural activity, similar to Johnson et al. (2017), across different social contexts.

We have taken a broad approach in our first attempt at teasing apart function in the naked mole-rat SDMN. For this reason, we did not break down groups by subcaste or focus on a single sex for stimulus animal. Subordinates are pre-pubertal, do not exhibit sex differences in behavior (Lacey and Sherman, 1991), and take a week to begin demonstrating behaviors characteristic of reproductive maturation (Margulis et al., 1995; Clarke and Faulkes, 1997; Mooney et al., 2015b; Swift-Gallant et al., 2015; Toor et al., 2015). Thus, it is likely that the sex of stimulus animals would cause a negligible effect given the very short period of removal from colony. It, of course, also warrants mention that our experimental design was likely insufficiently powered to robustly examine OT manipulation by behavioral paradigm interactions. Our sample size was limited by the challenges of rearing naked mole-rats in captivity (taking 1 year to reach adulthood coupled with unpredictable breeding). Low statistical power might also explain why we did not detect any

significant effects of OT manipulation on behavior or significant relationships between c-Fos activity and behavior, though the lack of behavioral results following central administration of OT is not necessarily unexpected. While we have previously shown that peripheral OT administration changes in-colony behavior (e.g., time spent huddling) and proximity to novel conspecifics, we did not measure in-colony behavior in the current study and also used a different testing apparatus for outpairing (single chamber vs. double chamber) (Mooney et al., 2014). To address these limitations, we provide the raw data in **Supplementary Information** with the hope that future work by us and others will build on this sample.

Here we report the first formal investigation of activation of the SDMN in the highly social naked mole-rat. We have demonstrated that coordinated neural activity within this network and with related regions varies according to social context, and that this coordinated activation is altered by manipulation of the OT system. The pattern of connectivity associated with each behavioral paradigm suggests a role for the NAcc in social valence and sociosexual interaction, the mPFC in assessing/establishing social dominance, and the hippocampus in pup recognition.

AUTHOR CONTRIBUTIONS

MH and SM contributed conception and design of the study. SM and MF-M collected the data. MF-M performed statistical analyses and wrote the first draft of the manuscript. SM performed the experiments and wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This work was funded by a NSERC CGS M and NSERC PGS D to MF-M, QEII-GSST OGS to SM, and NSERC grants (RGPIN 2011-402633, RGPIN 2018-04780, and RGPAS 2018-522465) and an Ontario Early Researcher Award to MH.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00272/full#supplementary-material

REFERENCES

Anacker, A. M. J., and Beery, A. K. (2013). Life in groups: the roles of oxytocin in mammalian sociality. *Front. Behav. Neurosci.* 7:185. doi: 10.3389/fnbeh.2013.00185

Barrett, C. E., Keebaugh, A. C., Ahern, T. H., Bass, C. E., Terwilliger, E. F., and Young, L. J. (2013). Variation in vasopressin receptor (Avpr1a) expression creates diversity in behaviors related to monogamy in prairie voles. *Horm. Behav.* 63, 518–526. doi: 10.1016/j.yhbeh.2013.01.005

Beery, A. K., Bicks, L., Mooney, S. J., Goodwin, N. L., and Holmes, M. M. (2016). Sex, social status, and CRF receptor densities in naked mole-rats. *J. Comp. Neurol.* 524, 228–243. doi: 10.1002/cne.23834

Beery, A. K., Lacey, E. A., and Francis, D. D. (2008). Oxytocin and vasopressin receptor distributions in a solitary and a social species of tuco-tuco (*Ctenomys haigi* and *Ctenomys sociabilis*). *J. Comp. Neurol.* 507, 1847–1859. doi: 10.1002/cne.21638

Bicks, L. K., Koike, H., Akbarian, S., and Morishita, H. (2015). Prefrontal cortex and social cognition in mouse and man. *Front. Psychol.* 6:1805. doi: 10.3389/fpsyg.2015.01805

Brennan, P. A., and Kendrick, K. M. (2006). Mammalian social odours: attraction and individual recognition. *Philos. Trans. R. Soc. B Biol. Sci.* 361, 2016–2078. doi: 10.1098/rstb.2006.1931

Brett, R. A. (1991a). "The ecology of naked mole-rat colonies: burrowing, food, and limiting factors.(pepper braude lacey Sherman 1991)," in *The Biology of the Naked Mole-Rat*, eds P. W. Sherman, J. U. M. Jarvis, and R. D. Alexander (Princeton: Princeton University Press), 137–184.

Brett, R. A. (1991b). "The population structure of naked mole-rat colonies," in *The Biology of the Naked Mole-Rat*, eds P. W. Sherman, J. U. M. Jarvis, and R. D. Alexander (Princeton: Princeton University Press), 97–136.

Champagne, F., Diorio, J., Sharma, S., and Meaney, M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12736–12741. doi: 10.1073/pnas.221224598

Chang, S. W. C., Fagan, N. A., Toda, K., Utevsky, A. V., Pearson, J. M., and Platt, M. L. (2015). Neural mechanisms of social decision-making in the primate amygdala. *Proc. Natl. Acad. Sci. U.S.A.* 112, 16012–16017. doi: 10.1073/pnas.1514761112

Choleris, E., Little, S. R., Mong, J. A., Puram, S. V., Langer, R., and Pfaff, D. W. (2007). Microparticle-based delivery of oxytocin receptor antisense DNA in the medial amygdala blocks social recognition in female mice. *Proc. Natl. Acad. Sci. U.S.A.* 104, 4670–4675. doi: 10.1073/pnas.0700670104

Clarke, F. M., and Faulkes, C. G. (1997). Dominance and queen succession in captive colonies of the eusocial naked mole-rat, *Heterocephalus glaber*. *Proc. Biol. Sci.* 264, 993–1000. doi: 10.1098/rspb.1997.0137

Consiglio, A. R., Borsoi, A., Pereira, G. A. M., and Lucion, A. B. (2005). Effects of oxytocin microinjected into the central amygdaloid nucleus and bed nucleus of stria terminalis on maternal aggressive behavior in rats. *Physiol. Behav.* 85, 354–362. doi: 10.1016/j.physbeh.2005.05.002

Curtis, J. T., Liu, Y., and Wang, Z. (2001). Lesions of the vomeronasal organ disrupt mating-induced pair bonding in female prairie voles (*Microtus ochrogaster*). *Brain Res.* 901, 167–174. doi: 10.1016/S0006-8993(01)02343-5

Faykoo-Martinez, M., Ashley Monks, D., Zovkic, I. B., and Holmes, M. M. (2018). Sex- and brain region-specific patterns of gene expression associated with socially-mediated puberty in a eusocial mammal. *PLoS One* 13:e0193417. doi: 10.1371/journal.pone.0193417

Ferguson, J. N., Aldag, J. M., Insel, T. R., and Young, L. J. (2001). Oxytocin in the medial amygdala is essential for social recognition in the mouse. *J. Neurosci.* 21, 8278–8285. doi: 10.1523/JNEUROSCI.21-20-08278.2001

Ferguson, J. N., Young, L. J., Hearn, E. F., Matzuk, M. M., Insel, T. R., and Winslow, J. T. (2000). Social amnesia in mice lacking the oxytocin gene. *Nat. Genet.* 25, 284–288. doi: 10.1038/77040

Francis, D. D., Champagne, F. C., and Meaney, M. J. (2000). Variations in maternal behaviour are associated with differences in oxytocin receptor levels in the rat. *J. Neuroendocrinol.* 12, 1145–1148. doi: 10.1046/j.1365-2826.2000.00599.x

Geissmann, Q. (2013). OpenCFU, a new free and open-source software to count cell colonies and other circular objects. *PLoS One* 8:e50472. doi: 10.1371/journal.pone.0054072

Goodson, J. L., and Kabelik, D. (2009). Dynamic limbic networks and social diversity in vertebrates: from neural context to neuromodulatory patterning. *Front. Neuroendocrinol.* 30, 429–441. doi: 10.1016/j.yfrne.2009.05.007

Harrell, F. E. (2017). *CRAN – Package Hmisc. Hmisc Harrell Misc*. Available at: http://biostat.mc.vanderbilt.edu/Hmisc

Hathaway, G. A., Faykoo-Martinez, M., Peragine, D. E., Mooney, S. J., and Holmes, M. M. (2016). Subcaste differences in neural activation suggest a prosocial role

for oxytocin in eusocial naked mole-rats. *Horm. Behav.* 79, 1–7. doi: 10.1016/j.yhbeh.2015.12.001

Holmes, M. M., Van Mil, S., Bulkowski, C., Goldman, S. L., Goldman, B. D., and Forger, N. G. (2013). Androgen receptor distribution in the social decision-making network of eusocial naked mole-rats. *Behav. Brain Res.* 256, 214–218. doi: 10.1016/j.bbr.2013.08.025

Hyer, M. M., Hunter, T. J., Katakam, J., Wolz, T., and Glasper, E. R. (2016). Neurogenesis and anxiety-like behavior in male California mice during the mate's postpartum period. *Eur. J. Neurosci.* 43, 703–709. doi: 10.1111/ejn.13168

IBM Corp (2016). *IBM SPSS Statistics for Macintosh, Version 24.0*.

Jarvis, J. U. M. (1981). Eusociality in a mammal: cooperative breeding in naked mole-rat colonies. *Science* 212, 571–573. doi: 10.1126/science.7209555

Johnson, Z. V., Walum, H., Jamal, Y. A., Xiao, Y., Keebaugh, A. C., Inoue, K., et al. (2016). Central oxytocin receptors mediate mating-induced partner preferences and enhance correlated activation across forebrain nuclei in male prairie voles. *Horm. Behav.* 79, 8–17. doi: 10.1016/j.yhbeh.2015.11.011

Johnson, Z. V., Walum, H., Xiao, Y., Riefkohl, P. C., and Young, L. J. (2017). Oxytocin receptors modulate a social salience neural network in male prairie voles. *Horm. Behav.* 87, 16–24. doi: 10.1016/j.yhbeh.2016.10.009

Kalamatianos, T., Faulkes, C. G., Oosthuizen, M. K., Poorun, R., Bennett, N. C., and Coen, C. W. (2010). Telencephalic binding sites for oxytocin and social organization: â comparative study of eusocial naked mole-rats and solitary cape mole-rats. *J. Comp. Neurol.* 518, 1792–1813. doi: 10.1002/cne.22302

Kinsley, C. H., Madonia, L., Gifford, G. W., Tureski, K., Griffin, G. R., Lowry, C., et al. (1999). Motherhood improves learning and memory. *Nature* 402, 137–138. doi: 10.1038/45957

Kuznetsova, A., Brockhoff, P., and Christensen, R. (2016). *lmerTest: Tests in Linear Mixed Effects Models. R Packag. version*. doi: 10.18637/jss.v082.i13

Lacey, E. A., and Sherman, P. W. (1991). "Social organization of naked mole-rat colonies: evidence for divisions of labor," in *The Biology of the Naked Mole-Rat*, eds P. W. Sherman, J. U. M. Jarvis, and R. D. Alexander (Princeton: Princeton University Press).

Lee, E., Rhim, I., Lee, J. W., Ghim, J.-W., Lee, S., Kim, E., et al. (2016). Enhanced neuronal activity in the medial prefrontal cortex during social approach behavior. *J. Neurosci.* 36, 6926–6936. doi: 10.1523/jneurosci.0307-16.2016

Lee, V. K., and Harris, L. T. (2013). How social cognition can inform social decision making. *Front. Neurosci.* 7:259. doi: 10.3389/fnins.2013.00259

Lim, M. M., and Young, L. J. (2004). Vasopressin-dependent neural circuits underlying pair bond formation in the monogamous prairie vole. *Neuroscience* 125, 35–45. doi: 10.1016/j.neuroscience.2003.12.008

Margulis, S. W., Saltzman, W., and Abbott, D. H. (1995). Behavioral and hormonal changes in female naked mole-rats (*Heterocephalus glaber*) following removal of the breeding female from a colony. *Horm. Behav.* 29, 227–247. doi: 10.1006/hbeh.1995.1017

McCann, K. E., Rosenhauer, A. M., Jones, G. M. F., Norvelle, A., Choi, D. C., and Huhman, K. L. (2017). Histone deacetylase and acetyltransferase inhibitors modulate behavioral responses to social stress. *Psychoneuroendocrinology* 75, 100–109. doi: 10.1016/j.psyneuen.2016.10.022

Mooney, S. J., Coen, C. W., Holmes, M. M., and Beery, A. K. (2015a). Region-specific associations between sex, social status, and oxytocin receptor density in the brains of eusocial rodents. *Neuroscience* 303, 261–269. doi: 10.1016/j.neuroscience.2015.06.043

Mooney, S. J., Filice, D. C. S., Douglas, N. R., and Holmes, M. M. (2015b). Task specialization and task switching in eusocial mammals. *Anim. Behav.* 109, 227–233. doi: 10.1016/j.anbehav.2015.08.019

Mooney, S. J., Douglas, N. R., and Holmes, M. M. (2014). Peripheral administration of oxytocin increases social affiliation in the naked mole-rat (*Heterocephalus glaber*). *Horm. Behav.* 65, 380–385. doi: 10.1016/j.yhbeh.2014.02.003

Mooney, S. J., and Holmes, M. M. (2013). Social condition and oxytocin neuron number in the hypothalamus of naked mole-rats (*Heterocephalus glaber*). *Neuroscience* 230, 56–61. doi: 10.1016/j.neuroscience.2012.11.014

Mooney, S. J., and Holmes, M. M. (2015). Successful intracerebroventricular cannulation of a eusocial mammal. *J. Neurosci. Methods* 239, 75–79. doi: 10.1016/j.jneumeth.2014.09.026

Nakajima, M., Görlich, A., and Heintz, N. (2014). Oxytocin modulates female sociosexual behavior through a specific class of prefrontal cortical interneurons. *Cell* 159, 295–305. doi: 10.1016/j.cell.2014.09.020

Newman, S. (1999). The medial extended amygdala in male reproductive behavior. *Ann. N. Y. Acad. Sci.* 877, 242–257. doi: 10.1111/j.1749-6632.1999.tb09271.x

O'Connell, L. A., and Hofmann, H. A. (2011). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J. Comp. Neurol.* 519, 3599–3639. doi: 10.1002/cne.22735

O'Connell, L. A., and Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. *Science* 336, 1154–1157. doi: 10.1126/science.1218889

Oettl, L. L., Ravi, N., Schneider, M., Scheller, M. F., Schneider, P., Mitre, M., et al. (2016). Oxytocin enhances social recognition by modulating cortical control of early olfactory processing. *Neuron* 90, 609–621. doi: 10.1016/j.neuron.2016.03.033

Olazábal, D. E., and Young, L. J. (2006a). Oxytocin receptors in the nucleus accumbens facilitate "spontaneous" maternal behavior in adult female prairie voles. *Neuroscience* 141, 559–568. doi: 10.1016/j.neuroscience.2006.04.017

Olazábal, D. E., and Young, L. J. (2006b). Species and individual differences in juvenile female alloparental care are associated with oxytocin receptor density in the striatum and the lateral septum. *Horm. Behav.* 49, 681–687. doi: 10.1016/j.yhbeh.2005.12.010

Pepper, J. W., Braude, S. H., Lacey, E. A., and Sherman, P. W. (1991). "Vocalizations of the naked mole-rat," in *The Biology of the Naked Mole-Rat*, eds P. W. Sherman, J. U. M. Jarvis, and R. D. Alexander (Princeton: Princeton University Press), 243–274.

Pinheiro, J., Bates, D., DebRoy, S., and Sarkar, D. (2016). *nlme: Linear and Nonlinear Mixed Effects Models. R Packag. version*.

R Development Core Team (2011). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Rasband, W. (2012). *ImageJ*. Bethesda, MD: U.S. National Institutes of Health.

Reddon, A. R., Voisin, M. R., O'Connor, C. M., and Balshine, S. (2014). Isotocin and sociality in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*. *Behaviour* 151, 1389–1411. doi: 10.1163/1568539X-00003190

Rosen, G. J., De Vries, G. J., Goldman, S. L., Goldman, B. D., and Forger, N. G. (2007). Distribution of vasopressin in the brain of the eusocial naked mole-rat. *J. Comp. Neurol.* 500, 1093–1105. doi: 10.1002/cne.21215

Rosen, G. J., de Vries, G. J., Goldman, S. L., Goldman, B. D., and Forger, N. G. (2008). Distribution of oxytocin in the brain of a eusocial rodent. *Neuroscience* 155, 809–817. doi: 10.1016/j.neuroscience.2008.05.039

Ruscio, M. G., Sweeny, T. D., Hazelton, J. L., Suppatkul, P., Boothe, E., and Carter, C. S. (2008). Pup exposure elicits hippocampal cell proliferation in the prairie vole. *Behav. Brain Res.* 187, 9–16. doi: 10.1016/j.bbr.2007.08.028

Samuelsen, C. L., and Meredith, M. (2011). Oxytocin antagonist disrupts male mouse medial amygdala response to chemical-communication signals. *Neuroscience* 180, 96–104. doi: 10.1016/j.neuroscience.2011.02.030

Shahrokh, D. K., Zhang, T. Y., Diorio, J., Gratton, A., and Meaney, M. J. (2010). Oxytocin-dopamine interactions mediate variations in maternal behavior in the rat. *Endocrinology* 151, 2276–2286. doi: 10.1210/en.2009-1271

Shannon, P., Markiel, A., Owen Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303.metabolite

Swift-Gallant, A., Mo, K., Peragine, D. E., Monks, D. A., and Holmes, M. M. (2015). Removal of reproductive suppression reveals latent sex differences in brain steroid hormone receptors in naked mole-rats, *Heterocephalus glaber*. *Biol. Sex Differ.* 6:31. doi: 10.1186/s13293-015-0050-x

Takahashi, A., Nagayasu, K., Nishitani, N., Kaneko, S., and Koide, T. (2014). Control of intermale aggression by medial prefrontal cortex activation in the mouse. *PLoS One* 9:e94657. doi: 10.1371/journal.pone.0094657

Teles, M. C., Almeida, O., Lopes, J. S., and Oliveira, R. F. (2015). Social interactions elicit rapid shifts in functional connectivity in the social decision-making network of zebrafish. *Proc. R. Soc. B Biol. Sci.* 282:20151099. doi: 10.1098/rspb.2015.1099

Tobin, V. A., Hashimoto, H., Wacker, D. W., Takayanagi, Y., Langnaese, K., Caquineau, C., et al. (2010). An intrinsic vasopressin system in the olfactory bulb is involved in social recognition. *Nature* 464, 413–417. doi: 10.1038/nature08826

Toor, I., Clement, D., Carlson, E. N., and Holmes, M. M. (2015). Olfaction and social cognition in eusocial naked mole-rats, *Heterocephalus glaber*. *Anim. Behav.* 107, 175–181. doi: 10.1016/j.anbehav.2015.06.015

Wang, F., Zhu, J., Zhu, H., Zhang, Q., Lin, Z., and Hu, H. (2011). Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex. *Science* 334, 693–697. doi: 10.1126/science.1209951

Wei, T., and Simko, V. (2016). *The Corrplot package*. Vienna: R Foundation for Statistical Computing.

Williamson, C. M., Klein, I. S., Lee, W., and Curley, J. P. (2018). Immediate early gene activation throughout the brain is associated with dynamic changes in social context. *Soc. Neurosci.* 31, 1–13. doi: 10.1101/275495

Withers, P. C., and Jarvis, J. U. M. (1980). The effect of huddling on thermoregulation and oxygen consumption for the naked mole-rat. *Comp. Biochem. Physiol. Part A Physiol.* 66, 215–219. doi: 10.1016/0300-9629(80)90154-1

Zheng, D. J., Larsson, B., Phelps, S. M., and Ophir, A. G. (2013). Female alternative mating tactics, reproductive success and nonapeptide receptor expression in the social decision-making network. *Behav. Brain Res.* 246, 139–147. doi: 10.1016/j.bbr.2013.02.024

Conflict of Interest Statement: 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: 21 November 2018
doi: 10.3389/fnbeh.2018.00267

Sexually-Relevant Visual and Chemosensory Signals Induce Distinct Behaviors and Neural Activation Patterns in the Social African Cichlid, *Astatotilapia burtoni*

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Across vertebrates, the use of multimodal (multiple sensory modalities) signals has evolved to convey important information to receivers. Information content of multimodal signals can be the same as or different from information in each unimodal signal, and are classified as redundant or non-redundant, respectively, based on receivers' behavioral responses. Despite the prevalence and importance of multimodal signaling across taxa, relatively little is known about how and where these signals are processed in the brains of receivers. We used the social African cichlid fish, *Astatotilapia burtoni*, to investigate how sexually-relevant visual and chemosensory uni- and multimodal signals from gravid (full of eggs) females influence behavior, brain activation patterns, and physiology in dominant males. We presented both visual and chemosensory signals either alone or together and found that males need sexually-relevant visual signals to engage in stereotypical courtship behaviors such as body quivers, waggles, and leads into spawning territories. However, the number of courtship behaviors was greater when males were exposed to multimodal visual-chemosensory signals, compared to either unimodal signal alone. When a female visual signal was absent, males increased swimming and overall activity in response to female-conditioned water compared to control water, suggesting that female-released chemosensory signals may stimulate male searching behavior and motivation. Importantly, we also tested anosmic (olfactory ablated) males to demonstrate that this behavior is primarily mediated by the olfactory system rather than gustation. Using the immediate early gene *cfos* as a proxy for neural activation, we also demonstrate differential activation in social and olfactory-relevant brain regions of dominant males exposed to unimodal and multimodal visual-chemosensory signals. We found at least one region that is preferentially activated by reception of signals from each sense, as well as regions that exhibit an additive effect on activation with multimodal visual-chemosensory stimulation. These data provide insight on how multimodal signals are processed in the brain and integrated with internal physiology of receivers to produce social behaviors, and lay the groundwork for future studies on the evolution of sensory perception.

Keywords: behavior, brain, multimodal, olfaction, sensory, social decision-making network, teleost, vision

OPEN ACCESS

Edited by:
James P. Curley, University of Texas at Austin, United States

Reviewed by:
Leonard Maler, University of Ottawa, Canada
Yong Gu, Institute of Neuroscience, Shanghai Institutes for Biological Sciences (CAS), China

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Received: *29 May 2018*
Accepted: *22 October 2018*
Published: *21 November 2018*

Citation:
Field KE, McVicker CT and Maruska KP (2018) Sexually-Relevant Visual and Chemosensory Signals Induce Distinct Behaviors and Neural Activation Patterns in the Social African Cichlid, Astatotilapia burtoni. Front. Behav. Neurosci. 12:267. doi: 10.3389/fnbeh.2018.00267

INTRODUCTION

Across taxa, animals must constantly assess their environment to make behavioral decisions. Signals sent via different sensory modalities, such as visual, chemosensory, mechanosensory, touch, and sound are often delivered together and reception of this information by a receiver is integrated with the animal's own internal physiology to elicit context-dependent behaviors (Bradbury and Vehrencamp, 2000). This use of multimodal communication is prevalent across vertebrates, particularly during reproduction, providing receivers with varying types of information about the signaler such as breeding condition, motivation, and fitness qualities. Despite the importance of multimodal communication for survival and reproductive success, our understanding of how different sensory signals are processed in the brain of receivers to produce specific behavioral outputs is limited (Partan and Marler, 2005; Ronald et al., 2012).

The use of visual-chemosensory multimodal communication is widespread, with numerous examples from both invertebrate and vertebrate taxa (Kotrschal, 2000; Isogai et al., 2011; Mori, 2014). While vision is often the dominant sense mediating reproductive behaviors, chemosensory communication is also commonly used across the animal kingdom. It is particularly prevalent in fishes, where it functions in prey detection, predator avoidance, and social communication (Hara, 1994; Kotrschal, 2000). For example, females are often senders of potent chemical signals that provide important information for coordinating reproductive events, and in several fish species, these chemosensory signals can induce robust reproductive behavioral responses in male receivers (Stacey, 2011). However, the neural links between multisensory inputs and receiver behavioral output remains poorly understood (Ronald et al., 2012; Partan, 2013). Further, the physiology and/or reproductive state of receivers can influence how such sensory signals are processed (Insel, 2010). Thus, examining communication from a perspective that goes beyond behavioral responses to include receiver physiology and neural processing mechanisms is crucial for understanding the function and evolution of context-dependent signaling.

We used the social African cichlid fish, *Astatotilapia burtoni*, to investigate how visual and chemosensory signals alone and in combination from reproductively-receptive females influence the behavior, brain activation patterns, and hormonal responses of dominant males. *A. burtoni* is ideally suited for this inquiry because dominant males engage in elaborate, specific courtship behaviors in the presence of receptive females that includes sending information via multiple sensory channels (Maruska and Fernald, 2018), and males alter their courtship efforts based on distinguishing receptive from non-receptive females (Fernald and Hirata, 1977). Importantly, both males and females actively control urine release as a means of social communication in both aggressive and reproductive contexts, providing evidence for true chemosensory communication in this species (Maruska and Fernald, 2012; Field and Maruska, 2017). During reproduction, males increase urination in the presence of receptive females, while receptive females also increase urine release towards courting dominant males. Thus, while vision is the main sensory modality for communication in *A. burtoni*, chemosensory signals provide additional information for both males and females to modify context-dependent social decisions (Maruska and Fernald, 2012; Field and Maruska, 2017). How unimodal and multimodal signals from these two senses influence male behavior, physiology and brain activation patterns in socially-relevant nuclei, however, remains unexplored in this and the majority of fish species.

How relevant sensory and social information is integrated with an animal's own internal physiology to elicit context-specific behaviors is a key goal of behavioral neuroscience (Insel, 2010). The social decision making network (SDMN) is a collection of highly conserved brain nuclei proposed as a framework for examining where and how this information leads to adaptive behaviors (Newman, 1999; O'Connell and Hofmann, 2011), but it is increasingly clear that many other brain regions outside of this network are also involved in social decisions. Our current knowledge of how multimodal sensory signals are processed in the brain of receivers to induce behavioral responses is limited. By associating specific behavioral output with neural activation patterns of receivers in response to unimodal and multimodal signals, we can help bridge this knowledge gap in social neuroscience. Further, little is known about where sexually-relevant chemosensory signals are processed in the brain above primary olfactory processing regions, especially in fishes (Nikonov and Caprio, 2005; Yaksi et al., 2009; Yabuki et al., 2016). Thus, by examining neural activation patterns in socially-relevant brain regions as a complement to behavioral responses in receivers, we can provide a framework to expand the current knowledge of the neural substrates that link sensory inputs to behavioral outputs.

To investigate how sexually-active males respond to unimodal and multimodal visual-chemosensory reproductive signals, we exposed dominant courting males to visual and chemosensory signals from gravid females either alone or combined, and recorded males' behavioral and physiological responses. Further, we used *in situ* hybridization for the immediate early gene *cfos* to test for differences in neural activation of relevant brain nuclei in males receiving unimodal and multimodal signals from receptive females. This approach allows us to elucidate the brain regions important for processing visual and chemosensory signals alone, and those involved in integrating information from both sensory channels when presented together in a naturalistic reproductive context.

MATERIALS AND METHODS

Experimental Animals

Adult *Astatotilapia burtoni* (Günther, 1894) from a wild-caught stock were kept in aquaria under water and lighting conditions that are similar to their natural habitat in Lake Tanganyika, Africa (28°C; pH 8.0; 12 h light:12 h dark cycle). These fish were bred in laboratories since original collection in the 1970s and exhibit behaviors similar to those in wild populations (Fernald and Hirata, 1977). Aquaria contained gravel-covered floors and halved terra cotta pots to serve as shelters and spawning territories. Fish were fed cichlid flakes (AquaDine, Healdsburg,

CA, USA) daily and supplemented with brine shrimp twice a week. All experiments were performed in accordance with the recommendations and guidelines provided by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 2011. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA, USA.

Experimental Protocol

Dominant males (standard length (SL): 43.19 ± 2.19 mm (mean ± SD)) were housed in aquaria in mixed broods prior to being selected for experiments. Experiments were conducted in 37.85 L aquaria that were divided into three equal sized compartments (16.7 × 25.3 × 30.8 cm each) by clear, acrylic barriers permanently sealed into the tank (**Figure 1**) and verified to block transmission of water and chemosensory cues as in our previous study (Field and Maruska, 2017). Each compartment contained a layer of gravel at the bottom, an air stone, and a territory/shelter (half terracotta pot). All experimental compartments were drained, cleaned, and refilled between experiments to ensure no cross-experiment contamination of odorants. Prior to experiments, focal dominant males were selected based on bright coloration and behaviors typical of dominance such as defending territories and actively courting females for at least three consecutive days. Males were then moved to the center experimental compartment and allowed to acclimate for 48 h while visually exposed to a community consisting of one male (smaller than focal dominant male) and three females in the right compartment while a movable black opaque barrier visually blocked the empty left compartment. The center experimental compartment contained the chemosensory delivery tube throughout the entire acclimation period of the focal male.

To examine responses of focal males to unimodal and multimodal visual and chemosensory signals, we presented visual and chemosensory stimuli in different combinations. Visual signals were provided in the left compartment and consisted of the presence of a gravid female or no fish (empty compartment). Chemosensory signals were delivered through a tube connected to a gravity feed bottle and consisted of 850 mL of gravid female-conditioned water or 850 mL of reverse osmosis (RO)-filtered water as a control (see below). The flow rate of the chemosensory stimulus was tested the day before and morning of experiments and was verified to be 0.325 ± 0.25 L/min. The stimuli were presented in the following combinations (visual in left compartment/chemosensory in center compartment): (1) no fish/RO-filtered water (termed "control"); (2) gravid female/RO-filtered water (termed "vision only"); (3) no fish/female-conditioned water (termed "chemosensory only"); and (4) gravid female/female-conditioned water (termed "vision and chemosensory"). For clarity, **Figure 1B** outlines terminology of the stimulus delivery used throughout this study.

To determine whether focal male responses were mediated by olfaction or gustation (taste), we also tested anosmic (ablation of olfactory sense) focal males in the same experimental paradigm in the chemosensory only condition. Males (n = 3; SL: 43.2 ± 0.70 mm; gonadosomatic index (GSI) > 0.70) were rendered anosmic 2 days prior to behavioral experiments by sedating and immobilizing the fish via gradual cooling in ice-cold cichlid-system water and then bilaterally severing the olfactory nerves between the olfactory epithelia and olfactory bulbs. Anosmia was verified by lack of *cfos* expression in the olfactory bulb and reduced *cfos* expression in the posterior nucleus of the dorsal telencephalon (Dp), a forebrain region important in olfactory processing. Three sham-handled males were included to ensure effects in anosmic males were not due to the handling procedure. Sham males had the tissue covering their olfactory nerve cut as anosmic males did, but without causing any damage to the olfactory nerve. Incisions for both anosmic and sham-handled fish were sealed with Vetbond™. For behavioral testing, anosmic and sham males received only chemosensory signals from gravid females (no visual signal). The behavior of sham-handled males was verified as not different from intact males exposed to the same sensory conditions (chemosensory stimulus only).

Gravid (ripe with eggs) females (SL: 37.5 ± 2.1 mm, GSI > 7.0) used as visual stimuli were selected the morning of experiments prior to feeding based on the presence of a distended abdomen due to the presence of large ova. GSI ([gonad mass/(body mass-stomach mass)*100]) and ovulation state of females were verified after experiments. For female-conditioned water used for chemosensory stimuli, four gravid females were selected in the morning prior to feeding and placed in a bucket with an air stone and allowed to soak in RO water for 5 h. All solid materials (feces and/or algae) were removed from the water before stimulus delivery. The control RO-filtered water stimulus was also soaked for 5 h in an identical (but separate) bucket with an air stone for 5 h to match handling of female-conditioned water.

On the morning of experiments (08:30–09:30 a.m.), the focal male and visual stimulus female (if present) were fed two flakes of cichlid food, and the chemosensory stimuli were prepared. Following the 5 h. soaking period (at 1:30–2:30 p.m.), the gravid females (if present) were removed from the soaking bucket, and the stimulus water was transferred into a gravity feed bottle. The black barrier was simultaneously removed and placed between the experimental and community compartments such that the focal male was visually exposed to the left stimulus compartment and visually blocked from the community on the right. Experiments lasted 40 min from the start of chemosensory stimulus delivery.

Behavioral Quantification

Focal male behaviors during the first 15 min of each experiment were scored using BORIS software[1]. Only the first 15 min were quantified because behaviors declined after this point and were therefore not representative of the stimulus-evoked response of the focal male. The male behaviors quantified were number of courtship behaviors and time spent performing searching behavior (increased swimming activity/arousal). Courtship behaviors included body quivers, tail waggles, and leads towards the shelter. Searching was defined by increased swimming speed

[1]http://www.boris.unito.it/

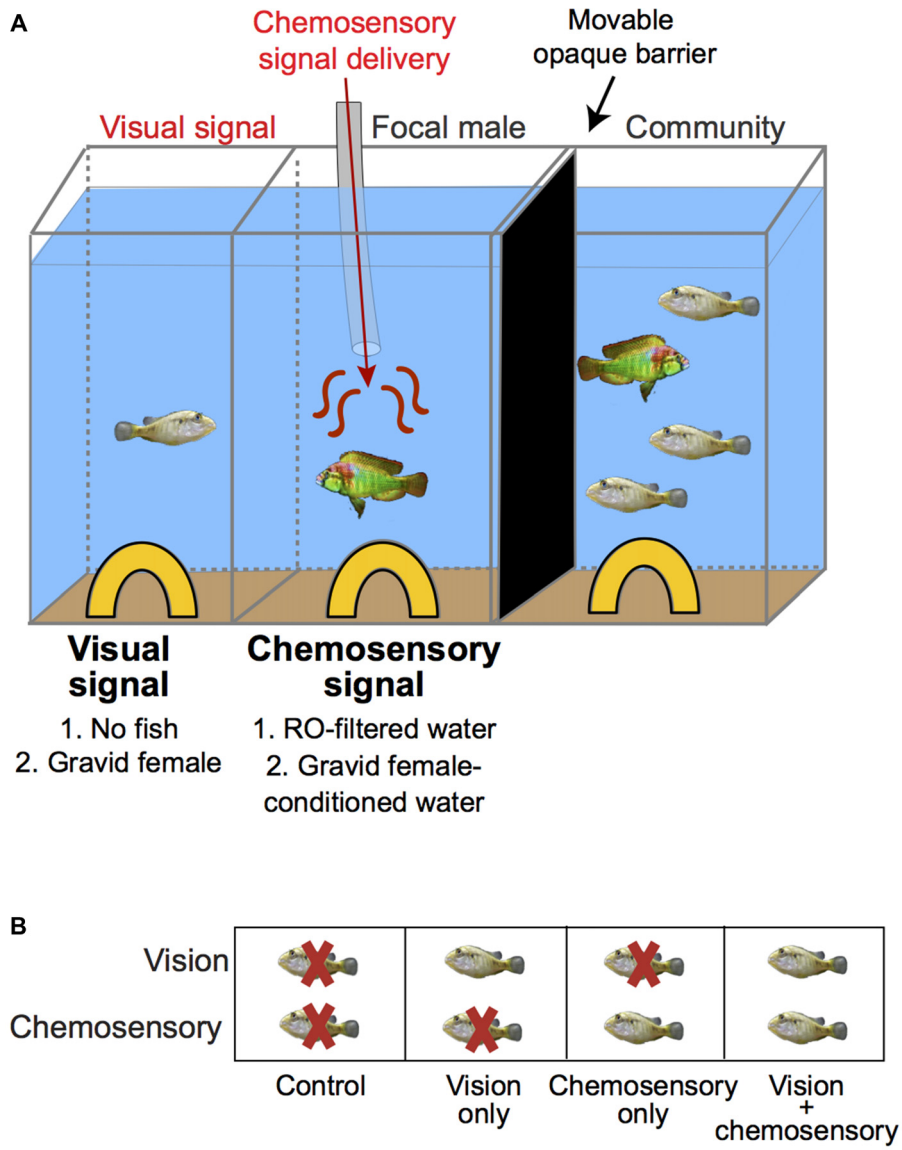

FIGURE 1 | Experimental paradigm to examine behavioral responses and neural activation patterns in dominant male *A. burtoni* exposed to unimodal and multimodal visual and chemosensory signals from females. **(A)** Focal males were acclimated to the center compartment with chemosensory stimulus delivery tube present. A movable black barrier visually blocked the left visual stimulus compartment while allowing visual exposure to a fish community consisting of one male and three females in the right compartment for 24–48 h before experiments. Immediately before experiments, the black barrier was moved to visually block the community on the right and expose the left compartment. Visual signals were presented in the left compartment and chemosensory signals were simultaneously delivered to the center compartment via a gravity-feed bottle at a controlled flow rate. **(B)** The combinations of visual and chemosensory stimuli presented to males are shown with terminology used to describe each condition indicated below boxes: "Control" refers to no fish/reverse osmosis (RO)-filtered water, "Visual only" refers to gravid female/RO-filtered water; "Chemosensory only" refers to no fish/gravid female-conditioned water, and "Visual and chemosensory" refers to gravid female/gravid female-conditioned water.

with at least one change in direction in the water column (up or down) that lasted at least 3 s. Bouts of searching were separated by pauses in swimming that lasted at least 2 s.

Tissue Preparation

Focal males were collected after 40 min of stimulation, sedated and immobilized in ice-cold cichlid-system water, and sacrificed by rapid cervical transection. SL, body mass, stomach mass, gonad mass and GSI were recorded. All dominant focal males had a GSI > 0.70 and visual stimulus females had a GSI > 7.0. Blood was collected from the caudal vein and centrifuged at 8,000 rpm for 10 min to isolate serum, and then stored at $-80°C$. Brains were removed and fixed overnight in 4% paraformaldehyde (PFA) made in 1× PBS, rinsed overnight in 1× PBS, and cryoprotected in 30% sucrose for 1–5 nights prior to sectioning (all at 4°C). Brains were embedded in OCT media (TissueTek),

sectioned coronally at 20 μm with a cryostat, collected on alternate charged slides (Superfrost Plus, VWR), dried overnight at room temperature, and stored at −80°C.

In situ Hybridization for *cfos*

To examine differences in neural activation in the brains of behaving focal males, we performed colorimetric *in situ* hybridization for the immediate early gene *cfos* using riboprobes specific for *A. burtoni cfos* mRNA as previously described (Butler and Maruska, 2016). We chose *cfos* as a marker for this study because our focus was to examine which brain regions received unimodal and multimodal visual-chemosensory inputs, rather than the brain regions involved in the expression of behavioral outputs. It was previously shown in zebrafish that *cfos* is an ideal marker for determining neural activity associated with processing perceptual stimuli from the social environment (Teles et al., 2015). Briefly, slides of sectioned brains were rinsed at room temperature in 1× PBS, fixed with 4% PFA, rinsed with 1× PBS, treated with proteinase K (10 μg/mL), rinsed with 1× PBS, fixed with 4% PFA, rinsed with 1× PBS followed by milliQ water, treated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCL (pH 8.0), and rinsed with 1× PBS. Slides were then incubated in warmed pre-hybridization buffer at 60–65°C for 3 h. Subsequently, slides were then incubated with warmed hybridization buffer containing *cfos* riboprobe at 60–65°C for 12–16 h sealed with HybriSlip covers in sealed humidified chambers. Then, at this same temperature, sections were washed in 2× SSC in 50% formamide with 0.1% Tween-20, followed by a 1:1 mixture of 2× SSC and maleic acid buffer with 0.1% Tween-20 (MABT). MABT washes were then performed again at room temperature. Non-specific binding was blocked with MABT with 2% bovine serum albumin (BSA) for 3 h and then slides were incubated with alkaline phosphatase (AP) anti-DIG fragments (Sigma Aldrich) overnight at 4°C in a sealed humidified chamber. Slides were then rinsed in MABT at room temperature, incubated in AP buffer, and then developed with nitro-blue tetrazolium/5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP; Sigma Aldrich) substrate at 37°C in darkness for 2–3 h. Slides were then rinsed in 1× PBS, fixed in 4% PFA, washed in 1× PBS, and coverslipped with aquamount aqueous mounting media (Fisher Scientific).

Quantification of *cfos*-Expressing Cells

To quantify differences in *cfos* staining in the brain, slides were visualized on a Nikon Eclipse Ni microscope and photographed with a color digital camera controlled by Nikon NIS-Elements software. Brightfield and phase contrast were used to visualize neuroanatomical markers and brain nuclei in relation to stained cells. Quantifications were done by an observer blind to experimental condition. *cfos*-positive cells were easily identifiable by dark purple staining inside the cell with a clear, discernible border. Final images were adjusted for levels, contrast, and brightness in Adobe Illustrator CC v21.10. Neuroanatomical structures were identified using a cresyl violet stained *A. burtoni* reference brain and *A. burtoni* brain atlas. Stereotactic and neuroanatomical markers were used to designate the borders and rostro-caudal extent of each region to ensure consistency across animals. The following socially-relevant regions of the brain were quantified: ventral nucleus of the ventral telencephalon (Vv), supracomissural nucleus of the ventral telencephalon (Vs), dorsal part of the ventral telencephalon (Vd), granular zone of lateral part of the dorsal telencephalon (Dl-g), fourth and fifth subdivisions of central part of the dorsal telencephalon (Dc-4 and Dc-5), anterior tuberal nucleus (ATn), anterior part of the periventricular preoptic area (nPPa). The posterior nucleus of the dorsal telencephalon (Dp) was also quantified, as it is an important olfactory processing region. Images were taken at the highest magnification (10× or 20× objective) that encompassed the entire area of interest. For 10× images (Vs, Dl-g, Dp and ATn), nuclei borders were outlined with either 50 μm × 50 μm gridlines (Dp, ATn) or 80 μm × 80 μm gridlines (Vs, Dl-g) applied to each image. *cfos*-expressing cells in five randomly chosen boxes per section were counted for Vs and Dl-g and four randomly chosen boxes for Dp and ATn and cell density calculated by dividing the number of cells within the boxes by the total area of the boxes. For 20× images (Vv, Vd, nPPa, Dc-4 and Dc-5), the same procedure was followed, except nuclei borders were overlaid with 50 μm × 50 μm grid lines and *cfos*-expressing cells were counted in three boxes. For all regions, four consecutive sections were quantified for each region and averaged together for a cell density value (#cells/μm^2) of that region for each animal. Alternate sections were used for quantification so that adjacent 20 μm sections were separated by 40 μm (and cell diameters are ∼<10–25 μm on average) ensuring no double counting of cells. Density values were then averaged across individuals exposed to the same sensory stimulus conditions.

Steroid Hormone Assays

To test for differences in circulating sex-steroid hormones among visual-chemosensory conditions, plasma 11-ketotestosterone (11-KT) and estradiol (E_2) were measured using Enzyme ImmunoAssay (EIA) kits (Cayman Chemical Inc.), as previously described and validated for *A. burtoni* (Maruska and Fernald, 2010b). For both steroids, 4.4 μl of plasma from each focal male was extracted three times using 220 μl of ethyl ether and evaporated under a fume hood prior to re-constitution in assay buffer (1:50 dilution). Kit protocols were then strictly followed, plates were read at 405 nm using a spectrophotometer microplate reader and steroid concentrations determined based on standard curves.

Statistical Analysis

Behavior and neural activation data were compared with one-way ANOVAs. Focal male courtship and searching behavior could not be normalized by transformation and were compared with non-parametric Kruskal-Wallis (KW) one-way ANOVA on Ranks with Dunn's *post hoc* tests (α =0.05). Behavior and neural activation in anosmic and intact males were compared using student's *t*-tests. To compare searching behavior in anosmic and intact males, data were log-transformed to pass equal variance. To test for differences in neural activation across sensory stimulus conditions, the density of *cfos* expressing cells in each brain nucleus was compared with parametric one-way

ANOVA with SNK *post hoc* tests. Cell density data that could not be transformed (one brain region only, Dc-5) were compared with KW one-way ANOVA on Ranks with Dunn's *post hoc* test. Pearson correlation tests were used to test for relationships among *cfos*-labeled cell densities across brain regions, and with courtship and search behavior to generate co-activation heat maps for each stimulus combination. Factor analyses were done using principal component extractions with Eigenvalues >1 and components plotted in rotated space (varimax rotation). For discriminant function analysis (DFA), any missing values were replaced with the group mean (Dc-5 only). Significant outliers detected by Grubb's test were removed prior to all comparisons (Dc-5: one outlier removed from *cfos* quantification). Steroid hormone levels were analyzed across stimulus condition for intact and anosmic males using ANCOVA with body size as a covariate. Statistical comparisons were performed in SigmaPlot 12.3 or SPSS 24.

RESULTS

Behavioral Response to Unimodal and Multimodal Visual and Chemosensory Signals

Focal males did not perform any courtship behaviors (body quivers, tail waggles, leads to shelter) in control and chemosensory only conditions (**Figure 2A**). In vision only conditions, focal males performed more courtship behaviors (0–9 total courtship behaviors) than both control and chemosensory only conditions. However, the number of courtship behaviors was significantly higher when focal males received visual and chemosensory signals together (KW one-way ANOVA on Ranks, $H = 22.212$, $df = 3$, $P < 0.001$; Dunn's $P < 0.05$; **Figure 2A**). Thus, dominant *A. burtoni* males must see a receptive female to engage in specific courtship behaviors, but courtship is enhanced when visual signals are paired with chemosensory signals from females.

Searching behavior was observed in all focal males across all stimulus conditions. Focal males spent significantly more time searching in vision only, chemosensory only, and visual-chemosensory conditions compared to controls, but searching did not differ among the three non-control conditions (KW one-way ANOVA on Ranks, $H = 12.645$, $df = 3$, $P = 0.005$; Dunn's $P < 0.05$; **Figure 2B**).

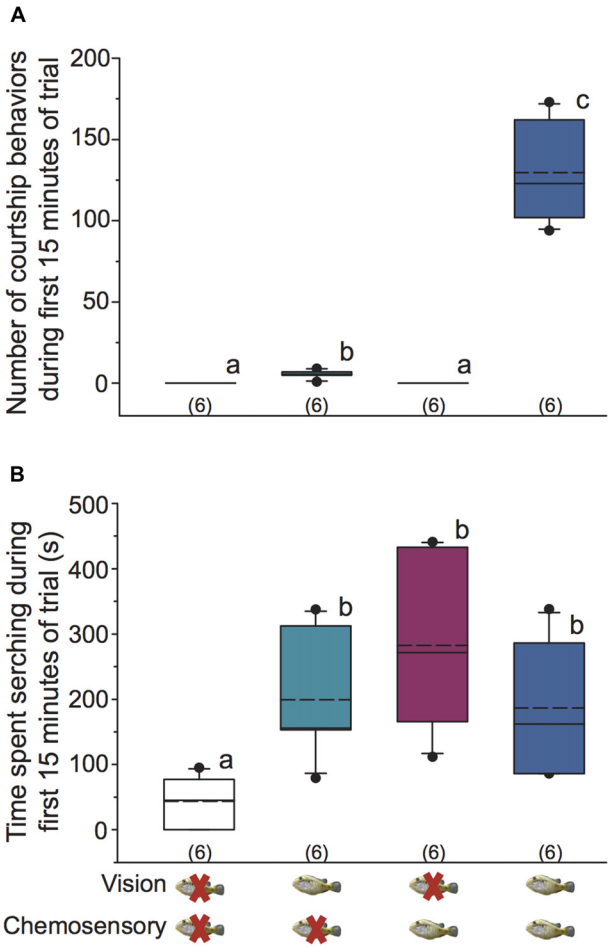

FIGURE 2 | Visual and chemosensory signals from gravid females induce distinct behaviors in dominant *A. burtoni* males. **(A)** Focal males require a visual signal from females to engage in courtship behaviors, but visual signals alone are sufficient to induce some courtship. Male courtship behaviors are dramatically increased when both visual and chemosensory signals from females are present. **(B)** Focal males spend more time searching when a visual, chemosensory, or paired visual-chemosensory signal from females is present compared to control conditions. Different letters indicate statistical differences at $P < 0.05$ (Kruskal-Wallis (KW) one-way ANOVA on Ranks, SNK *post hoc*). Box plots were used to represent data: solid line indicates the median and dashed line indicates the mean. The box extends to the furthest data points within the 25th and 75th percentile, and whiskers extend to the 10th and 90th percentile. Closed circles indicate data points outside the 5th and 95th percentile. Sample sizes are shown in parentheses.

Neural Activation

Figure 3 shows representative low magnification cresyl violet-stained transverse sections from *A. burtoni* with locations of relevant regions quantified for neural activation (measured as *cfos* cell density) in this study. Focal males exposed to unimodal and multimodal visual and chemosensory signals from females showed differential neural activation patterns in regions that process sensory inputs and mediate social decisions. For example, activation in Vv was significantly higher when a visual signal was present regardless of whether or not a chemosensory signal was present (one-way ANOVA $F_{(3,20)} = 8.516$, $P < 0.001$; SNK $P < 0.05$; **Figure 4A**). Activation in Vd also showed differences depending on stimuli, with greater neural activation when a chemosensory signal was present whether or not a visual signal was present (one-way ANOVA $F_{(3,20)} = 16.627$, $P < 0.001$; SNK $P < 0.05$; **Figure 4B**). Activation in Vs was higher in the chemosensory only condition compared to the control, but did not differ from the visual only condition. Further, Vs activation was greater when multimodal visual-chemosensory signals were presented compared to all other conditions (one-way ANOVA $F_{(3,20)} = 11.262$, $P < 0.001$; SNK $P < 0.05$; **Figure 4C**). Activation in Dl-g in chemosensory only and visual-chemosensory conditions was greater than that in controls, but

the visual only condition did not differ from either of these or the control condition (one-way ANOVA $F_{(3,20)} = 3.317$, $P = 0.041$; SNK $P < 0.05$; **Figure 5A**). Dp had greater activation when a chemosensory stimulus was present, regardless of whether or not a visual signal was present, compared to controls (one-way ANOVA $F_{(3,20)} = 4.565$, $P = 0.014$; SNK $P < 0.05$; **Figure 5B**). For nPPa, activation was greater when visual or chemosensory unimodal signals were present compared to the control. Further, when visual and chemosensory signals were presented together there was greater activation in nPPa compared to all other conditions (one-way ANOVA $F_{(3,20)} = 41.205$, $P < 0.001$; SNK $P < 0.05$; **Figure 5C**). In ATn, activation was greater in all stimulus conditions compared to the control, but stimulus conditions did not differ from one another (one-way ANOVA $F_{(3,20)} = 7.967$, $P = 0.001$; SNK $P < 0.05$; **Figure 5D**). There was a significant difference in activation in Dc-5 across sensory stimulus conditions, but *post hoc* tests were unable to detect differences (KW one-way ANOVA on Ranks, $H = 9.778$, $df = 3$, $P = 0.021$, Dunn's $P > 0.05$). No significant differences in activation occurred among stimulus conditions in Dc-4 (one-way ANOVA $F_{(3,20)} = 0.424$, $P = 0.738$).

Behavior and Neural Activation in Anosmic Males

We used anosmic males to test whether behavioral responses were mediated by olfaction or taste. Anosmia was verified in males by absence of *cfos* staining (no neural activation) in the inner cellular layer of the olfactory bulb, indicating no transmission of sensory information from the olfactory epithelium to the olfactory bulb when the olfactory nerves were severed (see **Figure 6** for example *cfos* staining in the olfactory bulb). Anosmic males presented with only chemosensory signals from females (no visual signal) showed reduced *cfos* staining in the ICL compared to intact males that received the same stimulus (**Figure 6A**). Further, anosmic males spent less time searching compared to intact focal males (student's *t*-test, $P = 0.002$; **Figure 6B**) and also had fewer *cfos*-stained cells in Vd (student's *t*-test, $P = 0.039$) and Vs (student's *t*-test, $P = 0.009$) as well as in Dp (a known olfactory processing region) compared to intact focal males (student's *t*-test, $P = 0.003$; **Figures 6C–E**).

Hormone Responses of Intact and Anosmic Males

We measured circulating levels of 11-KT and E_2 in intact males exposed to uni- and multimodal visual and chemosensory signals, and in anosmic males. There was no difference in either hormone among males in any experimental group (11-KT: ANCOVA $F_{(5,22)} = 1.203$, $P = 0.34$; E_2: ANCOVA $F_{(6,19)=}$ 1.892, $P = 0.135$).

Correlations and Multivariate Analyses of Brain Regions and Social Behaviors

To investigate functional connectivity of the examined brain regions and how it relates to expression of social behaviors, we created heat maps from Pearson correlation coefficients of *cfos* cell density (**Figure 7**) and number of social behaviors

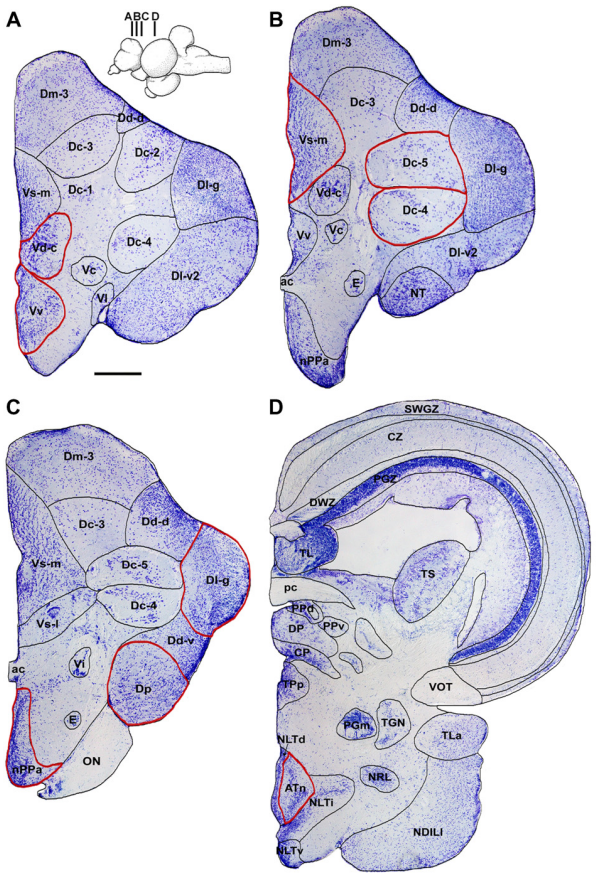

FIGURE 3 | Representative transverse sections from the *A. burtoni* brain showing the locations of relevant nuclei quantified in this study. Cresyl violet-stained sections are shown from rostral **(A)** to caudal **(D)** and nuclei are outlined and labeled. Only the right half of the brain is shown for each, relevant quantified regions are outlined in red, and inset shows the approximate locations of each section on a sagittal brain. Scale bar = 250 μm. Abbreviations: ac, anterior commissure; ATn, anterior tuberal nucleus; CP, central posterior thalamic nucleus; CZ, central zone of tectum; Dc-1–5, central part of the dorsal telencephalon, subdivisions 1–5; Dd-d, dorsal part of the dorsal telencephalon, dorsal subdivision; Dd-v, dorsal part of the dorsal telencephalon, ventral subdivision; Dl-g, granular zone of lateral zone of the dorsal telencephalon; Dl-v2, ventral part of the lateral zone of the dorsal telencephalon, subdivision 2; Dm-3, medial part of the dorsal telencephalon, subdivision 3; Dp, posterior part of the dorsal telencephalon; DP, dorsal posterior thalamic nucleus; E, entopeduncular nucleus; NDILl, lateral part of the diffuse nucleus of the inferior lobe; NLTd, lateral tuberal nucleus, dorsal part; NLTi, lateral tuberal nucleus, intermediate part; NLTv, lateral tuberal nucleus, ventral part; nPPa, parvocellular preoptic nucleus, anterior part; NRL, nucleus of the lateral recess; NT, nucleus taenia; ON, optic nerve; pc, posterior commissure; PGm, medial preglomerular nucleus; PGZ, periventricular gray zone of tectum; PPd, dorsal periventricular pretectal nucleus; PPv, ventral periventricular pretectal nucleus; SWGZ, superficial gray and white zone of tectum; TGN, tertiary gustatory nucleus; TL, torus longitudinalis; TLa, nucleus of the torus lateralis; TPp, periventricular nucleus of the posterior tuberculum; TS, torus semicircularis; Vc, central part of the ventral telencephalon; Vd-c, dorsal part of the ventral telencephalon, caudal subdivision; Vi, intermediate nucleus of the ventral telencephalon; Vl, lateral part of the ventral telencephalon; VOT, ventral optic tract; Vs-l, lateral part of the supracommissural nucleus of the ventral telencephalon; Vs-m, medial part of the supracommissural nucleus of the ventral telencephalon; Vv, ventral part of the ventral telencephalon.

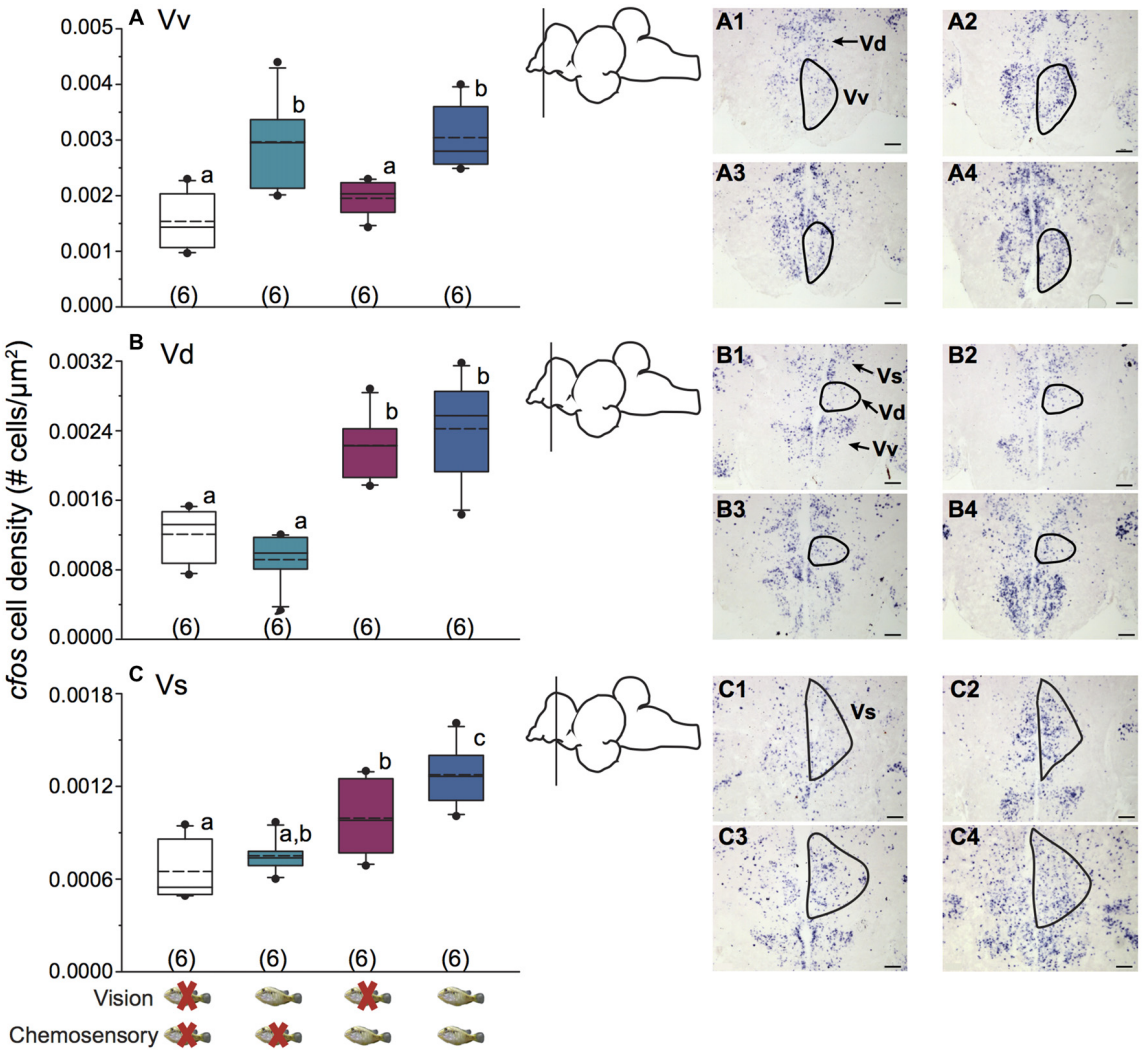

FIGURE 4 | Unimodal and multimodal visual and chemosensory signals from females elicit distinct neural activation patterns in ventral telencephalic brain regions of dominant *A. burtoni* males. **(A)** Focal males show greater *cfos* expression in Vv when a visual signal is present, regardless of whether a chemosensory signal is present or not. **(B)** There is greater *cfos* expression in Vd when a chemosensory signal is present, regardless of whether a visual signal is present or not. **(C)** Neural activation in Vs is greater in the unimodal chemosensory signal condition compared to control, but it did not differ from the visual only condition. Activation in Vs was also greater in visual-chemosensory compared to all other conditions. Photos show representative examples of *cfos* staining in each region for control conditions (1), visual only (2), chemosensory only (3), and visual and chemosensory (4). Outlines demonstrate approximate quantified area for each region. Scale bars represent 100 μm. Schematics show a lateral view of the brain with the approximate location of each region. Different letters indicate statistical significance at $P < 0.05$ (one-way ANOVA). See **Figure 2** for box plot descriptions.

for each sensory condition (**Figure 8**). In control and visual only conditions, there were no significant correlations between any brain regions (**Figures 7A,B**). In the visual only condition, courtship behavior positively correlated with activation in nPPa and Vv (**Figure 8**). When only chemosensory signals were present, negative correlations were observed between activation in Vv and Dp as well as between nPPa and Dl-g (**Figure 7C**). However, there was a positive correlation between searching behavior and neural activation in both nPPa and Dl-g (**Figure 8A**). When both visual and chemosensory signals were present, Vv, Vs and nPPa were positively correlated with each other (**Figure 7D**) and with courtship behaviors (**Figure 8B**). **Tables 1–4** show Pearson correlation coefficients and *P* values for *cfos* cell densities among brain regions for each condition. **Tables 5**, **6** show correlation coefficients and *P* values among brain regions and behaviors in relevant conditions.

To examine whether patterns of neural activation could correctly classify individual focal males into their respective sensory stimulus condition, we performed canonical DFA and principal component analysis (PCA) on neural activation data for all investigated brain regions (**Figure 9**). DFA weights variable inputs (activation of brain regions) and determines if animals can be sorted into groups based on these variables, and identifies which variables may contribute to this sorting. Our

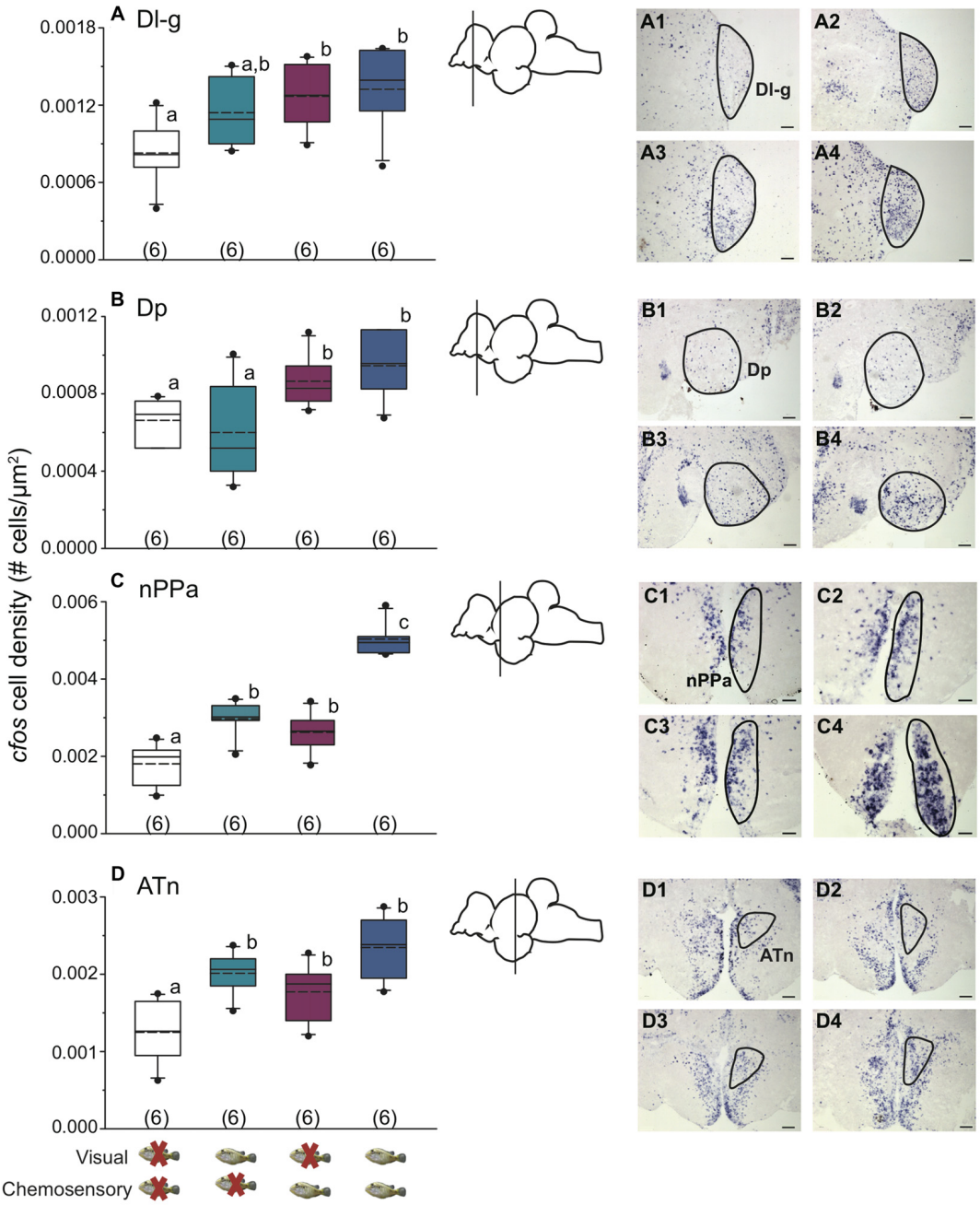

FIGURE 5 | Unimodal and multimodal visual and chemosensory signals from females elicit distinct neural activation patterns in dorsal telencephalic and hypothalamic brain regions of dominant *A. burtoni* males. **(A)** In Dl-g, there is greater *cfos* expression in chemosensory only and visual-chemosensory conditions compared to controls, with activation in vision only conditions not different from either of these conditions or controls. **(B)** There is higher *cfos* expression in Dp when a chemosensory signal is present, regardless of whether a visual signal is present or not. **(C)** nPPa shows the greatest neural activation when both visual and chemosensory signals from females are present. **(D)** In ATn, focal males show higher *cfos* expression in all visual-chemosensory sensory conditions compared to controls. Photos show representative examples of *cfos* staining in each region for control conditions (1), visual only (2), chemosensory only (3), and visual and chemosensory (4). Outlines demonstrate quantified area for each region. Scale bar in **(A)** represents 50 μm. Scale bars in **(B–D)** represent 100 μm. Schematics show a lateral view of the brain with approximate location of each region. Different letters indicate statistical significance at $P < 0.05$ (one-way ANOVA). See **Figure 2** for box plot descriptions.

DFA correctly classified 100% of animals into their respective groups (control, vision only, chemosensory only, vision and chemosensory; **Figure 9A**). Function 1 was driven positively by nPPa and Dp and explained 67.6% of the variance. Function 2 was driven positively by Vd and Dc-5 but negatively by nPPa and Vv, and accounted for 29.2% of the variance. Together

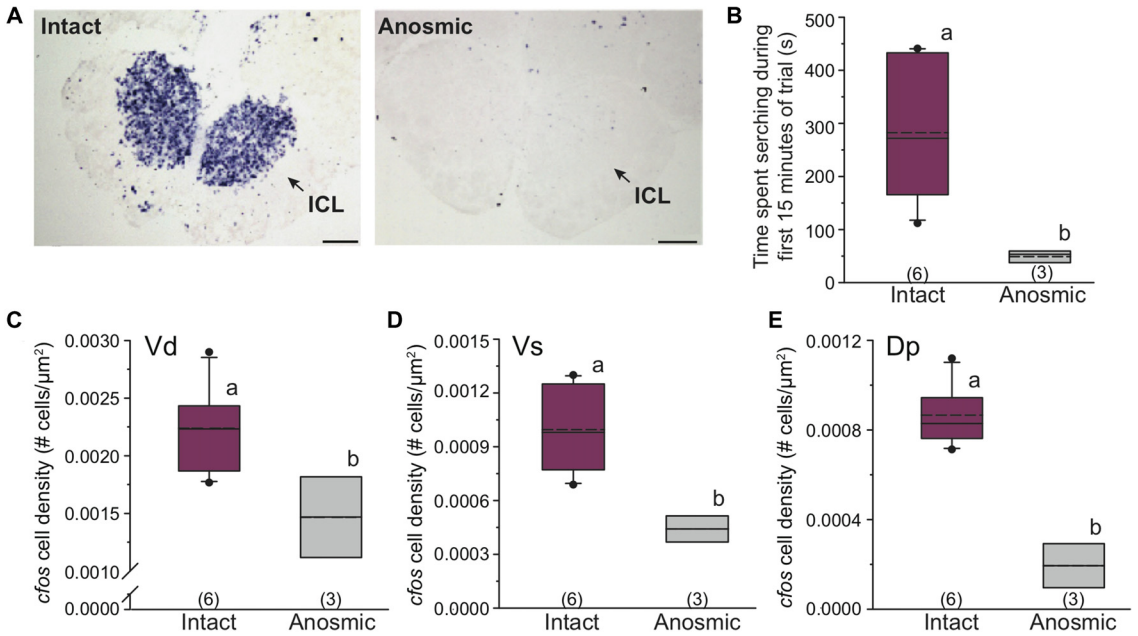

FIGURE 6 | Anosmic dominant males show altered searching behavior and reduced *cfos* expression in known olfactory processing regions of the brain. **(A)** Anosmic males show decreased *cfos* expression (purple staining) in the olfactory bulb compared to intact males. Arrows indicate inner cellular layer of the olfactory bulb. **(B)** Anosmic males receiving only chemosensory signals from gravid females spend less time searching compared to intact males. Anosmic males also have reduced neural activation (*cfos* cell density) in Vd **(C)**, Vs **(D)** and Dp **(E)** compared to intact males receiving the same chemosensory only stimulus. Scale bars in **(A)** represent 100 μm. Different letters indicate statistical significance at $P < 0.05$. See **Figure 2** for box plot descriptions.

functions 1 and 2 explained 96.8% of the variance and separated males into the four sensory stimulus groups based on neural activation patterns alone. PCA of *cfos* activation in examined brain regions produced two significant components driving variability in the data (**Figure 9B**; $N = 24$ animals; Kaiser-Meyer-Olkin measure of sampling adequacy = 0.577; Bartlett's test of sphericity chi-squared = 101.693, $df = 36$, $P < 0.001$). Component 1 accounted for 42.8% of variance and was strongly weighted by ATn, Vv and nPPa. Component 2 accounted for 17.982% of variance and was loaded by Dp and Vd. Based on the regions driving each node, components 1 and 2 likely represent visual and chemosensory inputs, respectively. A summary of differences in neural activation in each region with each unimodal and multimodal sensory input (compared to control conditions) is shown in the Venn diagram of **Figure 9C**.

DISCUSSION

We investigated behavioral, physiological and neural responses of dominant *A. burtoni* males to uni- and multimodal visual and chemosensory signals from reproductively-receptive females. Our results show that males need sexually-relevant visual signals from females to engage in stereotypical courtship behaviors such as body quivers, tail waggles, and leads into the spawning territory. However, the number of courtship behaviors was greater when males were simultaneously exposed to visual and chemosensory signals from females, compared to either sensory signal alone. When a female visual signal was absent, males showed increased searching activity in response to female-conditioned water compared to control water, suggesting that these chemosensory signals may stimulate male motivation. Importantly, we also tested anosmic (olfactory ablated) males to demonstrate that this searching behavior is primarily mediated by the olfactory system rather than gustation. Using the immediate early gene *cfos* as a proxy for neural activation, we also revealed that decision and olfactory processing regions show differential activation when dominant males are exposed to visual and chemosensory signals together compared to exposure of either sensory signal alone.

Behavioral and Physiological Responses to Uni- and Multimodal Signals

By examining behavioral responses to both uni- and multimodal visual-chemosensory signals, we show that male *A. burtoni* must see a female to perform courtship behaviors, but courtship is dramatically increased when chemosensory information is also available. It is well known that animals across taxa use multimodal signals for communication, particularly in courtship and reproductive contexts (Darwin and Prodger, 1998). A multimodal signal often benefits the receiver by allowing for better detection and localization of the signaler (Hasson, 1989), reduced habituation of individual signals (Todt and Fiebelkorn, 1980), and potential priming of one signal by another (Partan and Marler, 2005). The classification of

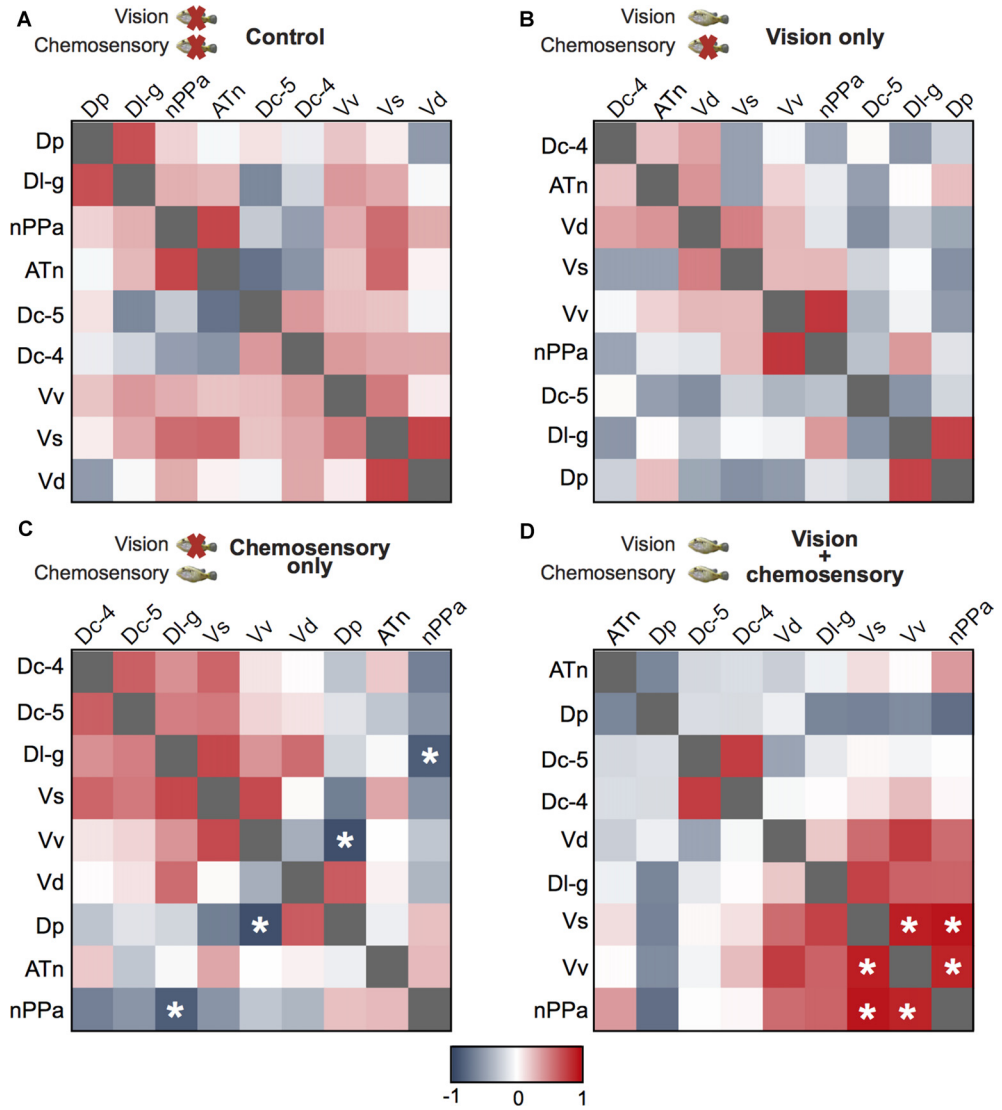

FIGURE 7 | Males exposed to unimodal and multimodal visual and chemosensory signals from gravid females show different co-activation patterns in the brain. Heat maps of Pearson correlation coefficients (R = color scale) of *cfos* staining in brain nuclei in **(A)** control conditions; **(B)** unimodal visual only; **(C)** unimodal chemosensory only; and **(D)** multimodal visual and chemosensory. Heat maps ordered based on hierarchical clustering of brain regions for each condition. See **Tables 1**–**4** for R and P values. Asterisks indicate significance at $P < 0.05$.

multimodal signals into two broad categories, redundant and non-redundant, is based on the receiver's response to each unimodal component separately and the response to the combined multimodal signal (Partan and Marler, 1999). In a simplified description, redundant signals are those that "mean the same thing" or result in the same response alone and together, while non-redundant signals "carry multiple messages" or result in altered responses (Moller and Pomiankowski, 1993; Johnstone, 1996; Partan and Marler, 2005). Based on these criteria, visual-chemosensory multimodal signals from gravid *A. burtoni* females are non-redundant because males' behavioral responses to each unimodal signal and to the combined multimodal signal are all different, demonstrating that they convey different information. Vision alone elicits courtship behaviors while smell alone does not, however smell alone does induce increased searching behavior. In addition, dual visual-chemosensory signals result in increased courtship behavior compared to visual only conditions, while maintaining the searching behavior. This altered behavioral response from a multimodal signal is called "modulation," and thus, visual-chemosensory signals in *A. burtoni* are classified as non-redundant modulatory signals. The processing of signals from multiple sensory modalities often results in responses that are larger than the sum of responses from the individual signals. This response profile, termed "super-additive response" is a generally common occurrence with even weak stimuli resulting in strong super-additive responses (Stein and Stanford, 2008; Angelaki et al., 2009). Thus, males likely get arousal

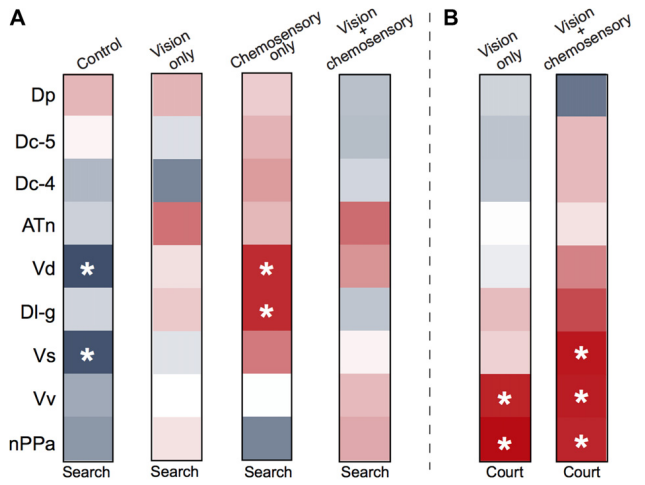

FIGURE 8 | Males exposed to unimodal and multimodal visual and chemosensory signals from gravid females show different neural co-activation patterns related to social behaviors. Heat maps of Pearson correlation coefficients (*R* = color scale of **Figure 6**) of *cfos* staining in brain nuclei and **(A)** searching behaviors across all sensory conditions, and **(B)** courtship behaviors in unimodal visual only and multimodal visual and chemosensory conditions. Control and unimodal chemosensory conditions are not included in **(B)** because no courtship behaviors occurred in these conditions. See **Tables 5**, **6** for *R* and *P* values. Asterisks indicate significance at $P < 0.05$.

and/or motivational information from chemosensory signals and visual signals elicit the courtship behaviors. In a similar study in *A. burtoni*, males exposed to the putative pheromone 17α-20β-dihydroprogesterone as a chemosensory signal showed no difference in the number of courtship displays when presented with and without a female visual signal (O'Connell et al., 2013). However, we used female-conditioned water that contains a "cocktail" of odorants as opposed to a single putative pheromonal compound. Indeed, specific behavioral and physiological responses to pheromones from conspecifics are typically due to a particular combination of compounds rather than a single odorant (Stacey, 2003; Derby and Sorensen, 2008). This mixed vs. single odorant application likely accounts for the different responses, especially since the identity of the pheromonal compounds released by *A. burtoni* are currently unknown.

We also examined male physiological responses to visual and chemosensory uni- and multimodal signals by measuring circulating 11-KT and E_2 levels and found no difference across any condition. Similarly, males exposed to reproductive contexts in another study showed no difference in levels of 11-KT after exposure to visual and chemical stimuli (O'Connell et al., 2013). All males used in our study were highly dominant and territorial, meaning sustained high levels of these circulating hormones. The lack of context-specific stimulus-induced changes in our experiment may be due to circulating levels already being at or close to their physiological maximum (Parikh et al., 2006; Maruska and Fernald, 2010a; Maruska et al., 2013b; Maruska, 2015).

Neural Activation in Response to Uni- and Multimodal Visual-Chemosensory Signals

Receiver behavior determines the use of true multimodal signaling in specific contexts (Partan and Marler, 2005), but there is a lack of information on how and where unimodal and multimodal signals are integrated in the brain to produce such behaviors (Partan, 2013). Using *in situ* hybridization for the IEG *cfos*, we examined neural activation patterns in social and olfactory-relevant brain regions as a result of uni- and multimodal signals. Some brain regions showed activation that was dependent on only one signal. For example, there was greater activation in Vv in males exposed to a visual signal from females, regardless of the presence of a chemosensory signal, while greater activation in Vd occurred in response to chemosensory signals regardless of vision.

Vv (homologous in part to the external globus pallidus (dorsal Vv) and mammalian septum (ventral Vv)) is well-known for its role in reproduction and courtship behaviors (Satou et al., 1984; Wullimann and Mueller, 2004; Ganz et al., 2012; Elliott et al., 2017). Component 1 of our PCA was driven strongly

TABLE 1 | Pearson correlation coefficients of *cfos* staining in brain nuclei of *A. burtoni* males exposed to control conditions.

		Dl-g	nPPa	ATn	Dc-5	Dc-4	Vv	Vs	Vd
Dp	R	0.703	0.176	−0.042	0.116	−0.090	0.233	0.073	−0.522
	P	0.119	0.739	0.937	0.852	0.866	0.657	0.891	0.288
Dl-g	R		0.310	0.278	−0.620	0.218	0.404	0.338	−0.033
	P		0.550	0.594	0.265	0.678	0.428	0.512	0.950
nPPa	R			0.743	−0.269	−0.496	0.318	0.578	0.330
	P			0.901	0.662	0.317	0.539	0.230	0.523
ATn	R				−0.731	−0.553	0.237	0.060	0.053
	P				0.160	0.255	0.651	0.911	0.920
Dc-5	R					0.406	0.255	0.234	−0.052
	P					0.497	0.679	0.704	0.933
Dc-4	R						0.399	0.349	0.346
	P						0.433	0.498	0.502
Vv	R							0.522	0.080
	P							0.288	0.880
Vs	R								0.751
	P								0.086

Data represent correlation coefficients (R) and P-values (P) from dominant males exposed to control conditions (N = 6). Data were used to create heat map in ***Figure 7A****.*

TABLE 2 | Pearson correlation coefficients of *cfos* staining in brain nuclei of *A. burtoni* males exposed to sexually-relevant unimodal visual signals.

		ATn	Vd	Vs	Vv	nPPa	Dc-5	Dl-g	Dp
Dc-4	R	0.243	0.360	−0.492	−0.037	−0.467	0.026	−0.543	−0.235
	P	0.694	0.552	0.400	0.953	0.428	0.968	0.345	0.703
ATn	R		0.419	−0.489	0.179	−0.101	−0.502	0.018	0.255
	P		0.408	0.325	0.734	0.849	0.310	0.973	0.626
Vd	R			0.504	0.282	−0.130	−0.582	−0.269	−0.455
	P			0.308	0.588	0.805	0.225	0.606	0.364
Vs	R				0.273	0.283	−0.223	−0.024	−0.573
	P				0.601	0.586	0.671	0.964	0.235
Vv	R					0.809	−0.375	−0.067	−0.523
	P					0.051	0.463	0.899	0.287
nPPa	R						−0.319	0.399	−0.146
	P						0.538	0.433	0.782
Dc-5	R							−0.552	−0.214
	P							0.256	0.684
Dl-g	R								0.755
	P								0.082

Data represent correlation coefficients (R) and P-values (P) from dominant males exposed to visual only conditions (N = 6). Data were used to create heat map in ***Figure 7B****.*

by Vv activation demonstrating the importance of sexually-relevant information to Vv. Neurons in Vv of the zebrafish also pool inputs from diverse mitral cells in the olfactory bulbs and respond more strongly to a mixture than to individual components of an odorant, suggesting that olfactory processing in this region may contribute to control of general behavioral or physiological state (Yaksi et al., 2009). One explanation for why there was not greater activation in Vv with chemosensory signals is that olfactory bulb projections to the medial olfactory terminal region (border of Vd and Vv; Sas et al., 1993), which contains GABAergic cells (Maruska et al., 2017) may inhibit activation of Vv cells, resulting in lower *cfos* cell density following exposure to chemosensory signals. Here, visual exposure to females resulted in greater activation in Vv, as well as an increased courtship response in males. While recent evidence supports a division of the Vv into dorsal and ventral regions with distinct homologs (see above), we did not distinguish them in this study. The route by which visual information may arrive at Vv in the cichlid is unknown, but tracing studies in zebrafish show inputs to the ventral telencephalon from visual centers such as preglomerular nuclei and the preoptic area (Rink and Wullimann, 2004). Here we provide additional evidence for the already well-documented role of Vv in decisions related to sexual behaviors.

The mammalian homolog of the teleost Vd is somewhat debated, but is considered in part to be the nucleus accumbens and/or striatal formation (O'Connell and Hofmann, 2011; Ganz et al., 2012; Elliott et al., 2017), both of which are involved in reward behavior. In teleosts, Vd is important for arousal (Forlano and Bass, 2011) and receives direct input from the olfactory bulbs (Meek and Nieuwenhuys, 1998). We previously showed that *A. burtoni* females have increased activation in Vd following reproductive interactions in full contact settings (when chemosensory signals were presumably released by males; Maruska and Fernald, 2012; Field and Maruska, 2017). Here, greater activation in Vd occurred when males were exposed to female-conditioned water regardless of whether a visual signal

TABLE 3 | Pearson correlation coefficients of *cfos* staining in brain nuclei of *A. burtoni* males exposed to sexually-relevant unimodal chemosensory signals.

		Dc-5	Dl-g	Vs	Vv	Vd	Dp	ATn	nPPa
Dc-4	R	0.635	0.435	0.609	0.107	0.016	−0.299	0.215	−0.655
	P	0.175	0.271	0.200	0.841	0.976	0.564	0.682	0.158
Dc-5	R		0.506	0.526	0.168	0.111	−0.144	−0.290	−0.546
	P		0.306	0.284	0.751	0.834	0.785	0.577	0.263
Dl-g	R			0.733	0.422	0.578	−0.212	−0.033	**−0.846**
	P			0.097	0.405	0.230	0.686	0.950	**0.034**
Vs	R				0.723	0.026	−0.663	0.351	−0.554
	P				0.104	0.961	0.151	0.495	0.254
Vv	R					−0.412	**−0.921**	0.075	−0.288
	P					0.417	**<0.01**	0.990	0.580
Vd	R						0.650	0.056	−0.375
	P						0.162	0.916	0.464
Dp	R							−0.081	0.246
	P							0.879	0.639
ATn	R								0.267
	P								0.609

Data represent correlation coefficients (R) and P-values (P) from dominant males exposed to chemosensory only conditions (N = 6). Data were used to create heat map in ***Figure 7C****. Bold indicates P < 0.05.*

TABLE 4 | Pearson correlation coefficients of *cfos* staining in brain nuclei of *A. burtoni* males exposed to sexually-relevant multimodal visual-chemosensory signals.

		Dp	Dc-5	Dc-4	Vd	Dl-g	Vs	Vv	nPPa
ATn	R	−0.625	0.200	−0.169	−0.252	−0.078	0.131	0.019	0.396
	P	0.185	0.704	0.750	0.630	0.884	0.804	0.972	0.437
Dp	R		−0.173	−0.180	−0.081	−0.633	−0.648	−0.597	−0.752
	P		0.743	0.733	0.879	0.177	0.164	0.211	0.085
Dc-5	R			0.777	−0.466	−0.111	0.034	−0.057	−0.007
	P			0.69	0.352	0.835	0.949	0.915	0.989
Dc-4	R				−0.040	0.012	0.122	0.262	0.036
	P				0.940	0.981	0.819	0.616	0.946
Vd	R					0.221	0.582	0.786	0.580
	P					0.674	0.225	0.064	0.227
Dl-g	R						0.757	0.621	0.618
	P						0.081	0.188	0.191
Vs	R							**0.915**	**0.948**
	P							**0.010**	**0.004**
Vv	R								**0.892**
	P								**0.017**

Data represent correlation coefficients (R) and P-values (P) from dominant males exposed to dual visual-chemosensory signals from females (N = 6). Data were used to create heat map in ***Figure 7D****. Bold indicates P < 0.05.*

was present or not, and there was reduced activation in anosmic males. Further, activation in Vd was positively correlated with searching behavior (indicator of motivation) in chemosensory only conditions in intact males. Thus, our results further support Vd in integrating sexually-relevant chemosensory signals that stimulate arousal/motivation in males, similar to the nucleus accumbens in mammals (Becker et al., 2001; Portillo and Paredes, 2004; Hosokawa and Chiba, 2005).

Dp (homologous to the mammalian primary olfactory cortex) also showed increased activation when smell was present, and reduced activation in anosmic males, confirming its role in olfactory processing (Satou, 1990; Meek and Nieuwenhuys, 1998; Kermen et al., 2013). Further, component 2 of our PCA was most strongly driven by activation in Dp. More than just primary odor detection, Dp is implicated in odor memory and deciphering quality of complex odor mixtures (Yaksi et al., 2009; Mori, 2014). In zebrafish, Dp neurons establish representations of complex odor objects, potentially for use in the formation and recall of odor memories (Yaksi et al., 2009). In Mozambique tilapia (*Oreochromis mossambicus*) gene expression in Dp changes depending on the odorants, providing information on social context of the odorant (i.e., from dominant male, subordinate male, receptive female; Simões et al., 2015). Investigation of which genes may be up- or down-regulated in Dp of *A. burtoni* following stimulation with female-conditioned water would provide further information on how sexually-relevant olfactory information may be processed to produce specific social behaviors.

Two regions we investigated showed an additive response to multimodal visual-chemosensory signals: nPPa and Vs. This

TABLE 5 | Pearson correlation coefficients of *cfos* staining in brain nuclei and searching behavior of *A. burtoni* males exposed to control conditions and sexually-relevant uni- and multimodal visual-chemosensory signals.

		Control	Vision only	Chemosensory only	Vision and chemosensory
Dp	R	0.288	0.296	0.198	−0.324
	P	0.580	0.569	0.708	0.531
Dc-5	R	0.048	−0.165	0.304	−0.338
	P	0.939	0.755	0.558	0.513
Dc-4	R	−0.362	−0.638	0.389	−0.210
	P	0.481	0.247	0.445	0.690
ATn	R	−0.237	0.557	0.281	0.581
	P	0.651	0.251	0.589	0.227
Vd	R	**−0.918**	0.124	**0.850**	0.425
	P	**0.01**	0.815	**0.032**	0.401
Dl-g	R	−0.222	0.215	**0.852**	−0.297
	P	0.673	0.682	**0.031**	0.568
Vs	R	**−0.900**	−0.143	0.527	0.052
	P	**0.014**	0.786	0.282	0.922
Vv	R	−0.433	0.001	−0.005	0.265
	P	0.391	0.998	0.993	0.612
nPPa	R	−0.537	0.117	−0.637	0.340
	P	0.272	0.825	0.173	0.509

Data represent correlation coefficients (R) and P-values (P) from dominant males exposed to each sensory condition (N = 6 for each condition). Data were used to create heat map in ***Figure 8A****. Bold indicates P < 0.05.*

TABLE 6 | Pearson correlation coefficients of *cfos* staining in brain nuclei and courtship behavior of *A. burtoni* males exposed to sexually-relevant unimodal visual signals and multimodal visual-chemosensory signals.

		Vision only	Chemosensory only
Dp	R	−0.225	−0.717
	P	0.668	0.109
Dc-5	R	−0.305	0.267
	P	0.556	0.609
Dc-4	R	−0.298	0.433
	P	0.627	0.391
ATn	R	0.009	0.114
	P	0.986	0.830
Vd	R	−0.090	0.493
	P	0.866	0.321
Dl-g	R	0.264	0.720
	P	0.613	0.107
Vs	R	0.180	**0.941**
	P	0.733	**0.005**
Vv	R	**0.896**	**0.918**
	P	**0.016**	**0.010**
nPPa	R	**0.976**	**0.882**
	P	**<0.001**	**0.020**

Data represent correlation coefficients (R) and P-values (P) from dominant males exposed to visual only and dual visual-chemosensory signals (N = 6 for each condition). Data were used to create heat map in ***Figure 8B****. Bold indicates P < 0.05.*

response suggests *integration* of sexually-relevant visual and chemosensory signals in these regions. nPPa is a sub-region of the pre-optic area (POA) which is widely viewed as a core brain center for reproduction and social behaviors across vertebrates (Forlano and Bass, 2011). It also plays a major role as a sensory integration center leading to motor and neuroendocrine responses in a variety of social contexts, including aggression, sexual arousal, and reproduction (Forlano and Bass, 2011). Thus, greater activation in nPPa with either of the sexually-relevant unimodal signals, and even greater activation with paired visual-chemosensory signals may be expected, but future studies that examine which neuronal phenotypes might be activated in different sensory conditions should provide further insights. Similarly, Vs (homologous to mammalian medial amygdala) is involved in processing the salience of sensory information (Gray, 1999; Newman, 1999), including sexually-relevant chemosensory signals (Kyle and Peter, 1982) making it essential for sexual motivation (reviewed in Forlano and Bass, 2011). Indeed, Vs is crucial for courtship and spawning in males of several fish species (Kyle et al., 1982; Satou, 1990), and receives visual, chemosensory, and acoustic information (Kyle et al., 1982; Gray, 1999; Butler and Maruska, 2016). Further, gene expression in developing zebrafish larvae identify Vs as homologous to the central amygdala (Ganz et al., 2012). In addition, Vs receives input from POA, Vd and Vv (Demski and Northcutt, 1983; Meek and Nieuwenhuys, 1998), each of which showed specific responses to uni- and multimodal visual-chemosensory signals in *A. burtoni*. Vs also has reciprocal connections with the olfactory bulbs (Demski and Northcutt, 1983; Forlano and Bass, 2011), and the greater activation observed in chemosensory only conditions was eliminated in anosmic males. Thus, the activation from multimodal visual-chemosensory signaling we observed further support Vs in processing sensory information that is important for courtship behavior in *A. burtoni* males.

In contrast to nPPa and Vs, ATn and Dl-g showed greater activation in response to at least one unimodal signal and the multimodal signal compared to control, and these responses were not different from one another. This suggests a broader role in mediating visual and chemosensory stimuli. The precise function of ATn (putative homolog in part of the mammalian ventromedial hypothalamus (VMH)) in teleosts is currently unknown, but this region has projections to POA, suggesting involvement in social behavior (Meek and Nieuwenhuys, 1998). In *A. burtoni* females, activation of ATn was increased following aggressive interactions (Field and Maruska, 2017), which is also observed in mammals (Kollack and Newman, 1992; Lin et al., 2011; Field and Maruska, 2017). In *A. burtoni* males, ATn is important in the transition between social statuses (Maruska et al., 2013a), as well as processing mechanosensory signals from lateral line stimulation during aggressive interactions (Butler and Maruska, 2016), thus implicating ATn in a variety of social behaviors. As well as mediating aggression, our data indicate a role in reproductive contexts. Similarly, sexually dimorphic neurons in the VMH of mice regulate both sexual and aggressive behaviors in males (Yang et al., 2013). In the current study, exposure to each reproductively-relevant sensory condition resulted in a similar activation response compared to control conditions, and component 1 of our PCA was driven strongly by ATn. However, ATn may not be explicitly involved in processing signals from specific sensory modalities, but rather mediate a more general response in courtship and reproduction. Examining neural activation with a different IEG, such as *egr-1*, may better identify regions involved in particular behavioral responses.

Dl-g showed greater activation in response to chemosensory only and multimodal signals in comparison to control conditions. Dl (putative homolog of the mammalian hippocampus) is involved in learning and memory (Rodríguez et al., 2002; Harvey-Girard et al., 2012; Elliott et al., 2017). In *A. burtoni* females, Dl-g had greater activation in response to social (reproductive and aggressive contexts) compared to non-social conditions (Field and Maruska, 2017). The general increase in *cfos* expression shown here may reflect a similar response to general social stimulation. Activation in Dl-g was positively correlated with searching behavior during exposure to unimodal chemosensory signals, but there is not currently any evidence to support Dl-g as an olfactory processing region. However, *A. burtoni* males must be able to locate females in order to spawn. In fact, males that successfully completed a spatial learning task that allowed them to access females showed greater activation in the Dl (Wood et al., 2011). Thus, correlation of activation in Dl-g with searching behavior during chemosensory only conditions may reflect some aspect of spatial cognition in males motivated to locate females and their territory shelters for spawning. Further, Dl has been implicated in pattern separation and completion in teleosts, and functions in a very similar way to the hippocampal circuits of mammals (Elliott et al., 2017). However, further studies investigating activation of other Dl sub-regions (in addition to Dl-g) are needed to better understand

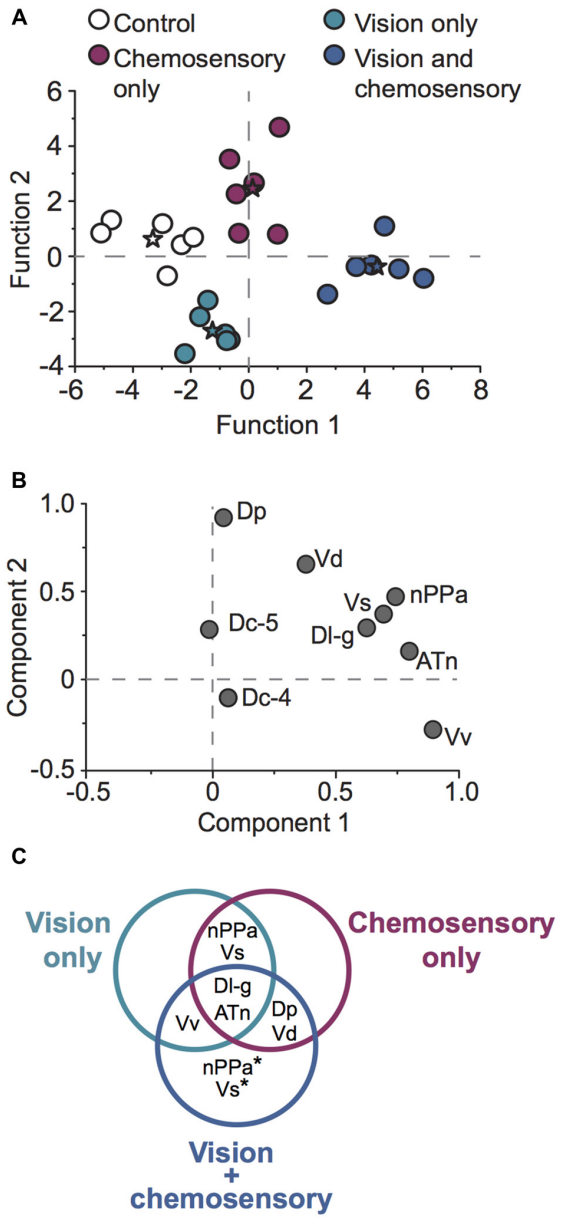

FIGURE 9 | *A. burtoni* males exposed to unimodal and multimodal visual and chemosensory signals show distinct neural activation patterns. **(A)** Discriminant function analysis (DFA) correctly classified 100% of focal males into their respective sensory stimulus categories based on brain activation patterns alone. Stars represent group centroids of focal males exposed to control conditions (white), unimodal visual only (green), unimodal chemosensory only (purple), and multimodal visual and chemosensory signals (blue). **(B)** Principal component analysis (PCA) of *cfos* staining in socially-relevant and sensory processing brain regions. **(C)** Venn diagram summarizing neural activation in males exposed to unimodal visual only, unimodal chemosensory only, and multimodal visual and chemosensory signals from females. Brain regions that exhibited greater activation in at least one of the sensory conditions compared to control conditions are shown. Regions in overlapping circles indicate similar activation in each of the relevant overlapping conditions. Asterisks indicate regions that showed greater activation in the multimodal vision-chemosensory condition compared to the other unimodal sensory conditions. Regions that showed no change in activation compared to control conditions (Dc-4 and Dc-5) are not shown.

its involvement in social behavior and sensory processing, as well as examination of visual and chemosensory inputs to Dl areas.

In addition to examining neural responses in individual regions, we also performed Pearson correlations among brain regions in response to uni- and multimodal visual-chemosensory signals to gain a better understanding of the functional connectivity, if any, of these regions (Teles et al., 2015). In control and visual only conditions, no significant correlations were observed among any investigated brain nuclei. When only chemosensory signals were present, however, there were negative correlations between Dp and Vv, as well as between nPPa and Dl-g. In response to multimodal signals, nPPa, Vv and Vs were all positively correlated with each other. While true functional connectivity of these regions could not be completely elucidated (possibly due to low sample size of 6 for each condition), the different neural activity patterns demonstrate unique responses to sexually-relevant visual, chemosensory, and combined visual-chemosensory signals in male receivers. Further, these regions exhibit anatomical connectivity and have each been implicated in courtship and reproductive displays (Demski and Northcutt, 1983; Meek and Nieuwenhuys, 1998; Forlano and Bass, 2011).

While our primary focus was to examine which brain regions might be involved in reception and processing of visual-chemosensory information, we also performed correlations of *cfos* cell density in each region with searching and courtship behaviors in male receivers. In control conditions, Vs and Vd were both negatively correlated with searching behavior, while in chemosensory only conditions Dl-g and Vd were both positively correlated with searching behavior, suggesting that chemosensory inputs may be driving a motivational response in males. No correlations with searching behavior were observed for any brain region in the vision only and visual-chemosensory multimodal contexts. However, males perform courtship behaviors during both of these conditions, and the drive to engage in reproductive behaviors likely overpowers searching. In other words, if a male can see a female he will engage in courtship behavior rather than searching behavior. It should also be noted that *cfos* expression is more associated with the reception of sensory information, rather than specific behavioral outputs (Teles et al., 2015). Although visual only conditions resulted in no correlations between neural activation and searching behavior, courtship behavior positively correlated with activation in Vv and nPPa. While these regions both have well-established roles in courtship, our data suggests that Vv and nPPa are likely involved in processing *visual* signals, at a neural level above the primary visual input centers, to produce specific reproductive behaviors.

In multimodal conditions, activation in Vv, nPPa and Vs, which all positively correlated with each other, also positively correlated with courtship behavior. These regions are all parts of the proposed SDMN (O'Connell and Hofmann, 2011); Vv and Vs are shared between the social behavior network and mesolimbic reward system that makes up the SDMN, and nPPa is part of the social behavior network (Newman, 1999; O'Connell and Hofmann, 2011). As such, all of these regions have well-established involvement in reproductive and courtship behavior in fishes (Demski and Knigge, 1971; Macey et al.,

1974; Kyle and Peter, 1982; Kyle et al., 1982; Satou et al., 1984; Satou, 1990). Further, we previously showed that *A. burtoni* females had greater activation in these same regions during reproductive contexts (Field and Maruska, 2017), demonstrating similar functions in reproduction across sexes. Here, the observed co-activation of these regions correlated with courtship behavior of males provides evidence of integration of visual and chemosensory information from gravid females that are vital to reproductive success.

SUMMARY

All animals must constantly integrate information from their environment with their own internal state to make important behavioral decisions. While we previously knew that visual and chemosensory signals were important for communication between sexes in *A. burtoni* (Maruska and Fernald, 2012; Field and Maruska, 2017), we show here that *combined* visual and chemosensory signals result in higher levels of male courtship behaviors, demonstrating a crucial role for chemosensory signals in reproduction. The behavioral responses in receiver males allow us to classify multimodal visual-chemosensory signals as non-redundant modulatory signals (Partan and Marler, 1999). By examining neural activation patterns with the IEG *cfos*, we identify decision centers involved in processing information from visual and chemosensory signals alone and together at an integration level above primary sensory processing. Vv and Vd, for example, both showed differential activation driven by one sensory modality, while ATn and Dl-g show greater activation in response to any and all sensory inputs. nPPa and Vs, however, show greatest activation with combined visual and chemosensory signals, suggesting sensory integration for behavioral decisions. By correlating neural activation in socially-relevant brain regions with courtship behaviors, we also show that activation in Vv, Vs and nPPa is associated with increased courtship behavior in males receiving multimodal visual-chemosensory signals from receptive females. Further, our DFA correctly classified 100% of males receiving no sexually-relevant signals (control conditions), visual signals only, chemosensory signals only, and multimodal visual-chemosensory signals based on neural activation alone. Thus, we demonstrate that multimodal visual-chemosensory signals are fundamentally different from either signal alone. These data provide insight on how different components of multimodal sensory inputs are received in the social brain, linked to essential behavioral outputs, and provide a framework for future studies on the evolution of sensory perception and multimodal signaling across species. This study, and others like it, will collectively help to better establish brain homologies and functional neural networks that shape context-dependent social behaviors.

AUTHOR CONTRIBUTIONS

KF, CM and KM had full access to the data and take full responsibility for the integrity of the data analysis, designed the experiments and wrote the manuscript. KF and CM performed the experiments and KF analyzed the data. KM provided funding, equipment and supplies. All authors reviewed and approved the final version of the manuscript.

FUNDING

Funding was provided by startup funds from the college of Science and Department of Biological Sciences at LSU (KM), Louisiana Board of Regents RCS Grant (KM), Powe Faculty Enhancement Award from ORAU (KM), the National Science Foundation (IOS-1456004 and IOS-1456558 to KM), and research awards from Sigma Xi (KF) and the Animal Behavior Society (KF). Financial support for publication was provided by the Louisiana State University Libraries Open Access Author Fund.

ACKNOWLEDGMENTS

We thank members of the Maruska lab for discussions and fish care, Julie Butler for assistance with figure design and insightful discussions and Makayla Voss and Kara Johnson for assistance with experimental design and conducting of behavioral experiments.

REFERENCES

Angelaki, D. E., Gu, Y., and DeAngelis, G. C. (2009). Multisensory integration: psychophysics, neurophysiology, and computation. *Curr. Opin. Neurobiol.* 19, 452–458. doi: 10.1016/j.conb.2009.06.008

Becker, J. B., Rudick, C. N., and Jenkins, W. J. (2001). The role of dopamine in the nucleus accumbens and striatum during sexual behavior in the female rat. *J. Neurosci.* 21, 3236–3241. doi: 10.1523/jneurosci.21-09-03236.2001

Bradbury, J. W., and Vehrencamp, S. L. (2000). Economic models of animal communication. *Anim. Behav.* 59, 259–268. doi: 10.1006/anbe.1999.1330

Butler, J. M., and Maruska, K. P. (2016). The mechanosensory lateral line system mediates activation of socially-relevant brain regions during territorial interactions. *Front. Behav. Neurosci.* 10:93. doi: 10.3389/fnbeh.2016.00093

Darwin, C., and Prodger, P. (1998). *The Expression of the Emotions in Man and Animals*. New York, NY: Oxford University Press.

Demski, L. S., and Knigge, K. M. (1971). The telencephalon and hypothalamus of the bluegill (Lepomis macrochirus): evoked feeding, aggressive and reproductive behavior with representative frontal sections. *J. Comp. Neurol.* 143, 1–16. doi: 10.1002/cne.901430102

Demski, L. S., and Northcutt, R. G. (1983). The terminal nerve: a new chemosensory system in vertebrates? *Science* 220, 435–437. doi: 10.1126/science.6836287

Derby, C. D., and Sorensen, P. W. (2008). Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *J. Chem. Ecol.* 34, 898–914. doi: 10.1007/s10886-008-9489-0

Elliott, S. B., Harvey-Girard, E., Giassi, A. C., and Maler, L. (2017). Hippocampal-like circuitry in the pallium of an electric fish: possible substrates for recursive pattern separation and completion. *J. Comp. Neurol.* 525, 8–46. doi: 10.1002/cne.24060

Fernald, R. D., and Hirata, N. R. (1977). Field study of haplochromis burtoni: quantitative behavioral observations. *Anim. Behav.* 25, 964–975. doi: 10.1016/0003-3472(77)90048-3

Field, K. E., and Maruska, K. P. (2017). Context-dependent chemosensory signaling, aggression and neural activation patterns in gravid female African cichlid fish. *J. Exp. Biol.* 220, 4689–4702. doi: 10.1242/jeb.164574

Forlano, P. M., and Bass, A. H. (2011). Neural and hormonal mechanisms of reproductive-related arousal in fishes. *Horm. Behav.* 59, 616–629. doi: 10.1016/j.yhbeh.2010.10.006

Ganz, J., Kaslin, J., Freudenreich, D., Machate, A., Geffarth, M., and Brand, M. (2012). Subdivisions of the adult zebrafish subpallium by molecular marker analysis. *J. Comp. Neurol.* 520, 633–655. doi: 10.1002/cne.22757

Gray, T. S. (1999). Functional and anatomical relationships among the amygdala, basal forebrain, ventral striatum and cortex. An integrative discussion. *Ann. N Y Acad. Sci.* 877, 439–444. doi: 10.1111/j.1749-6632.1999.tb09281.x

Günther, A. (1894). "Descriptions of the reptiles and fishes collected by Mr. E. Coode-Hore on Lake Tanganyika," in *Proceedings of the Zoological Society of London*, London, 628–632.

Hara, T. J. (1994). Olfaction and gustation in fish: an overview. *Acta Physiol. Scand.* 152, 207–217. doi: 10.1111/j.1748-1716.1994.tb09800.x

Harvey-Girard, E., Giassi, A. C., Ellis, W., and Maler, L. (2012). Organization of the gymnotiform fish pallium in relation to learning and memory: IV. Expression of conserved transcription factors and implications for the evolution of dorsal telencephalon. *J. Comp. Neurol.* 520, 3395–3413. doi: 10.1002/cne.23107

Hasson, O. (1989). Amplifiers and the handicap principle in sexual selection: a different emphasis. *Proc. R. Soc. Lond. B Biol. Sci.* 235, 383–406. doi: 10.1098/rspb.1989.0006

Hosokawa, N., and Chiba, A. (2005). Effects of sexual experience on conspecific odor preference and estrous odor-induced activation of the vomeronasal projection pathway and the nucleus accumbens in male rats. *Brain Res.* 1066, 101–108. doi: 10.1016/j.brainres.2005.10.036

Insel, T. R. (2010). The challenge of translation in social neuroscience: a review of oxytocin, vasopressin, and affiliative behavior. *Neuron* 65, 768–779. doi: 10.1016/j.neuron.2010.03.005

Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V. N., et al. (2011). Molecular organization of vomeronasal chemoreception. *Nature* 478, 241–245. doi: 10.1038/nature10437

Johnstone, R. A. (1996). Multiple displays in animal communication: 'backup signals' and 'multiple messages'. *Phil. Trans. R. Soc. Lond. B Biol. Sci.* 351, 329–338. doi: 10.1098/rstb.1996.0026

Kermen, F., Franco, L. M., Wyatt, C., and Yaksi, E. (2013). Neural circuits mediating olfactory-driven behavior in fish. *Front. Neural Circuits* 7:62. doi: 10.3389/fncir.2013.00062

Kollack, S. S., and Newman, S. W. (1992). Mating behavior induces selective expression of Fos protein within the chemosensory pathways of the male Syrian hamster brain. *Neurosci. Lett.* 143, 223–228. doi: 10.1016/0304-3940(92)90270-h

Kotrschal, K. (2000). Taste(s) and olfaction(s) in fish: a review of specialized sub-systems and central integration. *Pflugers Arch.* 439, R178–R180. doi: 10.1007/bf03376564

Kyle, A. L., and Peter, R. E. (1982). Effects of forebrain lesions on spawning behaviour in the male goldfish. *Physiol. Behav.* 28, 1103–1109. doi: 10.1016/0031-9384(82)90183-4

Kyle, A. L., Stacey, N. E., and Peter, R. E. (1982). Ventral telencephalic lesions: effects on bisexual behavior, activity, and olfaction in the male goldfish. *Behav. Neural Biol.* 36, 229–241. doi: 10.1016/s0163-1047(82)90855-x

Lin, D., Boyle, M. P., Dollar, P., Lee, H., Lein, E. S., Perona, P., et al. (2011). Functional identification of an aggression locus in the mouse hypothalamus. *Nature* 470, 221–226. doi: 10.1038/nature09736

Macey, M. J., Pickford, G. E., and Peter, R. E. (1974). Forebrain localization of the spawning reflex response to exogenous neurohypophysial hormones in the killifish, Fundulus heteroclitus. *J. Exp. Zool. A Ecol. Genet. Physiol.* 190, 269–279. doi: 10.1002/jez.1401900303

Maruska, K. P. (2015). Social transitions cause rapid behavioral and neuroendocrine changes. *Integr. Comp. Biol.* 55, 294–306. doi: 10.1093/icb/icv057

Maruska, K. P., Becker, L., Neboori, A., and Fernald, R. D. (2013a). Social descent with territory loss causes rapid behavioral, endocrine and transcriptional changes in the brain. *J. Exp. Biol.* 216, 3656–3666. doi: 10.1242/jeb.088617

Maruska, K. P., Zhang, A., Neboori, A., and Fernald, R. D. (2013b). Social opportunity causes rapid transcriptional changes in the social behavior network of the brain in an african cichlid fish. *J. Neuroendocrinol.* 25, 145–157. doi: 10.1111/j.1365-2826.2012.02382.x

Maruska, K. P., Butler, J. M., Field, K. E., and Porter, D. T. (2017). Localization of glutamatergic, GABAergic, and cholinergic neurons in the brain of the African cichlid fish, *Astatotilapia burtoni*. *J. Comp. Neurol.* 525, 610–638. doi: 10.1002/cne.24092

Maruska, K. P., and Fernald, R. D. (2010a). Behavioral and physiological plasticity: rapid changes during social ascent in an African cichlid fish. *Horm. Behav.* 58, 230–240. doi: 10.1016/j.yhbeh.2010.03.011

Maruska, K. P., and Fernald, R. D. (2010b). Steroid receptor expression in the fish inner ear varies with sex, social status, and reproductive state. *BMC Neurosci.* 11:58. doi: 10.1186/1471-2202-11-58

Maruska, K. P., and Fernald, R. D. (2012). Contextual chemosensory urine signaling in an African cichlid fish. *J. Exp. Biol.* 215, 68–74. doi: 10.1242/jeb.062794

Maruska, K. P., and Fernald, R. D. (2018). *Astatotilapia burtoni*: a model system for analyzing the neurobiology of behavior. *ACS Chem. Neurosci.* 9, 1951–1962. doi: 10.1021/acschemneuro.7b00496

Meek, J., and Nieuwenhuys, R. (1998). "Holosteans and teleosts," in *The Central Nervous System of Vertebrates*, eds R. Nieuwenhuys, H. J. Ten Donkelaar and C. Nicholson (Berlin: Springer-Verlag), 759–937.

Moller, A., and Pomiankowski, A. (1993). Why have birds got multiple sexual ornaments? *Behav. Ecol. Sociobiol.* 32, 167–176. doi: 10.1007/bf00173774

Mori, K. (2014). *The Olfactory System: From Odor Molecules to Motivational Behaviors*. Tokyo: Springer.

Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Ann. N Y Acad. Sci.* 877, 242–257. doi: 10.1111/j.1749-6632.1999.tb09271.x

Nikonov, A. A., and Caprio, J. (2005). Processing of odor information in the olfactory bulb and cerebral lobes. *Chem. Senses* 30, i317–i318. doi: 10.1093/chemse/bjh242

O'Connell, L. A., and Hofmann, H. A. (2011). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J. Comp. Neurol.* 519, 3599–3639. doi: 10.1002/cne.22735

O'Connell, L. A., Rigney, M. M., Dykstra, D. W., and Hofmann, H. A. (2013). Neuroendocrine mechanisms underlying sensory integration of social signals. *J. Neuroendocrinol.* 25, 644–654. doi: 10.1111/jne.12045

Parikh, V. N., Clement, T. S., and Fernald, R. D. (2006). Androgen level and male social status in the African cichlid, *Astatotilapia burtoni*. *Behav. Brain Res.* 166, 291–295. doi: 10.1016/j.bbr.2005.07.011

Partan, S. R. (2013). Ten unanswered questions in multimodal communication. *Behav. Ecol. Sociobiol.* 67, 1523–1539. doi: 10.1007/s00265-013-1565-y

Partan, S., and Marler, P. (1999). Communication goes multimodal. *Science* 283, 1272–1273. doi: 10.1126/science.283.5406.1272

Partan, S. R., and Marler, P. (2005). Issues in the classification of multimodal communication signals. *Am. Nat.* 166, 231–245. doi: 10.1086/431246

Portillo, W., and Paredes, R. G. (2004). Sexual incentive motivation, olfactory preference and activation of the vomeronasal projection pathway by sexually relevant cues in non-copulating and naive male rats. *Horm. Behav.* 46, 330–340. doi: 10.1016/j.yhbeh.2004.03.001

Rink, E., and Wullimann, M. F. (2004). Connections of the ventral telencephalon (subpallium) in the zebrafish (Danio rerio). *Brain Res.* 1011, 206–220. doi: 10.1016/j.brainres.2004.03.027

Rodríguez, F., López, J. C., Vargas, J. P., Gómez, Y., Broglio, C., and Salas, C. (2002). Conservation of spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. *J. Neurosci.* 22, 2894–2903. doi: 10.1523/jneurosci.22-07-02894.2002

Ronald, K. L., Fernández-Juricic, E., and Lucas, J. R. (2012). Taking the sensory approach: how individual differences in sensory perception can influence mate choice. *Anim. Behav.* 84, 1283–1294. doi: 10.1016/j.anbehav.2012.09.015

Sas, E., Maler, L., and Weld, M. (1993). Connections of the olfactory bulb in the gymnotiform fish, Apteronotus leptorhynchus. *J. Comp. Neurol.* 335, 486–507. doi: 10.1002/cne.903350403

Satou, M. (1990). Synaptic organization, local neuronal circuitry and functional segregation of the teleost olfactory bulb. *Prog. Neurobiol.* 34, 115–142. doi: 10.1016/0301-0082(90)90004-z

Satou, M., Oka, Y., Kusunoki, M., Matsushima, T., Kato, M., Fujita, I., et al. (1984). Telencephalic and preoptic areas integrate sexual behavior in hime salmon (landlocked red salmon, Oncorhynchus nerka): results of electrical

brain stimulation experiments. *Physiol. Behav.* 33, 441–447. doi: 10.1016/0031-9384(84)90167-7

Simões, J. M., Barata, E. N., Harris, R. M., O'Connell, L. A., Hofmann, H. A., and Oliveira, R. F. (2015). Social odors conveying dominance and reproductive information induce rapid physiological and neuromolecular changes in a cichlid fish. *BMC Genomics* 16:114. doi: 10.1186/s12864-015-1255-4

Stacey, N. (2003). Hormones, pheromones and reproductive behavior. *Fish Physiol. Biochem.* 28, 229–235. doi: 10.1023/b:fish.0000030540.99732.2c

Stacey, N. (2011). "Chapter 9–Hormonally derived sex pheromones in fishes," in *Hormones and Reproduction of Vertebrates*, eds O. N. David and H. L. Kristin (London: Academic Press), 169–192.

Stein, B. E., and Stanford, T. R. (2008). Multisensory integration: current issues from the perspective of the single neuron. *Nat. Rev. Neurosci.* 9, 255–266. doi: 10.1038/nrn2331

Teles, M. C., Almeida, O., Lopes, J. S., and Oliveira, R. F. (2015). Social interactions elicit rapid shifts in functional connectivity in the social decision-making network of zebrafish. *Proc. Biol. Sci.* 282:20151099. doi: 10.1098/rspb.2015.1099

Todt, D., and Fiebelkorn, A. (1980). Display, timing and function of wing movements accompanying antiphonal duets of Cichladusa guttata. *Behaviour* 72, 82–105. doi: 10.1163/156853980x00069

Wood, L. S., Desjardins, J. K., and Fernald, R. D. (2011). Effects of stress and motivation on performing a spatial task. *Neurobiol. Learn. Mem.* 95, 277–285. doi: 10.1016/j.nlm.2010.12.002

Wullimann, M. F., and Mueller, T. (2004). Teleostean and mammalian forebrains contrasted: evidence from genes to behavior. *J. Comp. Neurol.* 475, 143–162. doi: 10.1002/cne.20183

Yabuki, Y., Koide, T., Miyasaka, N., Wakisaka, N., Masuda, M., Ohkura, M., et al. (2016). Olfactory receptor for prostaglandin F2α mediates male fish courtship behavior. *Nat. Neurosci.* 19, 897–904. doi: 10.1038/nn.4314

Yaksi, E., von Saint Paul, F., Niessing, J., Bundschuh, S. T., and Friedrich, R. W. (2009). Transformation of odor representations in target areas of the olfactory bulb. *Nat. Neurosci.* 12, 474–482. doi: 10.1038/nn.2288

Yang, C. F., Chiang, M. C., Gray, D. C., Prabhakaran, M., Alvarado, M., Juntti, S. A., et al. (2013). Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males. *Cell* 153, 896–909. doi: 10.1016/j.cell.2013.04.017

Conflict of Interest Statement: 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

BRIEF RESEARCH REPORT
published: 09 January 2019
doi: 10.3389/fnbeh.2018.00334

Dopamine and Serotonin Are Both Required for Mate-Copying in *Drosophila melanogaster*

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Mate-copying is a form of social learning in which the mate-choice decision of an individual (often a female) is influenced by the mate-choice of conspecifics. *Drosophila melanogaster* females are known to perform such social learning, and in particular, to mate-copy after a single observation of one conspecific female mating with a male of one phenotype, while the other male phenotype is rejected. Here, we show that this form of social learning is dependent on serotonin and dopamine. Using a pharmacological approach, we reduced dopamine or serotonin synthesis in adult virgin females with 3-iodotyrosine (3-IY) and DL-para-chlorophenylalanine (PCPA), respectively, and then tested their mate-copying performance. We found that, while control females without drug treatment copied the choice of the demonstrator, drug-treated females with reduced dopamine or serotonin chose randomly. To ensure the specificity of the drugs, the direct precursors of the neurotransmitters, either the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) or the serotonin precursor 5-L-hydroxytryptophan (5-HTP) were given together with the drug, (respectively 3-IY and PCPA) resulting in a full rescue of the mate-copying defects. This indicates that dopamine and serotonin are both required for mate-copying. These results give a first insight into the mechanistic pathway underlying this form of social learning in *D. melanogaster*.

Keywords: fruit fly, mate choice, social learning, social memory, 3-iodotyrosine (3-IY), DL-para-chlorophenylalanine (PCPA), L-3,4-dihydroxyphenylalanine (L-DOPA), 5-L-hydroxytryptophan (5-HTP)

OPEN ACCESS

Edited by:
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Reviewed by:
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Received: *29 June 2018*
Accepted: *19 December 2018*
Published: *09 January 2019*

Citation:
Monier M, Nöbel S, Danchin E and Isabel G (2019) Dopamine and Serotonin Are Both Required for Mate-Copying in Drosophila melanogaster. Front. Behav. Neurosci. 12:334. doi: 10.3389/fnbeh.2018.00334

INTRODUCTION

Many animal species from a vast array of taxa can learn from others (i.e., social learning), particularly in the context of mate-choice (Avital and Jablonka, 2000; Danchin et al., 2004; Galef and Laland, 2005). Such observational learning can lead to mate-copying (Pruett-Jones, 1992), when females mate preferentially with a male showing similar characteristics as the male they saw being chosen by another female (trait-based copying, Bowers et al., 2012).

In *Drosophila melanogaster*, females are able to perform mate-copying (Mery et al., 2009) after watching only a single live demonstration of one female copulating with a male of a given phenotype and one male of another phenotype being rejected (Dagaeff et al., 2016; Danchin et al., 2018; Nöbel et al., 2018).

Despite some promising studies, research about the mechanisms of social learning in general and observational social learning in particular are still at the beginning (Burke et al., 2010; Debiec and Olsson, 2017; Kavaliers et al., 2017; Allsop et al., 2018). While social learning mechanisms are poorly known in any organism, *D. melanogaster* with its mini yet highly

structured brain (100,000 cells) is one of the most favorable model species to dissect the neuronal processes of learning. Mostly, studies focused on simple kinds of learning tasks, where flies can learn from their own experience (non-social learning task), that are easier to standardize and historically well studied, like olfactory or visual associative learning (Quinn et al., 1974; Vogt et al., 2014, 2016; Cognigni et al., 2018). Thus, while the mechanisms of non-social learning in *Drosophila* are now well-described, the neurotransmitters and neural structures involved in observational social learning in *Drosophila* are unknown.

The formation of non-social associative memory requires dopamine in *D. melanogaster*: during the olfactory or visual learning process, it mediates aversive or appetitive unconditional stimuli (Riemensperger et al., 2005, 2011; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Vogt et al., 2014), while serotonin is required for aversive place memory (Sitaraman et al., 2008), and for olfactory learning and memory (Johnson et al., 2011; Lee et al., 2011). Based on the fact that visual and olfactory learning share common neurotransmitters and neural structures (Vogt et al., 2014), we hypothesized that our model of observational social learning, mate-copying, involves the same two neurotransmitters. To address this, we used a pharmacological approach to reduce dopamine or serotonin synthesis with specific inhibitors of the limiting-step-enzyme of the synthetic pathway: 3-iodotyrosine (3-IY) inhibits tyrosine hydroxylase that catalyzes L-DOPA formation from tyrosine, and DL-para-chlorophenylalanine (PCPA) inhibits tryptophan hydroxylase that catalyzes 5-HTP formation from tryptophan, respectively. Young sexually mature virgin females were fed one of these drugs and their mate-copy ability was tested after a single demonstration. To ensure specificity of the drugs, we also had two rescue treatments in which the female received the drug (3-IY or PCPA) together with the immediate precursor of the neurotransmitter (L-DOPA or 5-HTP, respectively), so that the level of dopamine or serotonin was less reduced than with 3-IY or PCPA alone.

MATERIALS AND METHODS

Fly Maintenance

Wild-type Canton-S flies were raised in 30 ml food vials containing standard corn flour-yeast-agar medium. The room was maintained at 25 ± 0.8°C, 60 ± 3% humidity, with a 12 h:12 h light:dark cycle. Virgin flies were collected daily for the experiments and sexed without anesthesia, by gentle aspiration using a glass pipette, tubing and gauze. Flies were then kept in single-sex groups in food vials until the experiment started. As *D. melanogaster* females are reluctant to re-mate (Chapman et al., 2003), each female was used only once as demonstrator or observer.

Drug Treatment

The solutions were freshly prepared every week in vehicle (sucrose 5% in mineral water Vittel®) and 200 ml were poured on a Kimwipe paper (1.5 cm × 3.5 cm) deposited in a 15 ml Falcon tube. Nine 1-day-old virgin females were introduced in the tube for the length of the treatment, at 18°C, 12 h:12 h light:dark cycle. To explore dopamine effect, flies were fed with 3-IY (10 mg/ml, Sigma I8250) and/or L-DOPA (1 mg/ml, Sigma D9628) for 36–40 h (Bainton et al., 2000; Seugnet et al., 2008). To explore serotonin effect, flies were fed with PCPA (10 mg/ml, Sigma C6506) and/or 5-HTP (16 mg/ml, Sigma H9772) for 3 days, with papers being changed once during the 3 days period (Dierick and Greenspan, 2007; Plaçais et al., 2012). We used a high 5-HTP concentration (30% more than in Dierick and Greenspan, 2007) to ensure rescued serotonin levels in PCPA-treated flies in our conditions. This concentration did not affect mate-copying in flies fed with 5-HTP (**Figure 2**). The treatment "vehicle" consisted of vehicle solution given during 36–40 h or 3 days.

Mate-Copying Experiment

Flies were tested 3–4 days after eclosion. Experiments were conducted in the same conditions as fly maintenance. We used the same tubes set-up (double plastic tube (0.8 × 3 cm each) separated by a thin glass partition) and the speed-learning protocol as described in Dagaeff et al. (2016) except that mate-choice tests were run either immediately to test learning, or 3 h 20 ± 15 min after the demonstration, a time when associative memory in drosophila is composed of consolidated and labile memories (Folkers et al., 1993), two memories with independent pathways (Isabel et al., 2004; Scheunemann et al., 2012) so that we could detect a learning and/or memory defect. Artificial male phenotypes were obtained by randomly dusting them in green or pink (neutral trait) using colored powders (green: Shannon Luminous Materials, Inc., #B-731; red: BioQuip Products, Inc., #1162R). Demonstrations in tube set-ups showed a demonstrator female choosing between the two male phenotypes while the treated female could observe through a transparent partition. After the end of the copulation of demonstrator flies, each observer female was either directly tested or placed individually in a food vial until the test. The mate-choice test then involved two new virgin green and pink males placed in a tube with the observer female. Time when courtship began (first wing vibration) and color of the male, as well as time when copulation started and color of the chosen male were recorded.

Mate-Copying Index

Observer females that chose the same male color as the demonstrator for copulation (copied) were given a mate-copying score of 1, and females that chose the opposite color were given a score of 0. A mate-copying index (MCI) was calculated as the mean mate-copying score per treatment. Samples in which only one male courted the female before she initiated mating were not used for the analysis of the mate-copying performance because only when both males showed their interest the female was unambiguously in a position of choice. Samples in which no copulation occurred after 30 min were excluded from the analyses.

Ethics Statement

Behavioral observations of *D. melanogaster* required no ethical approval and complied with French laws regarding animal welfare. We kept the number of flies used in this study as small as

possible. We handled flies by gentle aspiration without anesthesia to minimize damage and discomfort. After the experiments, individuals were euthanized in a freezer.

Analyses

Mate-copying scores were analyzed with the R software 3.4.0 (R Core Team, 2017). For each treatment, the difference from random choice was tested with a binomial test. For global comparisons, mate-copying scores were analyzed in a generalized linear mixed model (GLMM) with binary logistic regression (package lme4, Bates et al., 2014). A random block effect introduced into the models accounted for the non-independence of observer flies from the same block of 6 demonstrations and tests. The significance of fixed effects was tested using Wald chi-square tests implemented in the ANOVA function of the car package (Fox and Weisberg, 2011). Starting models included treatment, air pressure at the time of the test, and its variation within the 6 preceding hours, and interactions between these effects. We used a backward selection approach using *P*-values, removing the highest order interaction as soon as it was non-significant. The final model was always chosen as the one with the lowest Akaike Information Criteria (AIC, Akaike, 1969). Two-by-two comparisons between treatments were done using *post hoc* X^2 tests.

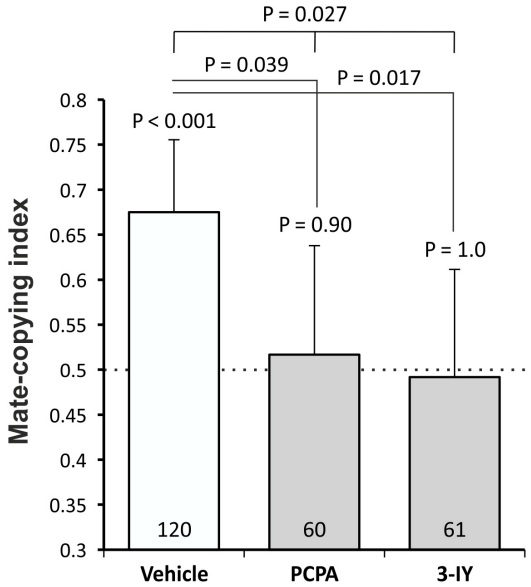

FIGURE 1 | Mate-copying index measured 3 h after the demonstration. Numbers inside the bars represent the sample size. Dashed line indicates random choice. Error bars: Agresti-Coull intervals. Just above bars: binomial tests. Two-by-two comparisons: *post hoc* chi-square tests. Test between all groups: GLMM.

RESULTS

PCPA and 3-IY Impair Learning or Memory in Mate-Copying

We first tested whether females' mate-copying performance was affected after a PCPA or a 3-IY treatment. We analyzed the mate-copying scores (**Figure 1**) and found that females fed with the vehicle mate-copied, while females lacking serotonin or dopamine did not. We then compared the three groups and found a significant difference (GLMM, N = 241, X^2 = 7.26, P = 0.027), which we also found when comparing PCPA- or 3-IY-treated flies to the vehicle (**Figure 1** and **Supplementary Table S1**). Thus, PCPA and 3-IY impaired mate-copying in these conditions. We also measured courtship duration in each group and found no statistical difference between them (**Supplementary Figure S1**).

Dopamine and Serotonin Are Both Required for Learning in a Mate-Copying Context

We then tested female mate-copying immediately after the demonstration, in order to study learning capacities only, and not memory retention. To ensure that the mate-copying defects observed in **Figure 1** depend on lacking dopamine or serotonin, and not to a side-effect of the drug, we added four more treatments: PCPA with 5-HTP, 5-HTP, 3-IY with L-DOPA and L-DOPA. We measured mate-copying scores in all groups (**Figure 2**) and found that all groups copied except PCPA and 3-IY treated females. We compared mate-copying scores in the five groups that copied and found no statistical difference (GLMM, N = 345, X^2 = 1.72, P = 0.79), indicating that 5-HTP and L-DOPA given alone did not alter mate-copying ability, and could rescue mate-copying in females treated with the inhibitor. Additionally, we found a significant difference between inhibitor-treated flies and flies fed with the vehicle or rescued flies (**Figure 2** and **Supplementary Table S2**). Thus, flies lacking dopamine

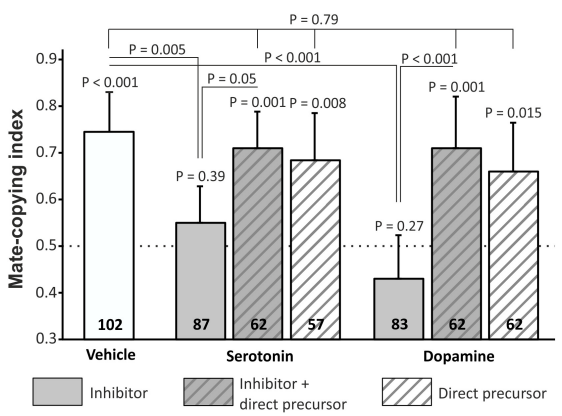

FIGURE 2 | Mate-copying index measured immediately after the demonstration. Flies received the following treatment (from left to right): vehicle, PCPA, PCPA + 5-HTP, 5-HTP, 3-IY, 3-IY + L-DOPA, L-DOPA. Numbers inside the bars represent the sample size. Dashed line indicates random choice. Error bars: Agresti-Coull intervals. Just above bars: binomial tests. Two-by-two comparisons: *post hoc* chi-square tests. Test between all groups that copied: GLMM.

or serotonin are not able to learn a mate preference from the demonstration.

DISCUSSION

We found that dopamine and serotonin are both required in learning during mate-copying. Observer females lacking these neurotransmitters were unable to learn the successful male phenotype in the demonstration while control females receiving the vehicle solution, females treated with 5-HTP or L-DOPA, and females treated with the precursor together with the inhibitor copied the choice of the demonstrator immediately after the demonstration. This is in accordance with other studies showing that dopamine and serotonin are required for learning. In an olfactory learning task, dopamine is required to mediate the unconditional stimulus after a single training phase (Riemensperger et al., 2011; Aso et al., 2012; Burke et al., 2012; Liu et al., 2012; Lin et al., 2014; Vogt et al., 2014). Alterations in behavioral tracking were reported in flies lacking dopamine (Andretic et al., 2005), but dopamine-deficient flies were shown to have no alteration in visual perception and display a normal electroretinogram (Riemensperger et al., 2011). Thus, the defects we observed are not due to deficient vision, although we cannot exclude attention deficiency in dopamine-depleted flies. Serotonin is necessary to form place memory (Sitaraman et al., 2008) and associative olfactory learning memory (Johnson et al., 2011; Lee et al., 2011). Mate-copying can be compared to associative learning with pairing between a conditional and an unconditional stimulus (Avarguès-Weber et al., 2015): the conditional stimulus would be the color of the male copulating with the demonstrator female while the unconditional reinforcing stimulus could be the observation of the copulation. Under these circumstances, dopamine would mediate the reinforcing stimulus. Our results provide one more indication that the pathways underlying memory formation are comparable for visual social information and for olfactory information, and it was shown that both share mushroom body circuits for memory consolidation (Vogt et al., 2014). Mate-copying was also described in many vertebrates (Dugatkin and Godin, 1993; White and Galef, 1999; Waynforth, 2007; Galef et al., 2008), so the mechanistic results discovered in *Drosophila* could be a starting point for such studies in vertebrates, as many vertebrate pathways and genes have homologs in *Drosophila*.

We showed that dopamine and serotonin are both required in mate-copying. This result paves the way for further studies of the neural pathways underlying social observational learning in *D. melanogaster*. The next step is now to dig into the role of each of these neurotransmitters, by assessing the neural structures and the receptors involved in this social learning task.

DATA AVAILABILITY

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

AUTHOR CONTRIBUTIONS

MM carried out the experiments, performed the analyses, and drafted the manuscript. SN contributed in the writing of the manuscript. ED and GI designed the experiments and jointly supervised all steps in the process. All authors gave final approval for publication.

FUNDING

This work was supported by the "Laboratoires d'Excellence (LABEX)" TULIP (ANR-10-LABX-41), the Toulouse Initiative of Excellence "IDEX UNITI" (ANR11-IDEX-0002-02) transversality grant, and the "MoleCulture" project from Agence Nationale pour la Recherche grant (ANR-18-CE37-0015) to GI and ED. ED and SN were also supported by the Soc-H^2 ANR project (ANR-13-BSV7-0007-01) to ED. MM's salary was provided by a grant from the French ministry of higher education and research. SN's salary was provided by Soc-H^2, a Marie Curie PRESTIGE grant (PRESTIGE-2014-1-0005) and received support from the ANR–Labex Institute for Advanced Study in Toulouse. GI benefited from a CNRS Excellence Chair.

ACKNOWLEDGMENTS

We would like to thank Nathalie Parthuisot and Tristan Lafont Rapnouil for help in fly care, Audrey Dussutour for providing food production equipment, and three referees for their valuable comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00334/full#supplementary-material

REFERENCES

Akaike, H. (1969). Fitting autoregressive models for prediction. *Ann. Inst. Stat. Math.* 21, 243–247. doi: 10.1007/BF02532251

Allsop, S. A., Wichmann, R., Mills, F., Burgos-Robles, A., Chang, C.-J., Felix-Ortiz, A. C., et al. (2018). Corticoamygdala transfer of socially derived information gates observational learning. *Cell* 173, 1329.e18–1342.e18. doi: 10.1016/j.cell.2018.04.004

Andretic, R., van Swinderen, B., and Greenspan, R. J. (2005). Dopaminergic modulation of arousal in *Drosophila*. *Curr. Biol.* 15, 1165–1175. doi: 10.1016/j.cub.2005.05.025

Aso, Y., Herb, A., Ogueta, M., Siwanowicz, I., Templier, T., Friedrich, A. B., et al. (2012). Three dopamine pathways induce aversive odor memories with different stability. *PLoS Genet.* 8:e1002768. doi: 10.1371/journal.pgen.1002768

Avarguès-Weber, A., Lihoreau, M., Isabel, G., and Giurfa, M. (2015). Information transfer beyond the waggle dance: observational learning

in bees and flies. *Front. Ecol. Evol.* 3:24. doi: 10.3389/fevo.2015.00024

Avital, E., and Jablonka, E. (2000). *Animal Traditions: Behavioural Inheritance in Evolution*. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511542251

Bainton, R. J., Tsai, L. T.-Y., Singh, C. M., Moore, M. S., Neckameyer, W. S., and Heberlein, U. (2000). Dopamine modulates acute responses to cocaine, nicotine and ethanol in *Drosophila*. *Curr. Biol.* 10, 187–194. doi: 10.1016/S0960-9822(00)00336-5

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2014). Fitting Linear Mixed-Effects Models using lme4. *arXiv* [preprint]. arXiv:1406.5823

Bowers, R. I., Place, S. S., Todd, P. M., Penke, L., and Asendorpf, J. B. (2012). Generalization in mate-choice copying in humans. *Behav. Ecol.* 23, 112–124. doi: 10.1093/beheco/arr164

Burke, C. J., Huetteroth, W., Owald, D., Perisse, E., Krashes, M. J., Das, G., et al. (2012). Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* 492, 433–437. doi: 10.1038/nature11614

Burke, C. J., Tobler, P. N., Baddeley, M., and Schultz, W. (2010). Neural mechanisms of observational learning. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14431–14436. doi: 10.1073/pnas.1003111107

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F., et al. (2003). The sex peptide of *Drosophila melanogaster*: female post-mating responses analyzed by using RNA interference. *Proc. Natl. Acad. Sci. U.S.A.* 100, 9923–9928. doi: 10.1073/pnas.1631635100

Cognigni, P., Felsenberg, J., and Waddell, S. (2018). Do the right thing: neural network mechanisms of memory formation, expression and update in *Drosophila*. *Curr. Opin. Neurobiol.* 49, 51–58. doi: 10.1016/j.conb.2017.12.002

Dagaeff, A.-C., Pocheville, A., Nöbel, S., Loyau, A., Isabel, G., and Danchin, E. (2016). *Drosophila* mate copying correlates with atmospheric pressure in a speed learning situation. *Anim. Behav.* 121, 163–174. doi: 10.1016/j.anbehav.2016.08.022

Danchin, E., Nöbel, S., Pocheville, A., Dagaeff, A.-C., Demay, L., Alphand, M., et al. (2018). Cultural flies: conformist social learning in fruit flies predicts long-lasting mate-choice traditions. *Science* 362, 1025–1030. doi: 10.1126/science.aat1590

Danchin, É, Giraldeau, L.-A., Valone, T. J., and Wagner, R. H. (2004). Public information: from nosy neighbors to cultural evolution. *Science* 305, 487–491. doi: 10.1126/science.1098254

Debiec, J., and Olsson, A. (2017). Social fear learning: from animal models to human function. *Trends Cogn. Sci.* 21, 546–555. doi: 10.1016/j.tics.2017.04.010

Dierick, H. A., and Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat. Genet.* 39, 678–682. doi: 10.1038/ng2029

Dugatkin, L. A., and Godin, J.-G. J. (1993). Female mate copying in the guppy (*Poecilia reticulata*): age-dependent effects. *Behav. Ecol.* 4, 289–292. doi: 10.1093/beheco/4.4.289

Folkers, E., Drain, P., and Quinn, W. G. (1993). Radish, a *Drosophila* mutant deficient in consolidated memory. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8123–8127. doi: 10.1073/pnas.90.17.8123

Fox, J., and Weisberg, S. (2011). *An {R} Companion to Applied Regression*. 2nd Edn. Thousand Oaks, CA: Sage Publishing.

Galef, B. G., and Laland, K. N. (2005). Social learning in animals: empirical studies and theoretical models. *Bioscience* 55, 489–499. doi: 10.1641/0006-3568(2005)055[0489:SLIAES]2.0.CO;2

Galef, B. G., Lim, T. C. W., and Gilbert, G. S. (2008). Evidence of mate choice copying in Norway rats, *Rattus norvegicus*. *Anim. Behav.* 75, 1117–1123. doi: 10.1016/j.anbehav.2007.08.026

Isabel, G., Pascual, A., and Preat, T. (2004). Exclusive consolidated memory phases in *Drosophila*. *Science* 304, 1024–1027. doi: 10.1126/science.1094932

Johnson, O., Becnel, J., and Nichols, C. D. (2011). Serotonin receptor activity is necessary for olfactory learning and memory in *Drosophila melanogaster*. *Neuroscience* 192, 372–381. doi: 10.1016/j.neuroscience.2011.06.058

Kavaliers, M., Matta, R., and Choleris, E. (2017). Mate-choice copying, social information processing, and the roles of oxytocin. *Neurosci. Biobehav. Rev.* 72, 232–242. doi: 10.1016/j.neubiorev.2016.12.003

Lee, P.-T., Lin, H.-W., Chang, Y.-H., Fu, T.-F., Dubnau, J., Hirsh, J., et al. (2011). Serotonin–mushroom body circuit modulating the formation of anesthesia-resistant memory in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 13794–13799. doi: 10.1073/pnas.1019483108

Lin, S., Owald, D., Chandra, V., Talbot, C., Huetteroth, W., and Waddell, S. (2014). Neural correlates of water reward in thirsty *Drosophila*. *Nat. Neurosci.* 17, 1536–1542. doi: 10.1038/nn.3827

Liu, C., Plaçais, P.-Y., Yamagata, N., Pfeiffer, B. D., Aso, Y., Friedrich, A. B., et al. (2012). A subset of dopamine neurons signals reward for odour memory in *Drosophila*. *Nature* 488, 512–516. doi: 10.1038/nature11304

Mery, F., Varela, S. A. M., Danchin, É, Blanchet, S., Parejo, D., Coolen, I., et al. (2009). Public versus personal information for mate copying in an invertebrate. *Curr. Biol.* 19, 730–734. doi: 10.1016/j.cub.2009.02.064

Nöbel, S., Danchin, E., and Isabel, G. (2018). Mate-copying for a costly variant in *Drosophila melanogaster* females. *Behav. Ecol.* 29, 1150–1156. doi: 10.1093/beheco/ary095

Plaçais, P.-Y., Trannoy, S., Isabel, G., Aso, Y., Siwanowicz, I., Belliart-Guérin, G., et al. (2012). Slow oscillations in two pairs of dopaminergic neurons gate long-term memory formation in *Drosophila*. *Nat. Neurosci.* 15, 592–599. doi: 10.1038/nn.3055

Pruett-Jones, S. (1992). Independent versus nonindependent mate choice: do females copy each other? *Am. Nat.* 140, 1000–1009. doi: 10.1086/285452

Quinn, W. G., Harris, W., and Benzer, S. (1974). Conditioned Behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 71, 708–712. doi: 10.1073/pnas.71.3.708

R Core Team (2017). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.

Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., et al. (2011). Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 108, 834–839. doi: 10.1073/pnas.1010930108

Riemensperger, T., Völler, T., Stock, P., Buchner, E., and Fiala, A. (2005). Punishment prediction by dopaminergic neurons in *Drosophila*. *Curr. Biol.* 15, 1953–1960. doi: 10.1016/j.cub.2005.09.042

Scheunemann, L., Jost, E., Richlitzki, A., Day, J. P., Sebastian, S., Thum, A. S., et al. (2012). Consolidated and labile odor memory are separately encoded within the *Drosophila* brain. *J. Neurosci.* 32, 17163–17171. doi: 10.1523/jneurosci.3286-12.2012

Seugnet, L., Suzuki, Y., Vine, L., Gottschalk, L., and Shaw, P. J. (2008). D1 Receptor activation in the mushroom bodies rescues sleep-loss-induced learning impairments in *Drosophila*. *Curr. Biol.* 18, 1110–1117. doi: 10.1016/j.cub.2008.07.028

Sitaraman, D., Zars, M., LaFerriere, H., Chen, Y.-C., Sable-Smith, A., Kitamoto, T., et al. (2008). Serotonin is necessary for place memory in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5579–5584. doi: 10.1073/pnas.0710168105

Vogt, K., Aso, Y., Hige, T., Knapek, S., Ichinose, T., Friedrich, A. B., et al. (2016). Direct neural pathways convey distinct visual information to *Drosophila* mushroom bodies. *eLife* 5:e14009. doi: 10.7554/eLife.14009

Vogt, K., Schnaitmann, C., Dylla, K. V., Knapek, S., Aso, Y., Rubin, G. M., et al. (2014). Shared mushroom body circuits underlie visual and olfactory memories in *Drosophila*. *eLife* 3:e02395. doi: 10.7554/eLife.02395

Waynforth, D. (2007). Mate choice copying in humans. *Hum. Nat.* 18, 264–271. doi: 10.1007/s12110-007-9004-2

White, D. J., and Galef, B. G. Jr. (1999). Mate choice copying and conspecific cueing in Japanese quail, *Coturnix coturnix* japonica. *Anim. Behav.* 57, 465–473. doi: 10.1006/anbe.1998.1015

Conflict of Interest Statement: 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: 25 January 2019
doi: 10.3389/fnbeh.2018.00317

Social Memory and Social Patterns Alterations in the Absence of STriatal-Enriched Protein Tyrosine Phosphatase

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STriatal-Enriched protein tyrosine Phosphatase (STEP) is a neural-specific protein that opposes the development of synaptic strengthening and whose levels are altered in several neurodegenerative and psychiatric disorders. Since STEP is expressed in brain regions implicated in social behavior, namely the striatum, the CA2 region of the hippocampus, cortex and amygdala, here we investigated whether social memory and social patterns were altered in STEP knockout (KO) mice. Our data robustly demonstrated that STEP KO mice presented specific social memory impairment as indicated by the three-chamber sociability test, the social discrimination test, the 11-trial habituation/dishabituation social recognition test, and the novel object recognition test (NORT). This affectation was not related to deficiencies in the detection of social olfactory cues, altered sociability or anxiety levels. However, STEP KO mice showed lower exploratory activity, reduced interaction time with an intruder, less dominant behavior and higher immobility time in the tail suspension test than controls, suggesting alterations in motivation. Moreover, the extracellular levels of dopamine (DA), but not serotonin (5-HT), were increased in the dorsal striatum of STEP KO mice. Overall, our results indicate that STEP deficiency disrupts social memory and other social behaviors as well as DA homeostasis in the dorsal striatum.

Keywords: social memory, social interaction, dominance, STEP KO mice, dopamine

OPEN ACCESS

Edited by:
Ellouise Leadbeater, Royal Holloway, University of London, United Kingdom

Reviewed by:
Michael Lukas, University of Regensburg, Germany
Antonia Manduca, Aix-Marseille Université, France

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Received: *02 August 2018*
Accepted: *04 December 2018*
Published: *25 January 2019*

Citation:
Blázquez G, Castañé A, Saavedra A, Masana M, Alberch J and Pérez-Navarro E (2019) Social Memory and Social Patterns Alterations in the Absence of STriatal-Enriched Protein Tyrosine Phosphatase. Front. Behav. Neurosci. 12:317. doi: 10.3389/fnbeh.2018.00317

INTRODUCTION

STriatal-Enriched protein tyrosine Phosphatase (STEP) is a neural-specific phosphatase that opposes the development of synaptic strengthening through the regulation of multiple kinases and glutamate receptor subunits critical for synaptic plasticity. It acts by dephosphorylating the GluN2B and GluA2 regulatory subunits of NMDA and AMPA receptors, respectively, leading to their internalization, and it promotes synaptic weakening by dephosphorylating the regulatory tyrosine (Tyr) of ERK1/2, Fyn or Pyk2 kinases resulting in their inactivation (Goebel-Goody et al., 2012a). Its dysregulation has been reported in several psychiatric and neurodegenerative diseases (Goebel-Goody et al., 2012a; Karasawa and Lombroso, 2014), and preclinical reports indicate that

genetic deletion or pharmacological inhibition of STEP improves cognitive deficits in mouse models of Alzheimer's disease (Zhang et al., 2010; Xu et al., 2014), fragile X syndrome (Goebel-Goody et al., 2012b; Chatterjee et al., 2018) and schizophrenia (Xu et al., 2018), as well as age-related memory decline (Castonguay et al., 2018).

STEP is highly expressed in the striatum and at lower levels in the cortex, hippocampus, amygdala and other brain regions, except in the cerebellum (Lombroso et al., 1991, 1993; Boulanger et al., 1995). The striatum plays a role in the computation of social behavior (van den Bos, 2015), being implicated in those behaviors that occur in a social context, like social reward behaviors and learning in social contexts (Báez-Mendoza and Schultz, 2013). The amygdala works as a hub to modulate a variety of brain networks that are important to normal social cognition (Bickart et al., 2014), and the cortex participates in the social reasoning (Bault et al., 2011). In the hippocampus, STEP is enriched in the CA2 (Shinohara et al., 2012; Kohara et al., 2014), a crucial region for socio-cognitive memory processing (Hitti and Siegelbaum, 2014; Stevenson and Caldwell, 2014), as well as for the modulation of further social patterns such as aggressive behavior (Pagani et al., 2015). It was previously reported that, like wild-type (WT) mice, STEP knockouts (KOs) show preference for a novel than for a known mouse, which was interpreted as mutant mice having intact social memory (Venkitaramani et al., 2011). Contradictorily, in another work, neither WT nor STEP KO mice spent more time exploring the novel mouse compared to the familiar one in the three-chamber sociability test (Goebel-Goody et al., 2012b) pointing at some procedural artifact. Given these controversial results and STEP expression profile, in the present work we sought to examine the role of STEP in social memory and further social patterns by thoroughly characterizing the social phenotype of STEP KO mice.

MATERIALS AND METHODS

Animals

Six months old male (C57BL/6J background) STEP KO (Venkitaramani et al., 2009) and WT mice were housed in groups of 2–5 animals per cage, maintained under standard housing conditions, 12 h light/dark schedule (lights on at 08:00 am) with food and water *ad libitum*, 22 ± 2°C room temperature and 50%–70% humidity. Mice were habituated to handling and given 1 h to habituate after transport to room before any tests were conducted. Experimental procedures were approved by the Local Ethical Committee of the University of Barcelona following European (2010/63/UE) and Spanish (RD53/2013) regulations for the care and use of laboratory animals. After behavioral assessment mouse genotype was confirmed by Western blot analysis (**Supplementary Figure S1**).

Behavioral Tests

Social memory and further social patterns were assessed by using the behavioral battery described below. Moreover, non-social memory abilities, sensorimotor and olfactory capabilities as well as anxiety levels were also studied to analyze their possible influence on social patterns. Two batches of animals were used, and the experimental timeline of the tests, from less to more aversive (McIlwain et al., 2001), is depicted in **Supplementary Figure S2**. Animal behavior was videotaped and analyzed using the SMART v3.0 software (Panlab, Barcelona, Spain). When appropriate, the arenas were cleaned with 5% ethanol between trials to remove any odor cues (Blázquez et al., 2014).

Three-Chamber Sociability Test

Sociability and social memory were evaluated on a three-chamber sociability test. Subjects were first habituated to the empty apparatus, a three-chamber box consisting of three interconnected lined compartments (DeVito et al., 2009) with open doors, for 10 min trial/day, for 3 days. Age and sex-matched mice to be explored were also habituated for 3 days to be caged in jails inside the apparatus. On the testing day, subject mice were habituated to the central compartment with closed doors for 5 min. After the habituation phase, subjects were tested in the sociability task, and 20 min later the social memory task was performed to evaluate preference for social novelty. The sociability task consisted in giving the subject mice the option to socialize with a conspecific mouse or explore a mouse dummy located in opposite external compartments. The social memory task performed 20 min later consisted in presenting to subjects, in the opposite compartment respect to the initial encounter, the known mouse (same as during the sociability phase) and a stranger mouse in the other external compartment. The trial tests lasted for 10 min and distance traveled, number of entrances, time spent in each compartment and time sniffing each cage were measured (DeVito et al., 2009).

Social Discrimination Test

Social memory was evaluated in the social discrimination test (Engelmann et al., 2011). Subjects were habituated to be individually caged in standard clean cages for 2 h before being tested. A mouse juvenile (C57BL/6J, 15–35 days old) was introduced in the subject's cage during a 4 min trial for the sampling phase. After a 1 h inter-trial interval (ITI) a 4 min choice phase was performed in which the previously encountered juvenile was presented to the subject. After another 1 h ITI, a new choice trial was performed in which a novel juvenile was introduced into the subject's cage. Interaction time (including anogenital and nose-to-nose sniffing as well as allogrooming) was evaluated, and the difference between time spent in social interaction during the sample and the choice phases was scored.

11-Trial Habituation/Dishabituation Social Recognition Test

Social memory was also assessed in the 11-trial habituation/dishabituation social recognition test. Subject male mice were habituated to a clean standard home cage for 5 h before the test. Age-matched C57BL/6J females in metestrus and diestrus estrous cycle were selected as stimulus for the social memory test (see procedure below). Female A and B belonged to different cages. The test consisted in a

habituation phase where female A was presented to subject male for 10 trials of 1 min each, and a 10 min ITI. In the 11th trial, an unknown female B was presented to subject male in the test phase. Interaction time (including anogenital and nose-to-nose sniffing, as well as allogrooming) was evaluated and the difference between time spent in social interaction during the habituation phase and the test phase was scored (Fergusson et al., 2000; Stevenson and Caldwell, 2014). Females (n = 8) were vaginal washed with 25 μl PBS flushed 4–5 times with a pipette tip introduced 1 mm in the vagina, until getting a turbid solution. One drop of the smear was put on a microscope slide, and once air dried it was stained by submersion during 3 min in a 0.1% crystal violet solution (Scharlau S.L., Spain), and rinsed twice during 1 min in distilled water. This protocol was repeated daily during a week, and only females in metestrus and diestrus were used as subject of interest for tested males (**Supplementary Figure S3**; McLean et al., 2012). Each female was presented just once a day to a subject male.

Novel Object Recognition Test (NORT)

Hippocampal-dependent learning and memory was analyzed using the novel object recognition test (NORT; Dere et al., 2007). Mice were first habituated to the arena (square white box: 59 cm lateral × 40 cm height) in the absence of objects, for 2 trials of 10 min duration with an ITI of 4 h. The second day, a training session was performed during 10 min by presenting two similar objects resembling eggs. After a 15 min ITI the testing session was performed, in which subjects were exposed for 5 min to a familiar (egg-like) and a new object (a plug). The object preference was measured as the time exploring each object × 100/total time exploring.

Olfactory Habituation/Dishabituation Test

Olfactory capabilities were assessed in the olfactory habituation/dishabituation test to elucidate if mice were able to smell and distinguish different social odors (Yang and Crawley, 2009). Subjects were first habituated to be individually housed in clean home cages 1 h before the experiment took place, and a wire ball (tea container of 2 cm diameter) containing a piece of cotton was introduced for a 30 min habituation. The test consisted in sequential presentations of non-social odor (clean bedding) and two different social odors that were obtained by impregnating a piece of cotton with dirty bedding (7 days old, from home cage of five male mice). Each odor was presented for three consecutive 2 min duration trials. The ITI was 1 min, the time needed to change the odor stimulus. Habituation was defined as the progressive decrease in olfactory investigation towards a repeated presentation of the same odor stimulus. Dishabituation was defined by a reinstatement of sniffing when a novel odor was presented. Time sniffing the wire ball was scored.

Sensorimotor Battery

The sensorimotor capabilities were evaluated by a SHIRPA standard task battery (Rogers et al., 1997) including motor coordination and equilibrium assessed in the iron rod and in the wire hanger tests, and prehensility and muscular strength in the wire hanger test, as previously described (Blázquez et al., 2014).

Nesting Behavior and Group Sleeping

Nesting behavior was assessed by using a protocol modified from Deacon (2006). Group-housed mice were transferred to new home cages with two pieces of soft paper for nesting, and nests were assessed 24 h later on a rating scale of 1–5. Group sleeping was scored 1 and individual sleeping was scored 0. Data was analyzed by a Fisher's test.

Corner Test

Neophobia or fearfulness to novelty was assessed in the corner test. Subjects were individually introduced in the center of a clean home cage (sides of 23 cm), and exploratory behavior was assessed as the number of rearings and corners explored during 30 s (Blázquez et al., 2014).

Open Field

To assess exploratory activity influenced by fearfulness to a novel environment, mice were individually placed in the center of an open round arena located in the center of an illuminated room. The open field apparatus was a white wooden arena of 38.5 cm diameter. Latency to initiate movement (initial freezing), distance traveled in cm, number of rearings and defecation boluses were measured in a single 5 min trial (Blázquez et al., 2014).

Plus Maze

Anxiety levels were assessed in the plus maze. The apparatus consisted of two opposing open arms (58 × 8 cm) crossed by two opposing enclosed arms (58 × 8 × 12 cm), and an open 8 × 8 cm square in the center. The maze was made of black plexiglass, and was elevated 50 cm above the floor. Mice were placed in the center of the plus maze facing an enclosed arm and behavior was measured during 5 min. The latency, number of entries, distance traveled into the open and closed arms, and number of defecation boluses were measured (Fernández-Teruel et al., 2002).

Dark-Light Box

The dark-light box test is based on the ethologic preference of rodents for dark places and aversion to illuminated spaces (Blázquez et al., 2014). The apparatus consisted of two compartments {black/dark: 13 × 14 × 27 cm; white/illuminated [with a white light bulb (390 luxes)]: 16 × 14 × 27 cm} separated by a wall with an opening (7 × 7 cm) that connected both spaces. Latency to the first entry into the white compartment, total number of entries and distance traveled into the white compartment were measured in a 5 min session.

Tail Suspension Test

The tail suspension test was used to evaluate behavioral despair (Can et al., 2012; Ye et al., 2016) by measuring time in immobile posture when mice were subjected to the short inescapable stress of being suspended by their tail. The mouse tail was introduced in a plastic cylinder (4 cm length × 1.3 cm diameter) to avoid tail climbing, and tape was subjecting 1–2 cm of the tail tip, suspending the mouse from the top of a white square plexiglass

box (60 cm sides × 40 cm depth, one side open to see the mouse), 60 cm above the floor. Latency to immobility and immobility time (none of the four paws moving) were measured during a 6 min trial.

Dominance Tube Test

Subjects were tested in a tube test for social dominance assessment (Lijam et al., 1997; Spencer et al., 2005). A transparent plexiglass 35 cm length × 3.5 cm diameter tube was used. After training the animals to cross the tube the day before, two subjects of different genotype were released simultaneously on opposite sides of the tube for a maximum of 2 min encounter. The match ended when one of the mice completely retreated from the tube. The subject remaining in the tube was the winner, scoring 1 point, and the retreated subject was scored with 0 points. Each mouse was matched in three trials with three different subjects of the opposite genotype.

Resident-Intruder Test

Social interaction was evaluated in the resident-intruder test as previously described (Lumley et al., 2000; Wood and Morton, 2015), with some modifications. The test had three steps: habituation, barrier and interaction phases. For the habituation phase, all mice but the "resident" were removed from their home cage (23 cm sides, containing dirty bedding from a few days to establish the territory). A wire net was introduced in the middle of the home cage dividing it into two equal spaces. The subject mouse was left for 5 min in one of the spaces to adapt to the barrier. Time interacting with the barrier was measured. After the habituation phase, an unknown C57BL/6J age-matched male intruder mouse was introduced into the other space for 5 min. Time interacting with the intruder in the presence of the barrier was scored in this phase. Finally, the barrier was removed and resident mouse could interact directly with the intruder. Time of interaction was measured during a 5 min trial.

In vivo Microdialysis

Extracellular serotonin (5-HT) and dopamine (DA) levels were measured by *in vivo* microdialysis as previously described (Castañé et al., 2008). Briefly, one concentric dialysis probe equipped with a Cuprophan membrane (1.5 mm long) was implanted in the dorsal striatum of anesthetized mice (sodium pentobarbital, 40 mg/kg, i.p.) at coordinates (in mm, from bregma and skull): AP +0.5; L −1.7; DV −4.5 (Franklin and Paxinos, 1997). Microdialysis experiments were performed in freely moving mice 24 h (day 1) and 48 h (day 2) after surgery. The artificial cerebrospinal fluid (aCSF) was pumped at 1.65 µl/min, and dialysate samples were collected every 20 min in microvials containing 5 µl of 10 mM perchloric acid. Following an initial 30 min stabilization period, six baseline samples were collected before local (reverse dialysis) veratridine (50 µM) or nomifensine (1, 10 and 50 µM) administration on day 1 and day 2, respectively. Veratridine (Tocris; Bristol, UK) was dissolved in dimethyl sulfoxide 99.9% (Sigma-Aldrich, Tres Cantos, Spain) to 5 mM (stock solution). Nomifensine maleate salt (Sigma-Aldrich) was dissolved in aCSF to 1 mM (stock solution). Stock solutions were stored at −20°C until use, and working solutions of veratridine and nomifensine were prepared by dilution in aCSF. 5-HT and DA concentration was analyzed by HPLC with amperometric detection at +0.6 V and +0.7 V, respectively, with a detection limit of 2 fmol/sample. Following sample collection, mice were sacrificed and brains were removed, sectioned and stained with neutral red to ensure proper probe placement.

Western Blot Analysis

Western blot analysis was performed as previously described (Saavedra et al., 2011). The primary antibodies used were: anti-STEP (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-DA D1 receptor (D1R; 1:500; Cell Signaling, Bevelly, MA, USA) and anti-DA D2 receptor (D2R; 1:1,000, Frontier Institute, Japan). Loading control was performed by reprobing the membranes with an anti-α-tubulin antibody (1:50,000; Sigma-Aldrich) for 15–20 min at room temperature. Then, membranes were washed with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), incubated for 1 h (15–20 min for loading controls) at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibody (1:2,000; Promega, Madison, WI, USA), and washed again with TBS-T. Immunoreactive bands were visualized using the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and quantified by a computer-assisted densitometer (Gel-Pro Analyzer, version 4, Media Cybernetics; Warrendale, PA, USA).

Statistical Analysis

Data are presented as mean ± SEM. Behavioral data were analyzed using the software SPSS Statistics 22, and biochemical data using the GraphPad Prism (v. 5.01, GraphPad Software Inc., San Diego, CA, USA). The statistical test applied in each experiment is detailed in the text/figure legends. Statistical significance was set at 95% confidence level.

RESULTS

STEP KO Mice Display Impaired Social Memory

To evaluate the effect of genetic deletion of STEP on social memory we first subjected WT and STEP KO mice to the three-chamber sociability test. During the socialization phase, STEP KO mice traveled less distance than WT mice (Student's *t*-test, WT: 3486.23 ± 135.69 cm and STEP KO: 3015.04 ± 111.69 cm, $t_{(1,21)} = 2.653$, $p < 0.05$), showing diminished levels of exploration compared to controls. Moreover, although WT and STEP KO mice spent more time exploring the mouse cage than the dummy cage (intragenotype comparison, Student's *t*-test, WT: $t_{(1,16.64)} = 5.79$, $p < 0.001$; STEP KO: $t_{(1,12.84)} = 9.33$, $p < 0.001$), indicating comparable levels of sociability and similar time spent for memory acquisition (**Figure 1A**), total exploration time of both cages was lower in STEP KO mice compared to WT group (WT: 92.99 ± 7.64 s and STEP KO: 70.71 ± 3.48 s, Student's *t*-test, $t_{(1,15.31)} = 2.65$, $p < 0.05$). When we analyzed social memory 20 min later, STEP KO mice showed reduced traveled distance (WT: 3831.05 ± 95.78 cm, STEP KO: 2895.38 ± 143.81 cm,

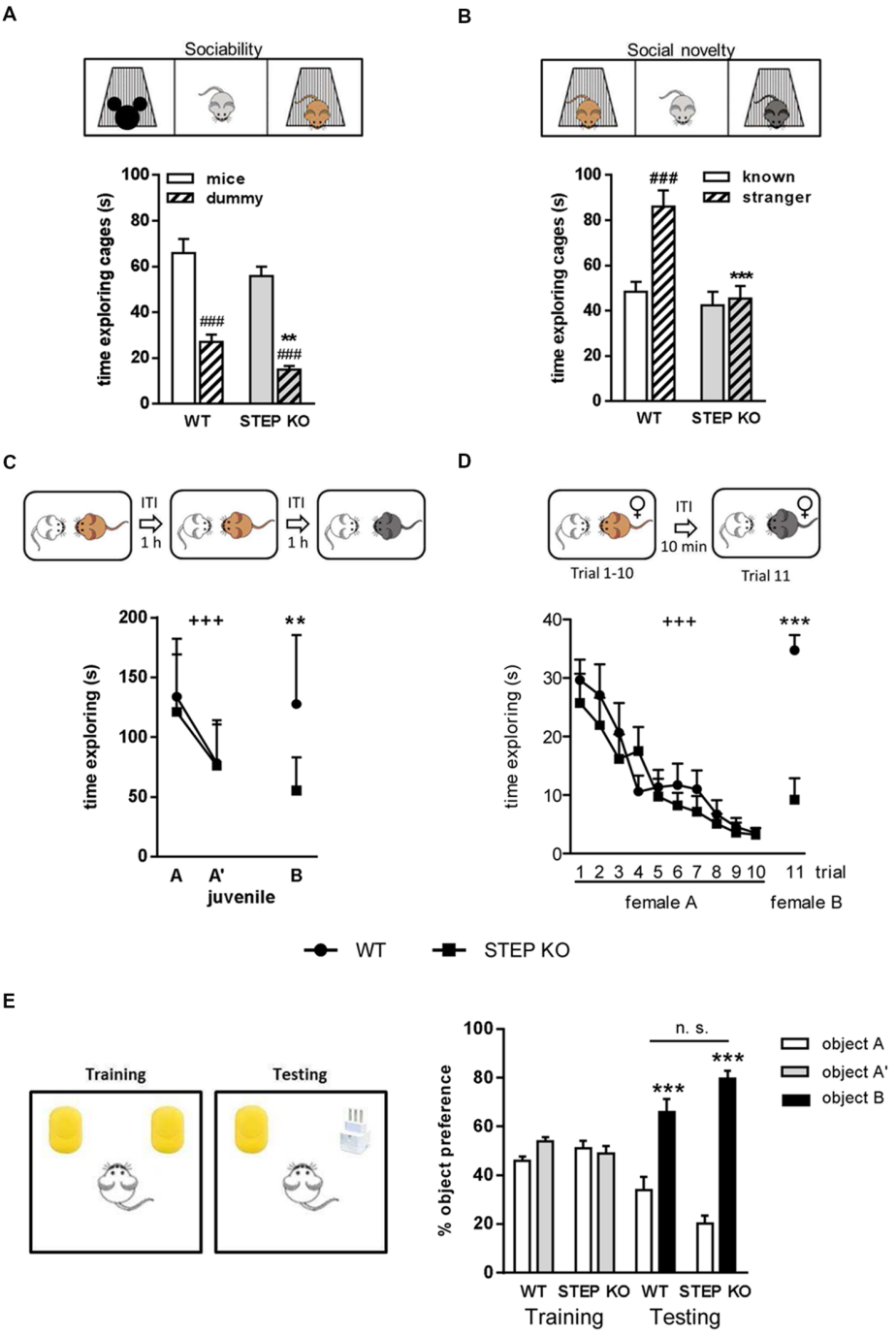

FIGURE 1 | Lack of STriatal-Enriched protein tyrosine Phosphatase (STEP) produces impairments in social memory. **(A)** Time exploring the mouse and dummy cages during the socialization in the three-chamber sociability test in wild-type (WT) and STEP knockout (KO) mice. Two-way ANOVA was performed indicating "genotype" and "cage" effect. $^{###}p < 0.001$, "cage" effect for each genotype (Student's dependent *t*-test), $^{**}p < 0.005$ "genotype" effect when analyzing the time exploring the dummy cage. **(B)** Time exploring the known and the stranger mouse cages in the short-term memory evaluation of the three-chamber sociability test in WT and STEP KO mice. Two-way ANOVA was performed indicating "genotype" and "cage" effect. $^{###}p < 0.001$, "cage" effect (Student's dependent *t*-test), $^{***}p < 0.001$ "genotype" effect when analyzing the time exploring the stranger mouse cage. **(C)** Time exploring the same juvenile in the first two trials, and a new juvenile in the third trial of the direct interaction test in WT and STEP KO mice. $^{+++}p < 0.001$, "trial" effect [repeated measures ANOVA (MANOVA)], $^{**}p < 0.005$ "genotype" effect (Student's independent *t*-test). **(D)** Time exploring the female A during the first 10 trials, and a new female B in the 11th trial of the 11-trial social memory test in WT and STEP KO mice. $^{+++}p < 0.001$, "trial" effect (repeated MANOVA), $^{***}p < 0.001$ "genotype" effect (Student's independent *t*-test). Results are presented as mean ± SEM [n = 11–12 for **(A,B)**; n = 9–10 for **(C,D)**]. **(E)** Percentage of time exploring each object during the training and testing phases of the novel object recognition test (NORT). Results are represented as mean ± SEM (n = 10–12). "Genotype" effect n.s., $^{***}p < 0.001$ "object" effect (two-way ANOVA).

Student's *t*-test, $t_{(1,21)} = 5.50$, $p < 0.001$), and less exploration of both cages (WT: 134.50 ± 8.62 s, STEP KO: 87.54 ± 8.24 s, $t_{(1,21)} = 3.92$, $p < 0.001$) compared to WT group. Importantly, while WT mice displayed social memory and spent more time exploring the "stranger" mouse than the "known" mouse cage (Student's *t*-test, $t_{(1,22)} = 4.46$, $p < 0.001$), STEP KO mice showed no preference for any mouse cage (Student's *t*-test, $t_{(1,20)} = 0.37$, n.s.) pointing at social memory alterations (**Figure 1B**).

To further characterize social memory performance of STEP KO mice, we next used the social discrimination test. When the same juvenile was presented in the second trial (A'), mice from both genotypes showed a habituation effect (repeated measures ANOVA (MANOVA) "trial": $F_{(1,17)} = 39.42$, $p < 0.001$), without differences between genotypes (repeated MANOVA "genotype × trial," $F_{(1,17)} = 0.67$, $p = 0.42$; "genotype," $F_{(1,17)} = 0.34$, $p = 0.56$; **Figure 1C**). In the third trial, when a new juvenile (B) was presented to the subject mouse, STEP KO mice explored the unknown mouse at similar levels as the previous habituation trial, whereas control mice explored longer, as during the first trial (Student's *t*-test "genotype" effect, $F_{(1,18)} = 11.93$, $p < 0.005$). Comparison between the first and the third trial also showed differences between genotypes (ANOVA, WT: 9.95 ± 17.33 s, STEP KO: 65.99 ± 11.73 s, $F_{(1,18)} = 6.85$, $p < 0.05$) in the dishabituation, thus indicating social memory alterations.

We also performed the 11-trial habituation/dishabituation social recognition test. Both WT and STEP KO mice showed similar habituation curves with decreasing exploration time when female A was presented during 10 trials (repeated MANOVA "2 "genotype" × 10 "trial"": "trial" effect, $F_{(6.38,108.55)} = 18.16$, $p < 0.001$; "genotype" effect, $F_{(1,17)} = 0.391$, $p = 0.54$). However, when a new female was presented to the subject mouse in the 11th trial there were differences between genotypes ($F_{(1,18)} = 34.45$, $p < 0.001$) since WT animals spent significantly more time exploring the new female than in the previous trials with the known one, which was not the case for STEP KO mice (**Figure 1D**). Altogether, these results showed that lack of STEP activity impairs social recognition memory.

To determine whether STEP KO have a general recognition memory deficit we evaluated their object recognition memory using the NORT. In the first habituation the traveled distance was similar in WT and STEP KO mice (Student's *t*-test, WT: 4178.57 ± 320.38 cm and STEP KO: 3493.39 ± 215.42 cm, $t_{(1,21)} = 1.74$, n.s.), while in the second habituation STEP KO mice traveled less distance than WT mice (Student's *t*-test, WT: 2534.48 ± 119.92 cm and STEP KO: 1638.02 ± 155.24 cm, $t_{(1,21)} = 4.61$, $p < 0.001$). No differences in object preference were found during the training phase when two identical objects were presented (ANOVA, "genotype" effect, $F_{(1,21)} = 1.11$, n.s.; "object" effect, $F_{(1,21)} = 0.72$, n.s.) although STEP KO mice explored less time both objects than WT mice (Student's *t*-test WT: 21.38 ± 3.32 s and STEP KO: 11.91 ± 2.78 s, $t_{(1,21)} = 2.16$, $p < 0.05$). Both genotypes explored longer the new object than the known one in the testing phase (ANOVA, "genotype" effect, $F_{(1,20)} = 2.58$, n.s.; "object" effect, $F_{(1,20)} = 48.76$, $p < 0.001$) and there were no significant differences in time of exploration of both objects (Student's *t*-test WT: 30.35 ± 5.74 s and STEP KO: 16.72 ± 4.99 s, $t_{(1,21)} = 1.17$, n.s.), indicating similar levels of object recognition memory (**Figure 1E**).

STEP KO Mice Have Unaltered Olfactory Function

Given that rodent social interactions largely depend on a functional olfactory system (Ropartz, 1968; Matochik, 1988; Popik et al., 1991), we asked whether STEP KO mice have affectations in the olfactory function. To address this possibility, WT and STEP KO mice were subjected to the olfactory habituation/dishabituation test. Mice from both genotypes showed habituation when each scent was presented during three consecutive trials (repeated MANOVA 3 "trial" × 2 "genotype," clean bedding: "trial" effect $F_{(1,34)} = 10.61$, $P < 0.001$, "genotype" effect $F_{(1,17)} = 7.39$, $p < 0.05$; social odor A: "trial" effect $F_{(1.38,23.50)} = 50.65$, $p < 0.001$; social odor B: "trial" effect $F_{(1.71,29.06)} = 39.01$, $p < 0.001$; **Figure 2**). There was also dishabituation when a new scent was presented, without differences between genotypes (Student's *t*-test "trial 1 social odor A—trial 3 clean bedding," WT vs. STEP KO mice: $t_{(1,17)} = 1.45$, n.s.; "trial 1 social odor B—trial 3 social odor A," WT vs. STEP KO mice: $t_{(1,17)} = 0.84$, n.s.; **Figure 2**), thus indicating that STEP KO mice have intact smell sense, and are able to distinguish different social odors.

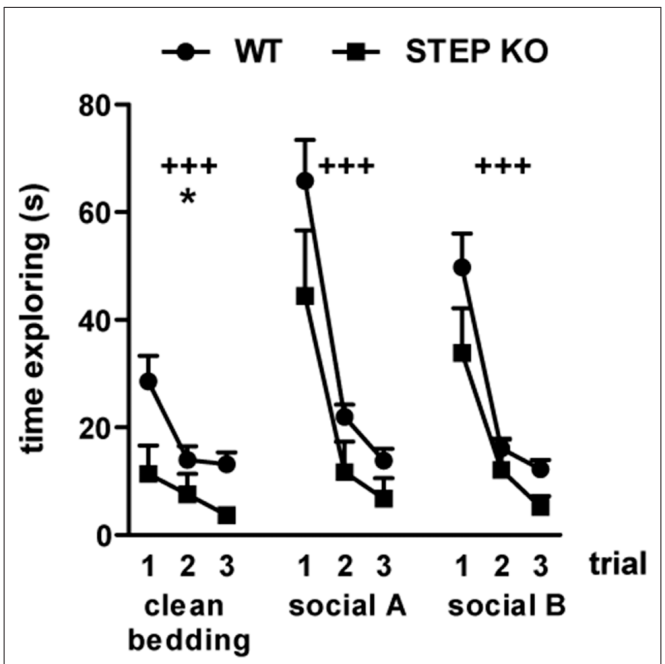

FIGURE 2 | STEP KO mice have normal olfactory function. Time exploring three different scents including clean bedding and two different social odors (social A and social B) in the olfactory habituation/dishabituation test. Results from WT and STEP KO mice are presented as mean ± SEM (n = 9–10). $^{+++}p < 0.001$, "trial" effect, $^{*}p < 0.05$ "genotype" effect (repeated MANOVA).

Lack of STEP Has No Effect on Sensorimotor Functions or Anxiety Levels

To know if the alterations found in social memory in STEP KO mice may be in part influenced by sensorimotor alterations and/or changes in anxiety levels, the animals were tested to assess these phenotypes. STEP KO mice did not show alterations in visual acuity, auditory reflex or escape reflex when assessed in a SHIRPA battery (data not shown). Nesting behavior, group sleeping, physical appearance, and absence of hind limb clasping were similar in both genotypes. As represented in **Table 1**, STEP KO mice did not show motor alterations in the wire hanger test or in the iron rod test, but they explored fewer corners than WT mice in the corner test. In line with the diminished exploratory levels seen in the corner test, STEP KO mice traveled less distance than WT mice in the open field (**Supplementary Figure S4**), performing more grooming behavior, and presenting more defecation boluses (**Table 1**). To assess possible alterations in anxiety levels, mice were tested in the plus maze and dark-light box. Data showed that both WT and STEP KO mice presented similar levels of activity in the more aversive areas, the open arms of the plus maze and the light compartment of the dark-light box. Actually, STEP KO mice showed diminished latency to enter the open arms of the plus maze compared to the control group (**Table 1**), thus indicating that they do not present altered anxiety levels. We also analyzed active vs. passive stress coping using the tail suspension test. STEP KO mice presented reduced latency to immobility ($t_{(1,17)} = 3.26$, Student's t-test; **Figure 3A**), and increased time of immobility ($t_{(1,17)} = 2.21$, Student's t-test; **Figure 3B**), pointing at diminished levels of active stress coping.

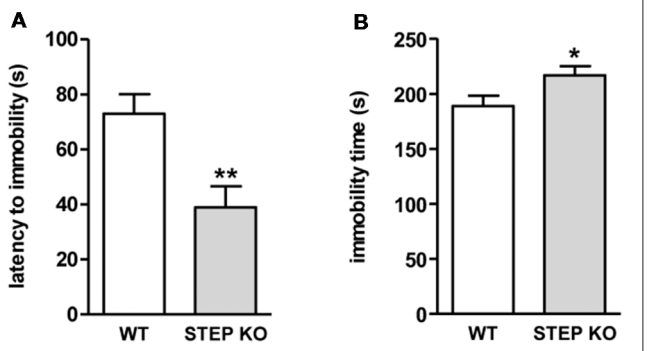

FIGURE 3 | STEP KO mice present behavioral despair. **(A)** Latency to first movement and **(B)** accumulated time of immobility in WT and STEP KO mice in the tail suspension test. Results are represented as mean ± SEM (n = 11–12). *$p < 0.05$, **$p < 0.01$ (Student's independent t-test).

STEP KO Mice Show Altered Social Patterns

Since mice with a genetic deletion of STEP displayed social memory impairment that could not be explained by the presence

TABLE 1 | Behavioral battery results when evaluating wild-type (WT) and STriatal-Enriched protein tyrosine Phosphatase knockout (STEP KO) mice (n = 11–12) in sensorimotor, exploratory and anxiety tests.

	WT	STEP KO	$t_{(1,21)}$	$p<$
Corner test				
Number of corners	9.42 ± 0.89	6.00 ± 0.77	2.87	0.01
Number of rearings	1.25 ± 0.37	2.45 ± 0.45	2.07	n.s
Iron rod				
Muscular strength (latency in s)	9.69 ± 1.59	10.46 ± 2.12	0.29	n.s.
Motor coordination (segments)	1.46 ± 0.61	0.54 ± 0.21	1.37	n.s.
Wire hanger test—60s				
Muscular strength (latency in s)	12.05 ± 2.63	6.66 ± 1.40	1.76	n.s.
Motor coordination (segments)	2.00 ± 0.45	1.23 ± 0.45	1.21	n.s.
Open field				
Distance (cm)	2185 ± 139.8	1374 ± 217.6	3.19	0.005
Number of groomings	1.42 ± 0.34	2.54 ± 0.39	2.20	0.05
Number of rearings	21.92 ± 2.16	17.27 ± 2.57	1.39	n.s.
Number of defecation boluses	0.58 ± 0.35	2.09 ± 0.47	2.56	0.05
Plus maze				
Latency of entry in the open arms (s)	109.7 ± 36.75	21.64 ± 13.93	2.16	0.05
Number of entries in the open arms	3.17 ± 0.82	3.00 ± 0.36	0.18	n.s.
Distance in the open arms (cm)	112.6 ± 29.51	129.1 ± 16.66	0.47	n.s.
% time in the open arms (s)	7.46 ± 1.80	10.79 ± 2.08	−1.21	n.s.
% time in the enclosed arms (s)	78.10 ± 3.39	76.88 ± 2.65	0.28	n.s.
Time in the open arms (s)	22.39 ± 5.42	32.38 ± 6.26	−1.21	n.s.
Time in the enclosed arms (s)	234.30 ± 10.17	230.63 ± 7.97	0.28	n.s.
Number of entries in the open arms	3.16 ± 0.82	3.00 ± 0.35	0.18	n.s.
Number of entries in the enclosed arms	8.41 ± 1.24	7.91 ± 0.49	0.38	n.s.
Dark-light box				
Latency of entry into the light compartment (s)	40.69 ± 15.42	60.67 ± 21.85	1.51	n.s.
Number of entrances into the light compartment	5.25 ± 0.69	4.36 ± 0.54	0.99	n.s.
Distance in the light compartment (cm)	258.1 ± 31.53	230.5 ± 32.07	0.61	n.s.
Time in the light compartment (s)	82.39 ± 13.75	46.39 ± 7.86	2.18	0.05

Results are presented as mean ± SEM. Data was analyzed by Student's t-test. n.s., non significant.

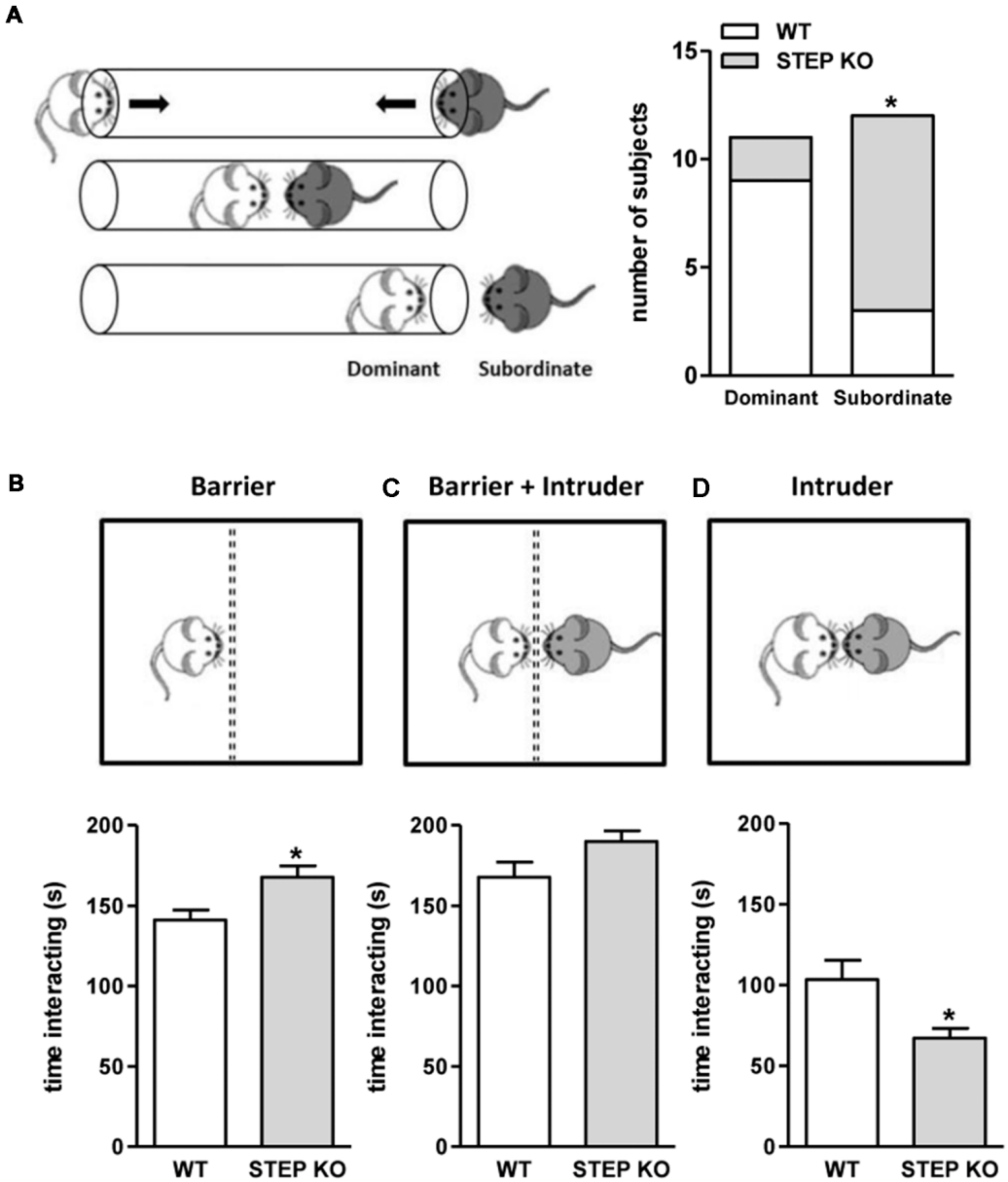

FIGURE 4 | STEP KO mice display altered social patterns. **(A)** Dominant and subordinate behavior of WT and STEP KO (n = 11–12) mice in the dominance tube test. Results are presented as number of subjects presenting each phenotype. *$p < 0.05$ "genotype" effect (Fisher's test). Time interacting with the barrier **(B)**, with the intruder behind the barrier **(C)** and directly with the intruder **(D)** during the resident-intruder test in WT and STEP KO mice. Results are presented as mean ± SEM (n = 11–12). *$p < 0.05$ "genotype" effect (Student's independent t-test).

of olfactory defects, alterations in sensorimotor functions or increased anxiety levels, we next sought to analyze whether social patterns were also affected. For that, we used the dominance tube test and the resident-intruder test. Data from the dominance tube test showed that the number of animals with subordinate behavior was higher in STEP KO mice group compared to controls (**Figure 4A**). The results from the resident-intruder test indicated that STEP KO mice showed more interest in interacting with the barrier than WT mice during the habituation to the barrier ("genotype" effect $t_{(1,21)} = 7.31$, $p < 0.05$; **Figure 4B**), but not when the intruder was introduced in the home cage behind the barrier ("genotype" effect $t_{(1,21)} = 2.86$, n.s.; **Figure 4C**). Nevertheless, and in line with the subordinate behavior observed in the dominance tube test, when the barrier was removed STEP KO mice showed reduced time of interaction with the intruder compared to the WT group ("genotype" effect $t_{(1,21)} = 6.44$, $p < 0.05$; **Figure 4D**).

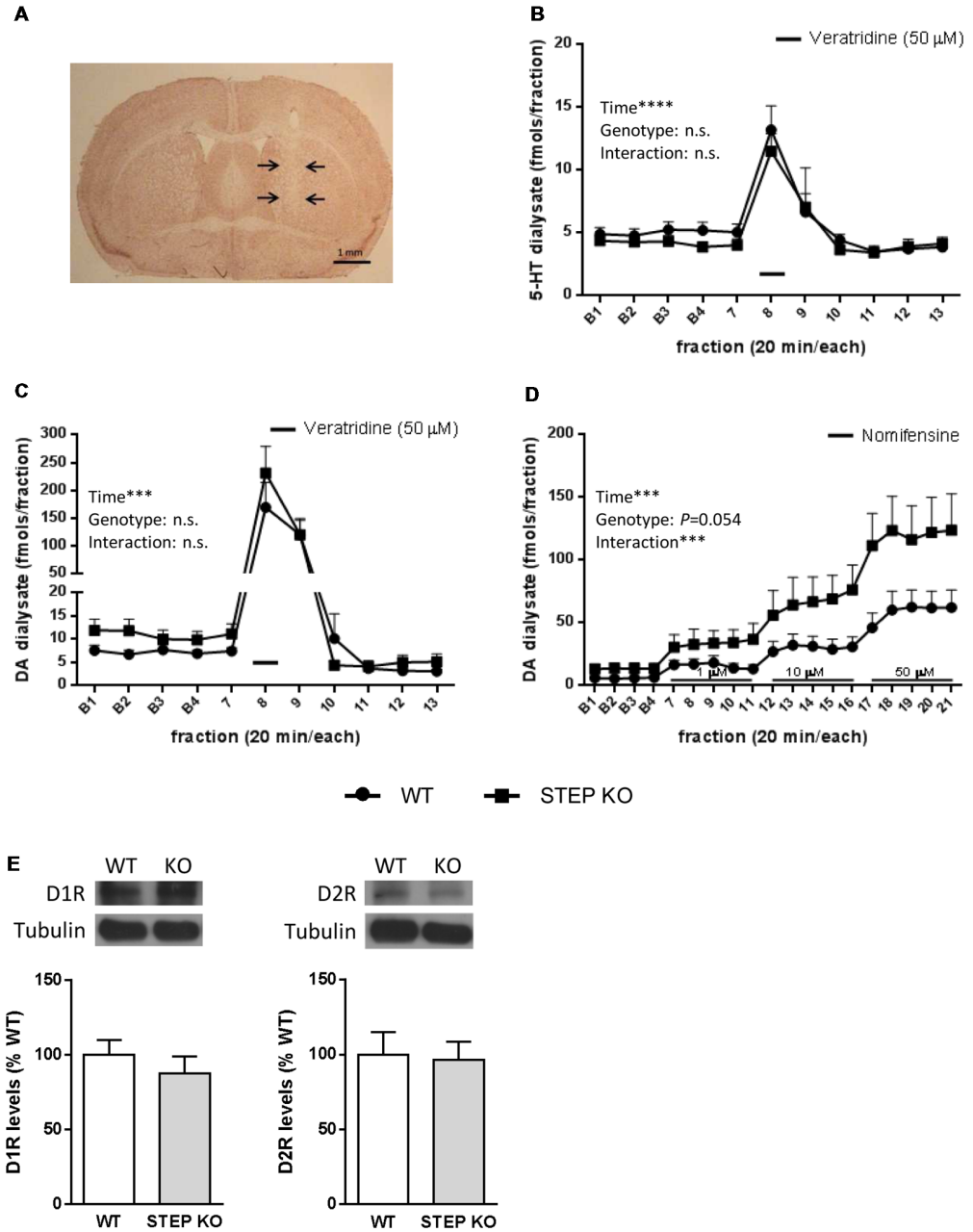

FIGURE 5 | Basal dopamine (DA) release in the dorsal striatum is increased in STEP KO mice. **(A)** Representative photomicrograph illustrating the location (arrows) of the dialysis probe after staining with neutral red. **(B)** Effect of local perfusion of 50 μM veratridine (line) on 5-HT output in the dorsal striatum of WT and STEP KO mice (two-way ANOVA, significant effect of time; $^{****}p < 0.001$). **(C)** Effect of local perfusion of 50 μM veratridine (line) on DA output in the dorsal striatum of WT and STEP KO mice (two-way ANOVA, significant effect of time; $^{***}p < 0.001$). **(D)** Effect of local perfusion of 1, 10 and 50 μM nomifensine (line) on DA output in the dorsal striatum of WT and STEP KO mice (two-way ANOVA, significant effect of time and time × genotype interaction; $^{***}p < 0.001$). Values are expressed as mean 5-HT/DA concentration (fmols/fraction) ± SEM (n = 9–11 mice). **(E)** DA D1 receptor (D1R) and DA D2 receptor (D2R) levels were analyzed by Western blot of protein extracts obtained from the striatum of WT and STEP KO mice. Representative immunoblots are shown. Values (obtained by densitometric analysis of Western blot data) are expressed as percentage of WT mice and shown as mean ± SEM (n = 10–11). Data were analyzed by Student's independent t-test. n.s., non significant.

STEP KO Mice Show Increased Basal DA Release in the Dorsal Striatum

Finally, we wondered whether the changes found in social behaviors were accompanied by alterations in neurotransmitter levels in the striatum of STEP KO mice. To answer this question, we implanted a dialysis probe in the dorsal striatum of freely moving WT and STEP KO mice (**Figure 5A**) to perform *in vivo* microdialysis, and determine extracellular 5-HT and DA levels by

HPLC. Basal 5-HT levels (fmols/fraction) were 5.34 ± 0.51 in WT ($n = 11$) and 4.67 ± 0.55 in STEP KO mice ($n = 11$; $t_{(1,20)} = 0.8933$; $p = 0.3823$), and basal 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) levels (pmols/fraction) were 1.27 ± 0.18 in WT ($n = 11$) and 1.27 ± 0.16 in STEP KO mice ($n = 11$; $t_{(1,20)} = 0$; $p = 1.00$). The analysis of the effect of local perfusion of the depolarizing drug veratridine (50 μM) on 5-HT levels indicated a significant effect of time ($F_{(13,247)} = 13.96$; $p < 0.0001$), but no genotype effect ($F_{(1,19)} = 0.6227$; $p = 0.4398$) or time × genotype interaction ($F_{(13,247)} = 0.3106$; $p = 0.9901$; **Figure 5B**). Thus, both basal levels and veratridine-induced release of 5-HT were similar in the dorsal striatum of WT and STEP KO mice.

On the other hand, basal extracellular DA levels (fmols/fraction) were 6.77 ± 1.00 in WT ($n = 15$) and 11.81 ± 2.76 in STEP KO mice ($n = 13$; $t_{(1,26)} = 2.326$; $p < 0.05$), and basal DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) values (pmols/fraction) were 1.22 ± 0.16 in WT ($n = 15$) and 2.15 ± 0.25 in STEP KO mice ($n = 13$; $t_{(1,26)} = 3.223$; $p < 0.01$). Thus, STEP KO mice had significantly increased basal extracellular concentrations of DA and DOPAC in the dorsal striatum compared to WT mice. Perfusion of veratridine (50 μM) increased DA output in the dorsal striatum similarly in both genotypes. Thus, two-way ANOVA showed a significant effect of time ($F_{(10,190)} = 33.44$; $p < 0.001$), but no genotype effect ($F_{(1,19)} = 0.657$; n.s.) or time × genotype interaction ($F_{(10,190)} = 0.670$; n.s.; **Figure 5C**). The local perfusion of the norepinephrine-DA reuptake inhibitor nomifensine (1, 10 and 50 μM) in the dorsal striatum produced an enhanced DA output in STEP KO mice compared to WT mice. Two-way ANOVA showed a significant effect of time ($F_{(18,306)} = 24.97$; $p < 0.001$), a quasi-significant effect of genotype ($F_{(1,17)} = 4.254$; $p = 0.054$) and a significant time × genotype interaction ($F_{(18,306)} = 2.929$; $p < 0.001$, **Figure 5D**), pointing at an increased release of DA in the STEP KO mice compared to the WT group. However, STEP KO mice did not show alterations in D1R or D2R striatal levels respect to WT mice (D1R: $t_{(1,19)} = 0.83$, n.s.; D2R: $t_{(1,18)} = 0.18$, n.s., **Figure 5E**).

DISCUSSION

It has been reported that STEP KO mice show improved cognitive performance in the Morris water maze and in the radial arm maze (Venkitaramani et al., 2011) as well as increased fear conditioning in the conditioning suppression food-motivated instrumental performance test (Olausson et al., 2012). The increased tyrosine phosphorylation of STEP substrates, and downstream targets, in distinct brain regions likely provides a potential molecular mechanism for those results (Venkitaramani et al., 2009, 2011; Zhang et al., 2010; Olausson et al., 2012). Indeed, based on previous works (Suzuki et al., 2011; Sinai et al., 2012), it could be expected that STEP KO mice show improved social memory. However, social memory and social behaviors have been barely studied in this mouse model, and there are also reports showing that alterations in the ERK pathway may underlie altered social behavior (Satoh et al., 2011; Faridar et al., 2014). In the present study, we focused on the social phenotype of STEP KO mice, and we show that STEP deficient mice present impaired social memory. Since WT and STEP KO mice showed similar levels of sociability (present results; Venkitaramani et al., 2011; Goebel-Goody et al., 2012b), and non-social hippocampal-dependent learning and memory is intact or improved (present results; Venkitaramani et al., 2011; Sukoff Rizzo et al., 2014; Castonguay et al., 2018), our results indicate that STEP deficiency specifically disturbs social memory.

In contrast to the present findings, a previous work reported that STEP KO mice had intact social memory (Venkitaramani et al., 2011). In that study, only a direct interaction test was performed, a smaller group of animals was tested, and data of social memory assessment was not provided (Venkitaramani et al., 2011). However, and in accordance with our results, these authors did report data of time spent in social interaction pointing at intact sociability levels in STEP KO mice. Strikingly, in another study, neither WT nor STEP KO mice showed social memory in the three-chamber sociability test (Goebel-Goody et al., 2012b). Probably, the lack of social memory in WT mice was due to the stimulus mice being from mixed genotypes. Indeed, it has been reported that social communication is affected by genotype, since social signaling from mice other than WT may elicit unusual social behaviors in the subject mice (Wood and Morton, 2015). To eliminate this confounding variable, in the present study all the stimulus mice used to assess social memory were WT animals.

As far as we have investigated, an impaired olfactory system or altered anxiety levels in STEP KO mice are unlikely to underlie the social memory deficits reported here. Indeed, STEP KO mice behave like WT mice in the olfactory habituation/dishabituation test, and no significant differences were observed in the plus maze or the dark-light box tests compared to WT mice, as previously described (Goebel-Goody et al., 2012b). In addition, STEP KO mice spent significantly more time in the center of the open field than WT mice, and did not display anxiety-related phenotypes in the stress induced hyperthermia test (Sukoff Rizzo et al., 2014).

The present results indicate that STEP KO mice consistently show reduced exploratory activity in the open field, in the socialization and test phases of the three-chamber sociability test, and in the habituation phase of NORT. Although this finding could have a confounding effect on the outcome in social tasks, several results suggest that this was not the case. During the socialization phase in the three-chamber sociability test, STEP KO mice explored the mouse similarly to WT mice, indicating similar levels of interest for social novelty as well as similar time for social learning acquisition compared to WT mice. In addition, the results obtained in the first trial of the social discrimination test and the 11-trial habituation/dishabituation test and their learning curves point to a similar interest and similar time for acquisition of a new social stimulus in both genotypes. Altogether, these results strengthen the idea that altered locomotion in STEP KO mice is unlikely to influence their outcome in social memory tests. In contrast to the present findings, previous data documented that STEP KO mice do not present alterations in spontaneous exploratory activity compared

to controls (Venkitaramani et al., 2011; Sukoff Rizzo et al., 2014; Legastelois et al., 2015). Given that STEP KO mice have intact motor capabilities (present results) and motor coordination (Venkitaramani et al., 2011; Sukoff Rizzo et al., 2014), this discrepancy might be explained by differences in methodological procedures. In our study, we assessed exploratory activity in a 5- and 10-min test, respectively, where a fear-to-novelty component of the first minutes might be diminishing STEP KO exploratory activity. In contrast, the previous studies evaluated longer trials, and thus the initial fear-to-novelty effect is likely diluted. In fact, it is noteworthy that the analysis of the initial 5–10 min in the open field reported in earlier studies suggests that STEP KO mice present a trend toward reduced exploratory activity respect to the control group (Venkitaramani et al., 2011; Sukoff Rizzo et al., 2014; Legastelois et al., 2015). Further supporting our hypothesis, the data of the corner test also pointed at increased fear-to-novelty in STEP KO mice. In agreement with our findings, it was reported that STEP deficient mice were hypoactive in the social novelty phase of the three-chamber test, where behavior is also evaluated in trials of 10 min duration (Goebel-Goody et al., 2012b). Actually, in a mouse model of fragile X syndrome, increased STEP levels promote locomotor hyperactivity that can be prevented by genetic deletion (Goebel-Goody et al., 2012b) or pharmacological inhibition of STEP using TC-2153 (Chatterjee et al., 2018). Nevertheless, no differences between STEP KO and WT group were found in a 5 min trial in the open field, probably due to the wide range of ages of the subjects (Goebel-Goody et al., 2012b). Overall, altered locomotion in STEP KO mice does not seem to play a major role in the social phenotype reported here, and STEP KO mice showed intact social memory in the form of habituation as indicated by the learning curves. However, STEP KO mice did show a compromised performance in the test phase. Nonetheless, we cannot rule out the contribution of lack of dishabituation to a new stimulus in the absence of STEP.

Neurotransmitters like 5-HT and DA are involved in the brain circuitry related to social behaviors (Miczek et al., 2002; Watanabe and Yamamoto, 2015; Lu et al., 2018). The dorsal striatum plays a role in internally guided social behavior, the ventral striatum regulates social behavior by the integration of external social stimuli (Báez-Mendoza and Schultz, 2013; van den Bos, 2015), and the balance of 5-HT and DA levels determines the role of each striatal region in social behaviors (van den Bos, 2015). Here, we reported that extracellular levels of 5-HT in the dorsal striatum are similar in both genotypes. However, we observed significantly increased basal extracellular concentrations of DA, and higher DA output in response to inhibition of DA reuptake in the dorsal striatum of STEP KO mice, without alterations in the total levels of D1R and D2R. The molecular mechanism underlying this result is currently unknown, but it could be related to the finding that phosphorylated $Pyk2^{Tyr402}$, a STEP substrate (Xu et al., 2012), has been implicated in DA release in PC12 cells (Zhang et al., 2014, 2016). Moreover, phosphorylation of synapsin I, which regulates the probability of vesicle release (Cesca et al., 2010), was found to be increased in STEP KO mice (Venkitaramani et al., 2011; Bosco et al., 2018).

It has been shown that high levels of DA in the dorsal striatum increase motivation, approach and reward behavior (Ikemoto et al., 2015), and social motivation and reward behaviors include those that happen in a social context (Báez-Mendoza and Schultz, 2013). Surprisingly, our data suggest that STEP KO present decreased interest/motivation as shown by the reduced exploratory activity (as discussed above), diminished active coping to stress in the tail suspension test, reduced exploration of the intruder in the resident intruder test, as well as diminished exploration of a new home cage in the corner test. Thus, our findings suggest that STEP KO mice present decreased motivation to cope with new environmental or social stimuli. On the other hand, it has been reported that mouse models with altered dopaminergic neural state also present social dominance and aggressive behavior (Rodriguiz et al., 2004; Adamczyk et al., 2012; McNamara et al., 2017). However, despite the presence of higher DA levels in STEP KO mice, they presented reduced dominance behavior in the dominance tube test and interacted less time with the intruder mouse in the resident-intruder test. Conversely, a previous study found that STEP KO and WT mice retreat from the social dominance tube test at a similar frequency (Goebel-Goody et al., 2012b), and the initial characterization of STEP KO mice reported greater dominance behavior than control mice scored based on holding down the other mouse against the cage floor or cage wall (Venkitaramani et al., 2011). It should be kept in mind that global genetic manipulation of STEP levels may promote compensatory mechanisms or developmental modifications. In fact, an acute pharmacological inhibition of STEP after intraperitoneal injection of 10 mg/Kg TC-2153 (Xu et al., 2014) in WT mice had no effect in most parameters analyzed in the open field, light-dark box task or three-chamber social test (Chatterjee et al., 2018). Moreover, we cannot rule out the contribution of other brain regions and neurotransmitters (van Erp and Miczek, 2000; Miczek et al., 2002). For instance, glutamate release is also increased in STEP deficient mice (Bosco et al., 2018), and it is known that glutamate and DA neurotransmission modulate each other in the striatum (Mora et al., 2008; Gardoni and Bellone, 2015).

In conclusion, the present results highlight that lack of STEP activity impairs social memory in the absence of affected olfactory function or altered anxiety levels, and produces changes in social patterns accompanied by dysregulation of striatal DA homeostasis.

AUTHOR CONTRIBUTIONS

GB, AS and EP-N conceptualized the study, and EP-N supervised it. GB designed the behavioral phenotyping, performed the experiments, data analysis and interpretation of results. AC designed the microdialysis studies, performed the experiments with GB and MM, and analyzed, interpreted data of microdialysis experiments, and wrote this section. GB and AS interpreted the results, wrote the manuscript and prepared the figures. All the authors critically reviewed the content and approved the final version.

FUNDING

This work was supported by Ministerio de Economia y Competitividad, Spain (SAF2016-08573-R to EP-N and SAF2017-88076-R to JA).

ACKNOWLEDGMENTS

We are very grateful to Ana López, Maite Muñoz, Leticia Campa and Pep Marimón for their technical support, and Garikoitz Azkona for the experimental design advises. We thank Dr. Paul J. Lombroso (Child Study Center, Yale School of Medicine, Yale University, New Haven, CT, USA) for providing the STEP KO mice to stablish our colony and Marta Garcia-Forn, Rafael Alcalá-Vida and Jordi Creus-Muncunill for the helpful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00317/full#supplementary-material

REFERENCES

Adamczyk, A., Mejias, R., Takamiya, K., Yocum, J., Krasnova, I. N., Calderon, J., et al. (2012). GluA3-deficiency in mice is associated with increased social and aggressive behavior and elevated dopamine in striatum. *Behav. Brain Res.* 229, 265–272. doi: 10.1016/j.bbr.2012.01.007

Báez-Mendoza, R., and Schultz, W. (2013). The role of the striatum in social behavior. *Front. Neurosci.* 7:233. doi: 10.3389/fnins.2013.00233

Bault, N., Joffily, M., Rustichini, A., and Coricelli, G. (2011). Medial prefrontal cortex and striatum mediate the influence of social comparison on the decision process. *Proc. Natl. Acad. Sci. U S A* 108, 16044–16049. doi: 10.1073/pnas.1100892108

Bickart, K. C., Dickerson, B. C., and Barrett, L. F. (2014). The amygdala as a hub in brain networks that support social life. *Neuropsychologia* 63, 235–248. doi: 10.1016/j.neuropsychologia.2014.08.013

Blázquez, G., Cañete, T., Tobena, A., Giménez-Llort, L., and Fernández-Teruel, A. (2014). Cognitive and emotional profiles of aged Alzheimer's disease (3xTgAD) mice: effects of environmental enrichment and sexual dimorphism. *Behav. Brain Res.* 268, 185–201. doi: 10.1016/j.bbr.2014.04.008

Bosco, F., Valente, P., Milanese, M., Piccini, A., Messa, M., Bonanno, G., et al. (2018). Altered intracellular calcium homeostasis underlying enhanced glutamatergic transmission in striatal-enriched tyrosine phosphatase (STEP) knockout mice. *Mol. Neurobiol.* 55, 8084–8102. doi: 10.1007/s12035-018-0980-5

Boulanger, L. M., Lombroso, P. J., Raghunathan, A., During, M. J., Wahle, P., and Naegele, J. R. (1995). Cellular and molecular characterization of a brain-enriched protein tyrosine phosphatase. *J. Neurosci.* 15, 1532–1544. doi: 10.1523/JNEUROSCI.15-02-01532.1995

Can, A., Dao, D. T., Terrillion, C. E., Piantadosi, S. C., Bhat, S., and Gould, T. D. (2012). The tail suspension test. *J. Vis. Exp.* 59:e3769. doi: 10.3791/3769

Castañé, A., Artigas, F., and Bortolozzi, A. (2008). The absence of 5-HT_{1A} receptors has minor effects on dopamine but not serotonin release evoked by MK-801 in mice prefrontal cortex. *Psychopharmacology* 200, 281–290. doi: 10.1007/s00213-008-1205-9

Castonguay, D., Dufort-Gervais, J., Ménard, C., Chatterjee, M., Quirion, R., Bontempi, B., et al. (2018). The tyrosine phosphatase STEP is involved in age-related memory decline. *Curr. Biol.* 28, 1079.e4–1089.e4. doi: 10.1016/j.cub.2018.02.047

Cesca, F., Baldelli, P., Valtorta, F., and Benfenati, F. (2010). The synapsins: Key actors of synapse function and plasticity. *Prog. Neurobiol.* 91, 313–348. doi: 10.1016/j.pneurobio.2010.04.006

Chatterjee, M., Kurup, P. K., Lundbye, C. J., Hugger Toft, A. K., Kwon, J., Benedict, J., et al. (2018). STEP inhibition reverses behavioral, electrophysiologic, and synaptic abnormalities in Fmr1 KO mice. *Neuropharmacology* 128, 43–53. doi: 10.1016/j.neuropharm.2017.09.026

Deacon, R. M. J. (2006). Assessing nest building in mice. *Nat. Protoc.* 1, 1117–1119. doi: 10.1038/nprot.2006.170

Dere, E., Huston, J. P., and De Souza Silva, M. A. (2007). The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. *Neurosci. Biobehav. Rev.* 31, 673–704. doi: 10.1016/j.neubiorev.2007.01.005

DeVito, L. M., Konigsberg, R., Lyyken, C., Sauvage, M., Scott, W. S. III., and Eichenbaum, H. (2009). Vasopressin 1b receptor knockout impairs memory for temporal order. *J. Neurosci.* 29, 2676–2683. doi: 10.1523/JNEUROSCI.5488-08.2009

Engelmann, M., Hädicke, J., and Noack, J. (2011). Testing declarative memory in laboratory rats and mice using the nonconditioned social discrimination procedure. *Nat. Protoc.* 6, 1152–1162. doi: 10.1038/nprot.2011.353

Faridar, A., Jones-Davis, D., Rider, E., Li, J., Gobius, I., Morcom, L., et al. (2014). Mapk/Erk activation in an animal model of social deficits shows a possible link to autism. *Mol. Autism* 5:57. doi: 10.1186/2040-2392-5-57

Fergusson, J. N., Young, L. J., Heran, E. F., Matzuk, M. M., Insel, T. R., and Winslow, J. T. (2000). Social amnesia in mice lacking the oxytocin gene. *Nat. Genet.* 25, 284–288. doi: 10.1038/77040

Fernández-Teruel, A., Escorihuela, R. M., Gray, J. A., Aguilar, R., Gil, L., Giménez-Llort, L., et al. (2002). A quantitative trait locus influencing anxiety in the laboratory rat. *Genome Res.* 4, 618–626. doi: 10.1101/gr.203402.

Franklin, K., and Paxinos, G. (1997). *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.

Gardoni, F., and Bellone, C. (2015). Modulation of the glutamatergic transmission by Dopamine: a focus on Parkinson, Huntington and Addiction diseases. *Front. Cell. Neurosci.* 9:25. doi: 10.3389/fncel.2015.00025

Goebel-Goody, S. M., Baum, M., Paspalas, C. D., Fernandez, S. M., Carty, N. C., and Kurup, P. (2012a). Therapeutic implications for striatal-enriched protein tyrosine phosphatase (STEP) in neuropsychiatric disorders. *Pharmacol. Rev.* 64, 65–87. doi: 10.1124/pr.110.003053

Goebel-Goody, S. M., Wilson-Wallis, E. D., Royston, S., Tagliatela, S. M., Naegele, S. R., and Lombroso, P. J. (2012b). Genetic manipulation of STEP reverses behavioral abnormalities in a fragile X syndrome mouse model. *Genes Brain Behav.* 11, 586–600. doi: 10.1111/j.1601-183x.2012.00781.x

Hitti, F. L., and Siegelbaum, S. A. (2014). The hippocampal CA2 region is essential for social memory. *Nature* 508, 88–92. doi: 10.1038/nature13028

Ikemoto, S., Yang, C., and Tan, A. (2015). Basal ganglia circuit loops, dopamine and motivation: a review and enquiry. *Behav. Brain Res.* 290, 17–31. doi: 10.1016/j.bbr.2015.04.018

Karasawa, T., and Lombroso, P. J. (2014). Disruption of striatal-enriched protein tyrosine phosphatase (STEP) function in neuropsychiatric disorders. *Neurosci. Res.* 89, 1–9. doi: 10.1016/j.neures.2014.08.018

Kohara, K., Pignatelli, M., Rivest, A. J., Jung, H., Kitamura, T., Suh, J., et al. (2014). Cell type-specific genetic and optogenetic tools reveal novel hippocampal CA2 circuits. *Nat. Neurosci.* 17, 269–279. doi: 10.1038/nn.3614

Legastelois, R., Darcq, E., Wegner, S. A., Lombroso, P. J., and Ron, D. (2015). Striatal-enriched protein tyrosine phosphatase controls responses to aversive stimuli: implication for ethanol drinking. *PLoS One* 10:e0127408. doi: 10.1371/journal.pone.0127408

Lijam, N., Paylor, R., McDonald, M. P., Crawley, J. N., Deng, C., Herrup, K., et al. (1997). Social interaction and sensorimotor gating abnormalities in mice lacking Dvl1. *Cell* 90, 895–905. doi: 10.1016/s0092-8674(00)80354-2

Lombroso, P. J., Murdoch, G., and Lerner, M. (1991). Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. *Proc. Natl. Acad. Sci. U S A* 88, 7242–7246. doi: 10.1073/pnas.88.16.7242

Lombroso, P. J., Naegele, J. R., Sharma, E., and Lerner, M. (1993). A protein tyrosine phosphatase expressed within dopaminoceptive neurons of the basal ganglia and related structures. *J. Neurosci.* 13, 3064–3074. doi: 10.1523/JNEUROSCI.13-07-03064.1993

Lu, D.-H., Liao, H.-M., Chen, C.-H., Tu, H.-J., Liou, H.-C., Gau, S. S.-F., et al. (2018). Impairment of social behaviors in Arhgef10 knockout mice. *Mol. Autism* 9:11. doi: 10.1186/s13229-018-0197-5

Lumley, L. A., Charles, R. F., Charles, R. C., Hebert, M. A., Morton, D. M., and Meyerhoff, J. L. (2000). Effects of social defeat and of diazepam on behavior in a resident-intruder test in male DBA/2 mice. *Pharmacol. Biochem. Behav.* 67, 433–447. doi: 10.1016/s0091-3057(00)00382-8

Matochik, J. A. (1988). Role of the main olfactory system in recognition between individual spiny mice. *Physiol. Behav.* 42, 217–222. doi: 10.1016/0031-9384(88)90073-x

McIlwain, K. L., Merriweather, M. Y., Yuva-Paylor, L. A., and Paylor, R. (2001). The use of behavioral test batteries: effects of training history. *Physiol. Behav.* 73, 705–717. doi: 10.1016/s0031-9384(01)00528-5

McLean, A. C., Valenzuela, N., Fai, S., and Bennett, S. A. L. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J. Vis. Exp.* 67:e4389. doi: 10.3791/4389

McNamara, G. I., Davis, B. A., Browne, M., Humby, T., Dalley, J. W., Xia, J., et al. (2017). Dopaminergic and behavioural changes in a loss-of-imprinting model of Cdkn1c. *Genes Brain Behav.* 2, 149–157. doi: 10.1111/gbb.12422

Miczek, K. A., Fish, E. W., De Bold, J. F., and De Almeida, R. M. (2002). Social and neural determinants of aggressive behavior: pharmacotherapeutic targets at serotonin, dopamine and γ-aminobutyric acid systems. *Psychopharmacology* 163, 434–458. doi: 10.1007/s00213-002-1139-6

Mora, F., Segovia, G., and del Arco, A. (2008). Glutamate-dopamine-GABA interactions in the aging basal ganglia. *Brain Res. Rev.* 58, 340–353. doi: 10.1016/j.brainresrev.2007.10.006

Olausson, P., Venkitaramani, D. V., Moran, T. D., Salter, M. W., Taylor, J. R., and Lombroso, P. J. (2012). The tyrosine phosphatase STEP constrains amygdala-dependent memory formation and neuroplasticity. *Neuroscience* 225, 1–8. doi: 10.1016/j.neuroscience.2012.07.069

Pagani, J. H., Zhao, M., Cui, Z., Williams Avram, S. K., Caruana, D. A., Dudek, S. M., et al. (2015). Role of the vasopressin 1b receptor in rodent aggressive behavior and synaptic plasticity in hippocampal area CA2. *Mol. Psychiatry* 20, 490–499. doi: 10.1038/mp.2014.47

Popik, P., Vetulani, J., Bisaga, A., and van Ree, J. M. (1991). Recognition cue in the rat's social memory paradigm. *J. Basic Clin. Physiol. Pharmacol.* 2, 315–327. doi: 10.1515/JBCPP.1991.2.4.315

Rodriguiz, R. M., Chu, R., Caron, M. G., and Wetsel, W. C. (2004). Aberrant responses in social interaction of dopamine transporter knockout mice. *Behav. Brain Res.* 148, 185–198. doi: 10.1016/s0166-4328(03)00187-6

Rogers, D. C., Fisher, E. M., Brown, S. D., Peters, J., Hunter, A. J., and Martin, J. E. (1997). Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. *Mamm. Genome* 8, 711–713. doi: 10.1007/s003359900551

Ropartz, P. (1968). The relation between olfactory stimulation and aggressive behaviour in mice. *Anim. Behav.* 16, 97–100. doi: 10.1016/0003-3472(68)90117-6

Saavedra, A., Giralt, A., Rué, L., Xifró, X., Xu, J., Ortega, Z., et al. (2011). Striatal-enriched protein tyrosine phosphatase expression and activity in Huntington's disease: a STEP in the resistance to excitotoxicity. *J. Neurosci.* 31, 8150–8162. doi: 10.1523/JNEUROSCI.3446-10.2011

Satoh, Y., Endo, S., Nakata, T., Kobayashi, Y., Yamada, K., Ikeda, T., et al. (2011). ERK2 contributes to the control of social behaviors in mice. *J. Neurosci.* 31, 11953–11967. doi: 10.1523/JNEUROSCI.2349-11.2011

Shinohara, Y., Hosoya, A., Yahagi, K., Ferecskó, A. S., Yaguchi, K., Sík, A., et al. (2012). Hippocampal CA3 and CA2 have distinct bilateral innervation patterns to CA1 in rodents. *Eur. J. Neurosci.* 35, 702–710. doi: 10.1111/j.1460-9568.2012.07993.x

Sinai, L., Mathew, R., and Roder, J. C. (2012). Impaired social memories in 129P2 inbred mice are rescued by reduced Csk expression. *Genes Brain Behav.* 11, 559–567. doi: 10.1111/j.1601-183x.2012.00775.x

Spencer, C. M., Alekseyenko, O., Serysheva, E., Yuva-Paylor, L. A., and Paylor, R. (2005). Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome. *Genes Brain Behav.* 4, 420–430. doi: 10.1111/j.1601-183x.2005.00123.x

Stevenson, E. L., and Caldwell, H. K. (2014). Lesions to the CA2 region of the hippocampus impair social memory in mice. *Eur. J. Neurosci.* 40, 3294–3301. doi: 10.1111/ejn.12689

Sukoff Rizzo, S. J., Lotarski, S. M., Stolyar, P., McNally, T., Arturi, C., Roos, M., et al. (2014). Behavioral characterization of striatal-enriched protein tyrosine phosphatase (STEP) knockout mice. *Genes Brain Behav.* 13, 643–652. doi: 10.1111/gbb.12169

Suzuki, A., Fukushima, H., Mukawa, T., Toyoda, H., Wu, L.-J., Zhao, M.-G., et al. (2011). Upregulation of CREB-mediated transcription enhances both short- and long-term memory. *J. Neurosci.* 31, 8786–8802. doi: 10.1523/JNEUROSCI.3257-10.2011

van den Bos, R. (2015). The dorsal striatum and ventral striatum play different roles in the programming of social behaviour: a tribute to Lex Cools. *Behav. Pharmacol.* 26, 6–17. doi: 10.1097/fbp.0000000000000110

van Erp, A. M., and Miczek, K. A. (2000). Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. *J. Neurosci.* 20, 9320–9325. doi: 10.1523/JNEUROSCI.20-24-09320.2000

Venkitaramani, D. V., Paul, S., Zhang, Y., Kurup, P. K., Ding, L., Tressler, L., et al. (2009). Knockout of STriatal enriched protein tyrosine phosphatase in mice results in increased ERK1/2 phosphorylation. *Synapse* 63, 69–81. doi: 10.1002/syn.20608

Venkitaramani, D. V., Moura, P. J., Picciotto, M. R., and Lombroso, P. J. (2011). Striatal-enriched protein tyrosin phosphatase (STEP) knockout mice have enhanced hippocampal memory. *Eur. J. Neurosci.* 33, 2288–2298. doi: 10.1111/j.1460-9568.2011.07687.x

Watanabe, N., and Yamamoto, M. (2015). Neural mechanisms of social dominance. *Front. Neurosci.* 9:154. doi: 10.3389/fnins.2015.00154

Wood, N. I., and Morton, A. J. (2015). Social behaviour is impaired in the R6/2 mouse model of Huntington's disease. *J. Huntingtons Dis.* 4, 61–73. doi: 10.3233/JHD-140132

Xu, J., Chatterjee, M., Baguley, T. D., Brouillette, J., Kurup, P., Ghosh, D., et al. (2014). Inhibitor of the tyrosine phosphatase STEP reverses cognitive deficits in a mouse model of Alzheimer's disease. *PLoS Biol.* 12:e1001923. doi: 10.1371/journal.pbio.1001923

Xu, J., Hartley, B. J., Kurup, P., Phillips, A., Topol, A., Xu, M., et al. (2018). Inhibition of STEP 61 ameliorates deficits in mouse and hiPSC-based schizophrenia models. *Mol. Psychiatry* 2, 271–281. doi: 10.1038/mp.2016.163

Xu, J., Kurup, P., Bartos, J. A., Patriarchi, T., Hell, J. W., and Lombroso, P. J. (2012). Striatal-enriched protein-tyrosine phosphatase (STEP) regulates Pyk2 kinase activity. *J. Biol. Chem.* 287, 20942–20956. doi: 10.1074/jbc.M112.368654

Yang, M., and Crawley, J. N. (2009). Simple behavioral assessment of mouse olfaction. *Curr. Protoc. Neurosci.* 8:8.24. doi: 10.1002/0471142301.ns0824s48

Ye, Y., Liu, C., Liu, X., and Huang, S. (2016). Ovariectomy changes the response to antidepressant drugs in tail suspension test in mice. *Gynecol. Endocrinol.* 12, 986–990. doi: 10.1080/09513590.2016.1197197

Zhang, Z., Chu, S. F., Mou, Z., Gao, Y., Wang, Z. Z., Wei, G. N., et al. (2016). Ganglioside GQ1b induces dopamine release through the activation of Pyk2. *Mol. Cell. Neurosci.* 71, 102–113. doi: 10.1016/j.mcn.2015.12.009

Zhang, Y., Kurup, P., Xu, J., Carty, N., Fernandez, S. M., Nygaard, H. B., et al. (2010). Genetic reduction of striatal-enriched tyrosine phosphatase (STEP) reverses cognitive and cellular deficits in an Alzheimer's disease mouse model. *Proc. Natl. Acad. Sci. U S A* 107, 19014–19019. doi: 10.1073/pnas.1013543107

Zhang, Z., Zhang, Y., Mou, Z., Chu, S., Chen, X., He, W., et al. (2014). Tyrosine 402 phosphorylation of Pyk2 is involved in ionomycin-induced neurotransmitter release. *PLoS One* 9:e94574. doi: 10.1371/journal.pone.0094574

Conflict of Interest Statement: 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: 11 April 2019
doi: 10.3389/fnbeh.2019.00063

Differential Effects of Novel Dopamine Reuptake Inhibitors on Interference With Long-Term Social Memory in Mice

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Edited by:
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Received: 28 August 2018
Accepted: 13 March 2019
Published: 11 April 2019

Citation:
Camats-Perna J, Kalaba P, Ebner K, Sartori SB, Vuyyuru H, Aher NY, Dragačević V, Singewald N, Engelmann M and Lubec G (2019) Differential Effects of Novel Dopamine Reuptake Inhibitors on Interference With Long-Term Social Memory in Mice. Front. Behav. Neurosci. 13:63. doi: 10.3389/fnbeh.2019.00063

In the laboratory, long-term social recognition memory (SRM) in mice is highly susceptible to proactive and retroactive interference. Here, we investigate the ability of novel designed dopamine (DA) re-uptake inhibitors (*rac*-CE-123 and *S*-CE-123) to block retroactive and proactive interference, respectively. Our data show that administration of *rac*-CE-123 30 min before learning blocks retroactive interference that has been experimentally induced at 3 h, but not at 6 h, post-learning. In contrast, *S*-CE-123 treatment 30 min before learning blocked the induction of retroactive interference at 6 h, but not 3 h, post-learning. Administration of *S*-CE-123 failed to interfere with proactive interference at both 3 h and 6 h. Analysis of additional behavioral parameters collected during the memory task implies that the effects of the new DA re-uptake inhibitors on retroactive and proactive interference cannot easily be explained by non-specific effects on the animals' general social behavior. Furthermore, we assessed the mechanisms of action of drugs using intracerebral *in vivo*-microdialysis technique. The results revealed that administration of *rac*-CE-123 and *S*-CE-123 dose-dependently increased DA release within the nucleus accumbens of freely behaving mice. Thus, the data from the present study suggests that the DA re-uptake inhibitors tested protect the consolidation of long-term social memory against interference for defined durations after learning. In addition, the data implies that DA signaling in distinct brain areas including the nucleus accumbens is involved in the consolidation of SRM in laboratory mice.

Keywords: cognitive enhancement, social recognition memory, retroactive interference, aggression social interaction, dopamine transport inhibitor, long-term memory

INTRODUCTION

Social recognition memory (SRM) is the ability to distinguish between familiar and unfamiliar conspecific individuals (Thor and Holloway, 1982; Steckler et al., 1998). More than 100 years ago, Müller and Pilzecker postulated that information acquired during learning require some time to become long lasting memories and coined for this process the term "consolidation."

The same authors have introduced the concept of retroactive interference by determining that acquired information can be "displaced" by the amnesic effect of subsequent newly acquired information (Müller and Pilzecker, 1900). In contrast to retroactive interference, proactive interference is considered when the past learned event interferes with the acquisition/consolidation/retrieval of new information (Camats Perna and Engelmann, 2017).

Previous studies have shown that SRM is highly susceptible to manipulations aimed at producing retroactive and proactive interference (Dantzer et al., 1987; Engelmann, 2009). In the course of these studies, it was shown that the nature and timing of defined stimuli after and before learning, respectively, are the prominent factors to determine whether interference occurs. SRM experiments performed in mice demonstrated that after learning, retroactive interference could be observed up to 15 h and proactive interference can be observed up to 9 h. After learning, protein synthesis required for consolidation of both memory traces seems first to collide, then to compete, and finally overwrite each other in a time-dependent manner. After 18 h, both "memory traces" seem to dissociate and consolidate independently from each other (Engelmann, 2009).

The neuronal processing of stimuli acquired by defined sensory modalities may cause interference in SRM. Experiments investigating the basis for retroactive interference revealed that exposure to stimuli activating audition, taction, vision and olfaction up to 6 h after learning affect memory (Noack et al., 2010; Perna et al., 2015). It was also shown that stimuli which simultaneously activate different sensory modalities cause a robust interference when compared to stimuli that activate fewer sensory modalities: transient retrograde amnesia triggered by 1% isoflurane was able to block retroactive interference induced by an object stimulus, but had no effect when a conspecific stimulus animal was used to produce interference (Camats Perna and Engelmann, 2017). Thus, the manipulation of interference phenomena in SRM may both help to develop new pharmacological tools for the treatment of memory decline ("cognitive enhancers") and provide new insight in the neuronal networks involved in the consolidation of this type of memory.

Modafinil is a wake-promoting drug which is used to treat sleep apnea, narcolepsy and shift work sleep disorders (Battleday and Brem, 2015; Kristofova et al., 2018). Recently, the synthesis and test in different behavioral paradigms of modafinil analogue 5-((benzhydrylsulfinyl)methyl)-thiazole (CE-123; Kalaba et al., 2017; Nikiforuk et al., 2017) was reported. CE-123 was structurally modified by substituting the carboxyl-amide moiety of modafinil with a heterocycle thiazole group attached to position five which may provide a high metabolic stability of CE-123. *In vitro* the racemate of CE-123 (*rac*-CE-123) blocks the dopamine transporter (DAT) with high specificity and no adverse side effects (Kalaba et al., 2017).

Pharmacokinetic studies showed that *rac*-CE-123 penetrates the blood-brain barrier and reaches its site of action in the brain within ∼30 min after intraperitoneal administration in rats. Intraperitoneal administration of *rac*-CE-123 into Sprague-Dawley rats enhanced the acquisition and retrieval of memory in spatial hole-board task (Kristofova et al., 2018). It improved working memory in the radial arm maze and seems to modulate also the DA receptor *in vivo* (Kristofova et al., 2018). Further, *S*-CE-123 has also proven to enhance the cognitive flexibility without triggering unnecessary impulsive responding (Nikiforuk et al., 2017).

The present study was designed to assess the impact of CE-123 on the phenomenon of memory interference in SRM. In addition to the racemate, we used *S*-CE-123. The social discrimination task was performed in mice, and two different time points after the 1st sampling (3 and 6 h) were selected to evaluate possible effects on retroactive or proactive interference during SRM consolidation. Further, additional parameters were monitored during the behavioral tests to allow a first identification of possible behavioral side effects of the treatment that might have affected the behavioral readout interpreted as "memory."

In addition, we used microdialysis to investigate the effects of a single systemic administration of *rac*-CE-123 and *S*-CE-123 on extracellular DA levels in the mouse nucleus accumbens. Previous studies have shown that this brain area might be involved in the correct processing of short-term SRM in rats (Ploeger et al., 1991).

MATERIALS AND METHODS

Animals and Housing Conditions

For behavioral testing, adult male C57BL/6JOlaHsd mice (Harlan-Winkelmann, Borchen, Germany) with an age group of 9–16 weeks were used as experimental subjects. If not stated otherwise, they were housed in groups of five per cage (size: 20 × 37 × 15 cm) for at least 1 week before starting the experiments under standard laboratory conditions (temperature 22 ± 1°C, humidity 60 ± 5% with a 12:12 h light-dark cycle lights on: 07:00 h). Stimulus animals were C57BL/6JOlaHsd mice of both sexes with an age of 25–35 days. For microdialysis experiments, adult male C57BL/6J mice were used. These animals were kept under similar conditions and experiments starting at 08:00–08:30 h. All experimental manipulations were approved by the Committee on Animal Health and Care of the local governmental body (Regierungspräsidium, Halle, registered and approved: 42502-2-1365 UniMD; microdialysis procedures were approved by the Austrian Animal Experimentation Ethics Board; Bundesministerium für Wissenschaft Forschung und Wirtschaft, Kommission für Tierversuchsangelegenheiten) and performed in strict compliance with the EEC recommendations for the care and use of laboratory animals (2010/63/EU).

Behavioral Procedure

The social discrimination test performed has been described in detail in Engelmann et al. (2011). In brief, experimental subjects were separated 2 h before starting the session by transferring them to small cages with fresh bedding. The test procedure consisted of two sampling sessions (4 min each) and one choice session (4 min) performed in the adult's cage under dimmed lighting conditions (during the light phase, i.e., between 8:00 and 15:00 h). During the 1st sampling, a given stimulus animal was exposed to the experimental subject and the behavior of the

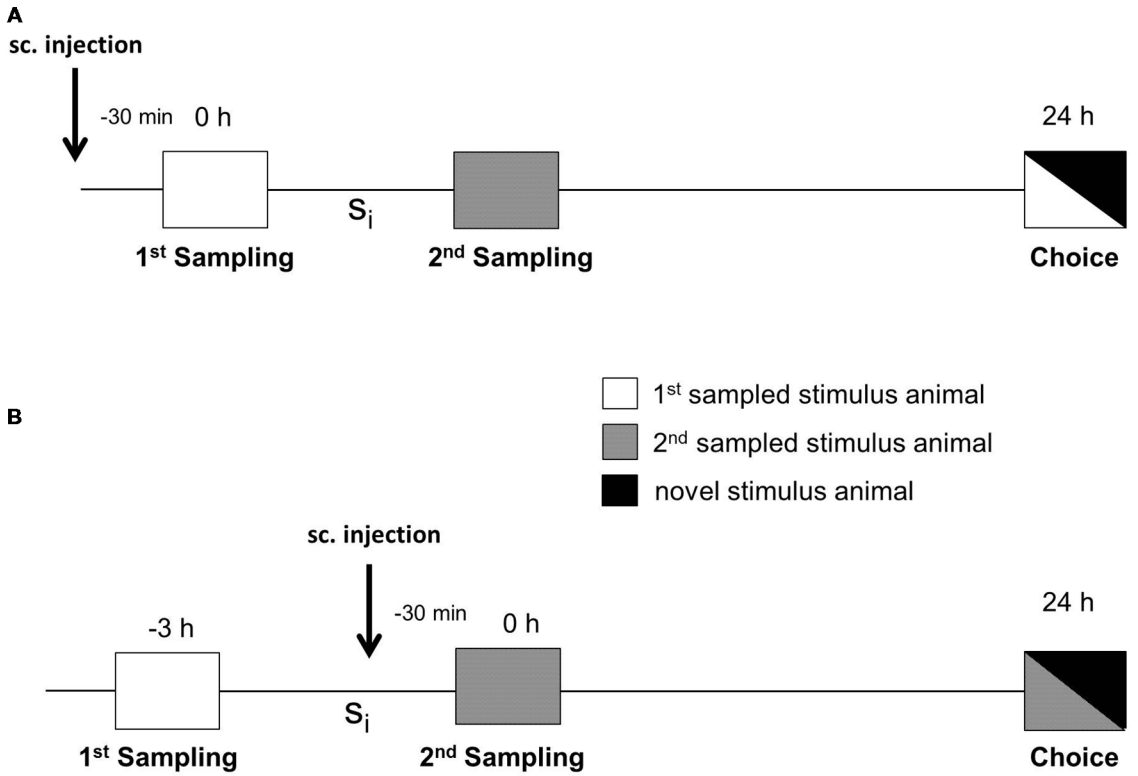

FIGURE 1 | Experimental protocol for testing the effects of the defined substances on interference in social recognition memory (SRM). **(A)** Subcutaneous (sc) injection was performed 30 min before the 1st sampling to measure the impact of administered substances on retroactive interference during choice. **(B)** Subcutaneous injection was performed 30 min before the 2nd sampling to measure the effect of administered compounds on proactive interference. The two samplings were separated by a defined sampling interval (S_i). Choice took place either 24 h after the 1st sampling, when testing retroactive interference **(A)**, or 24 h after the 2nd sampling when testing proactive interference **(B)**.

latter was monitored by pressing the pre-set key on a laptop by a trained observer unaware of the experimental subjects' treatment. The stimulus animal was then removed and kept individually in a new cage with food and water *ad libitum*. As illustrated in **Figure 1**, after a defined sampling interval (S_i), a second, previously not encountered stimulus animal was presented for 4 min to the experimental subject during the 2nd sampling session. To measure retroactive interference, during retrieval (choice), the stimulus animal encountered during the 1st sampling was presented to the experimental subject together with a novel stimulus animal 24 h after the 1st sampling. To measure the proactive interference, during the choice session, the 2nd sampled stimulus animal was presented to the adult together with a novel stimulus animal. Significant longer investigation of the novel stimulus animal compared to the already encountered stimulus animal during choice was taken as evidence for an intact recognition memory (Thor and Holloway, 1982; Engelmann et al., 2011). Earlier studies revealed that the consolidation of long-term SRM corresponds to two phases of anisomycin sensitivity with a gap at 3 h after sampling (Richter et al., 2005). We used both an S_i of 3 h (in the gap) and 6 h (after the gap) for our studies.

In addition to the investigation duration also the latency between the introduction of the stimulus animal in the experimental subject's cage and the first approach of the experimental subject towards the stimulus animal was monitored. Also, the duration of aggressive and sexual behavior of the experimental subject towards the given stimulus animal during the 1st and 2nd sampling was monitored.

Drug Treatment

The following drugs were used in the present study: *rac*-CE-123 = 5-((benzhydrylsulfinyl)methyl)-thiazole (Kristofova et al., 2018) and *S*-CE-123 = S-5-((benzhydrylsulfinyl)methyl)thiazole (Nikiforuk et al., 2017). The dosage of the drugs and the time point of administration were selected according to previous studies in which it was shown that 10 mg of the drugs per kg body weight administered 30 min before testing produces significant learning and memory effects without causing detectable undesired side effects in rats (Nikiforuk et al., 2017; Kristofova et al., 2018). The drugs were dissolved in 1% DMSO and 3.3% Tween 80 diluted in 0.9% NaCl. The solution contained 1 mg/ml and the dosage administered was 10 mg/kg body weight for all drugs. Vehicle contained the solvent (1% DMSO and 3.3% Tween 80 diluted in 0.9% NaCl) only. The experiments were performed in a double-blind cross-over design. Thus, all animals received both vehicle and the given drug in a random order. The code was broken after the end of the

behavioral experiments when the data was assigned to each treatment conditions and finally analyzed.

For retroactive interference, vehicle or drugs were administered subcutaneously (sc) 30 min before the 1st sampling session (**Figure 1A**). For proactive interference, sc administration was performed 30 min before the 2nd sampling session (**Figure 1B**). The testing of the effects of *S*-CE-123 on proactive vs. retroactive interference was incorporated to get a first insight into the timing and possible interactions of potentially DA signaling for early or late stabilization of an SRM trace.

Microdialysis

For the preparation of the microdialysis experiment, mice were anesthetized (5 mg/kg xylazine, 80 mg/kg ketamine, i.p., isoflurane) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). A guide cannula (MAB 4.15.IC, Microbiotech, Stockholm, Sweden; o.d. 0.48 mm) was implanted unilaterally 1 mm above the right nucleus accumbens (A/P = +1.0 mm, L/M = +0.8 mm, D/V = −3.6 mm) according to the mouse brain atlas by Franklin and Paxinos (2007) and fixed to the skull with dental acrylic cement and two stainless steel screws. Animals received buprenorphine (5 mg/kg, sc) and an analgesic *via* the drinking water (Meloxicam, 5 mg/kg, for 3 days) for post-surgery care and were housed individually. The evening before the microdialysis experiment, mice were shortly anesthetized with isoflurane and a microdialysis probe (MAB 4.15.1, Microbiotech, Stockholm, Sweden) with a molecular cutoff of 6 kDa (o.d. 0.2 mm, PES membrane 1 mm of length) was inserted into the guide cannula of mice reaching into the nucleus accumbens. The probe was connected to a CMA/Microdialysis Syringe pump (CMA-4004) and constantly superfused with sterile artificial cerebrospinal fluid (aCSF; NaCl 140 mM, KCl 3.0 mM, $CaCl_2$ 1.25 mM, $MgCl_2$ 1.0 mM and Na_2HPO_4 1.2 mM and NaH_2PO_4 0.3 mM; pH 7.4) at a flow rate of 0.5 µl/min. On the day of experiment, superperfusion rate was set to 1.0 µl/min and after 2 h of equilibration sequential microdialysis fractions were collected every 20 min into ice-cooled microtubes containing 6 µL of an antioxidative mixture (100 mM acetic acid, 0.27 mM Na_2EDTA and 12.5 µM ascorbic acid), vortexed and stored at −80°C until further analysis. After three baseline samples (collected from −60 to 0 min), vehicle or drugs (10 mg/kg, sc) were administered, and six samples were collected. Subsequently, vehicle or drugs were administered in a higher concentration (100 mg/kg, sc) and another six microdialysates were collected. For the last two dialysates aCSF containing 100 mM KCl was used as a positive control to elicit local depolarization in order to confirm the functionality of the system. At the end of the experiment, mice were euthanized by an overdose of thiopental and brains were removed for histological verification of the placement of microdialysis probes. Data were only used from subjects with correct probe displacement (see **Figure 5A**).

Analysis of Dopamine

Dopamine was analyzed in 5 µl microdialysate fractions by high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (CBM-20A), degassing unit (DGU-20A3R) and micro HPLC pump (LC-20ADXR) operated at a flow rate of 55 µl/min. The mobile phase consisted of 8% (v/v) methanol, 50 mM phosphoric acid, 50 mM citric acid, 2.36 mM octane-sulfonic acid, 0.1 mM Na_2EDTA at a pH of 5.6. Samples were injected *via* a SIL-20ACXR autosampler (Shimadzu, Japan) and separated on a C18 reversed-phase column (NeuroSep 105; 50 mm × 1.0 mm i.d.; 3 µM spherical particles; Antec, Zoeterwoude, Netherlands). The HPLC system was coupled to the DECADE II electrochemical detector (Antec SenCell, 2 mm glassy carbon working electrode, Ag/AgCl reference electrode, Antec Zoeterwoude, Netherlands). The column and detector cell were maintained at 35°C by a column oven as part of the electrochemical detector. The applied potential was set to +460 mV vs. reference electrode and was adjusted to a detection range of 100 pA/V with a filter frequency setting of 0.01 Hz. Substance amounts which yielded a detector signal corresponding to three times noise level were considered at detection limit. This allowed for the measurement of DA with a sensitivity of 0.25 fmol/5 µl sample. Instrument control and data acquisition were carried out by Lab Solution chromatography software (LabSolution CS, Shimadzu, Japan). Calibration curves were constructed in the range of 50 pM to 1 nM (0.25–5 fmol of DA injected) and were consistently linear with correlation coefficients higher than 0.999.

Statistics

Data are presented as mean + SEM. Statistical analysis of the behavioral data was performed by GraphPad Prism 6.05 (GraphPad Software, San Diego, CA, USA). Data obtained from the social discrimination experiments were analyzed using the paired Student's *t*-test. The additional behavioral parameters (latency from the experimental subject to investigate the stimulus animal after its introduction and duration of aggressive behavior) were analyzed using one-way ANOVA. For the microdialysis experiments, the DA content in each 20 min-microdialysate was expressed as a relative value to the mean content rates of the three samples preceding the administration of the drug or vehicle. Statistical analysis was carried out with Statistica Software v9 [StatSoft (Europe) GmbH, Hamburg, Germany] using two-way ANOVA for repeated measures followed by Fisher's test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Investigation durations measured during the 1st and 2nd sampling are presented in **Table 1**. When tested under vehicle conditions, in total two animals (for *rac*-CE-123 at a S_i = 6 h) had to be excluded from the analysis of the treatment conditions because the investigation duration during the 1st or 2nd sampling was <1 s and, thus, it is unreliable to assume that sufficient information was acquired for a successful recognition and interference, respectively. The data of the remaining animals show that the average investigation duration during both sampling sessions was sufficient to acquire the important

TABLE 1 | Investigation durations (means + SEM) during the 1st and 2nd sampling of the animals presented in **Figures 2–4**.

Corresponding Figure	S_i (type of interference)	Treatment	1st sampling	2nd sampling	*n*
2A	3 h (retroactive)	Vehicle	23.53 + 4.59	16.34 + 3.02	17
		rac-CE-123	26.20 + 3.34	15.83 + 4.19	17
2B	6 h (retroactive)	Vehicle	24.02 + 3.70	14.01 + 2.73	18
		rac-CE-123	27.09 + 3.46	15.53 + 2.07	20
3A	3 h (retroactive)	Vehicle	21.41 + 3.00	17.50 + 2.70	21
		S-CE-123	27.79 + 2.56	17.12 + 2.40	21
3B	6 h (retroactive)	Vehicle	26.04 + 3.82	28.68 + 4.82	21
		S-CE-123	25.35 + 4.60	27.30 + 4.15	21
4	3 h (proactive)	Vehicle	30.78 + 2.80	30.78 + 2.80	21
		S-CE-123	34.17 + 4.50	28.37 + 4.38	21

*It shows the investigation duration of experimental subjects towards presented stimulus animals during the 1st and 2nd sampling sessions of retroactive and proactive interference experiments (see **Figures 1–4**). n = number of animals per group.*

information essential to establish long-term SRM and to produce an interference, respectively (Engelmann et al., 2011).

When retroactive interference was introduced at a S_i = 3 h, *rac*-CE-123-, but not vehicle-administered experimental subjects showed significantly longer investigation durations towards the novel stimulus animal than towards the 1st sampled stimulus animal during choice (**Figure 2A**; paired Student's *t*-test; vehicle: $t = 0.73$, $p = 0.475$; drug: $t = 4.02$, $p = 0.001$). At a S_i of 6 h, *rac*-CE-123-treatment failed to significantly affect the investigation durations during choice (**Figure 2B**; paired Student's *t*-test: $t = 1.81$, $p = 0.087$). However, vehicle administered experimental subjects investigated the novel stimulus animal significantly longer than the 1st sampled stimulus animal during choice (**Figure 2B**; paired Student's *t*-test: $t = 2.15$, $p = 0.047$).

Neither administration of *S*-CE-123 nor that of vehicle caused a significant difference in the investigation of the 1st sampled and the novel stimulus animal during choice at a S_i = 3 h (**Figure 3A**; paired Student's *t*-test; vehicle: $t = 0.01$, $p = 0.993$; drug: $t = 0.21$, $p = 0.838$). If the same drug was administered at a S_i = 6 h, during the choice session experimental subjects investigated the novel stimulus animal longer than the 1st sampled stimulus animal (**Figure 3B**; paired Student's *t*-test: $t = 2.54$, $p = 0.020$). Vehicle treatment failed to affect significantly the investigation durations (paired Student's *t*-test: $t = 1.57$, $p = 0.131$).

When testing proactive interference and introduced at S_i of 3 h, neither vehicle (**Figure 4**; paired Student's *t*-test: $t = 0.96$, $p = 0.348$) nor *S*-CE-123 (**Figure 4**; paired Student's *t*-test: $t = 1.11$, $p = 0.279$) showed a significant difference in investigation duration between 1st sampled stimulus animal and novel stimulus animal during the choice session.

The data collected from the additional parameters monitored during the behavioral testing are shown in **Table 2**. Using S_i = 3 h, no significant effects on any of the additional behavioral parameters monitored were detected (*via* ANOVA), independently upon the administered substance (vehicle or drug). In contrast, ANOVA statistical test revealed a significant effect on the latency to start investigating the stimulus animal during the 2nd sampling at a S_i = 6 h in case of vehicle treatment only, for *rac*-CE-123 and its respective vehicle treatment only. Subsequent analysis *via* Student's *t*-test failed to detect significant differences between the 1st and 2nd sampling under a vehicle and the respective drug treatment (data not shown). Thus, the differences detected *via* ANOVA resulted from different values measured during the 1st

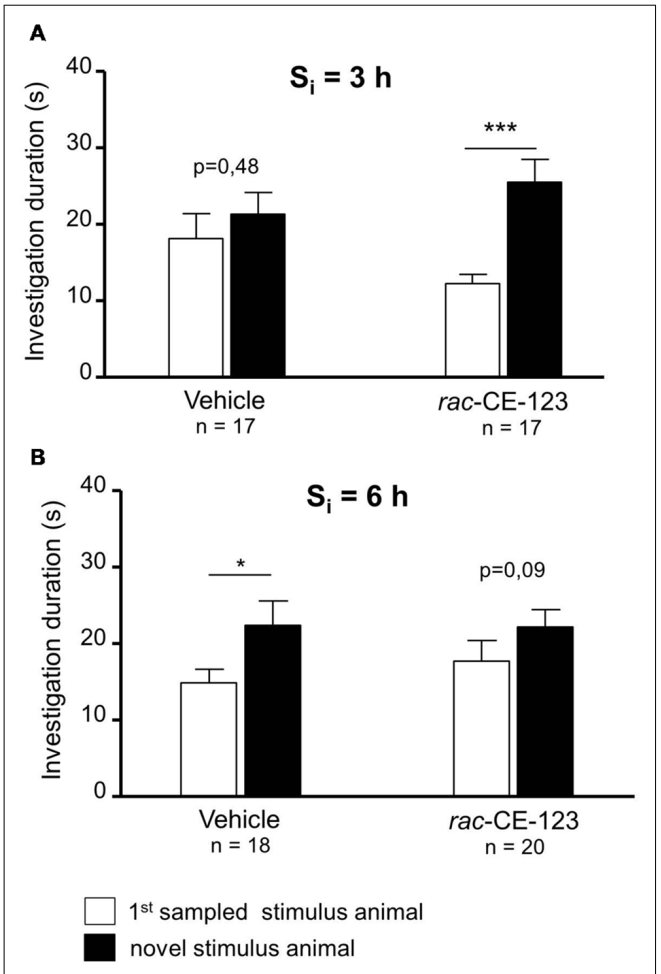

FIGURE 2 | Effect of subcutaneous injection of vehicle or *rac*-CE-123 on retroactive interference at S_i = 3 h **(A)** and 6 h **(B)** on social investigation. Recognition memory was tested during choice by exposing the stimulus animal presented during the 1st sampling (1st S) together with a novel stimulus animal mouse 24 h after the 1st sampling. $^*p < 0.05$ and $^{***}p < 0.01$ paired Student's *t*-test.

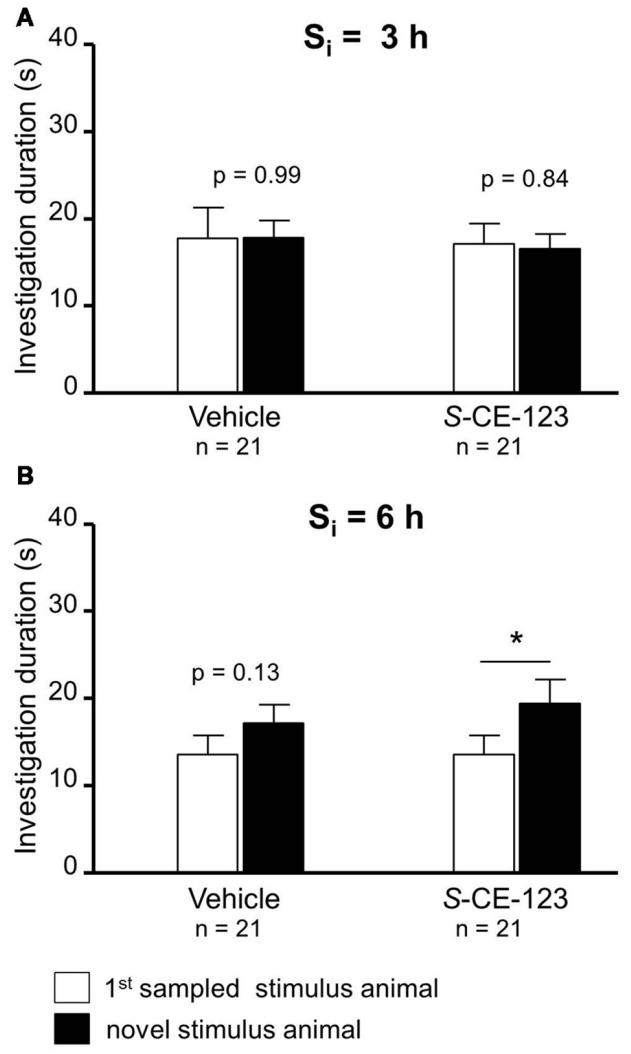

FIGURE 3 | Effect of subcutaneous injection of vehicle or *S*-CE-123 on retroactive interference at S_i 3 = h **(A)** and 6 h **(B)** on social investigation. Recognition memory was tested during choice by exposing the stimulus animal presented during the 1st sampling (1st S) together with a novel stimulus animal 24 h after the 1st sampling. $^*p < 0.05$ paired Student's *t*-test.

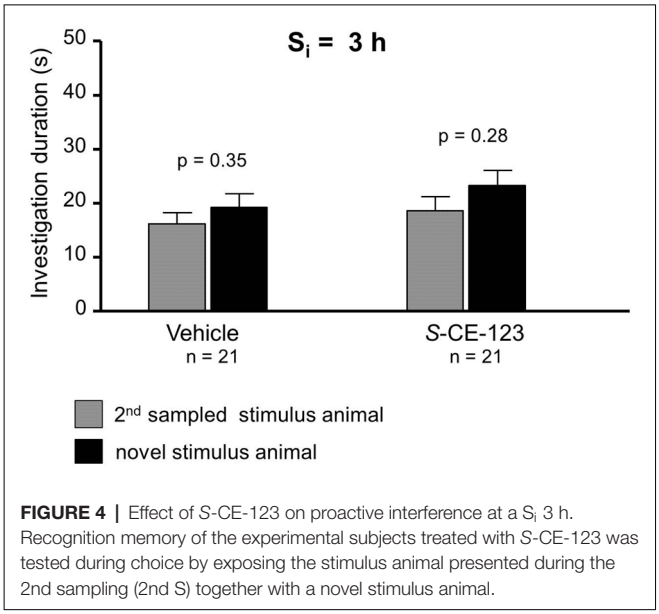

FIGURE 4 | Effect of *S*-CE-123 on proactive interference at a S_i 3 h. Recognition memory of the experimental subjects treated with *S*-CE-123 was tested during choice by exposing the stimulus animal presented during the 2nd sampling (2nd S) together with a novel stimulus animal.

sampling vs. the 2nd sampling and did not reflect a specific treatment effect.

A representative example of a correct placement of the microdialysis probe in the nucelus accumbens is shown in **Figure 5A**. DA levels in microdialysates reached stable baseline values of 0.54 ± 0.08 fmol/5 µl sample. No significant differences in basal dialysate concentrations of DA between vehicle-treated control group and drug treatment groups were found. Drug treatment significantly affected DA levels over time at both the low (drug × time interaction: $F_{(16,120)} = 1.949$, $p < 0.05$) and high doses (drug × time interaction: $F_{(16,120)} = 11.418$, $p < 0.001$). While vehicle treatment failed to alter the DA concentrations, sc injections of both *rac*-CE-123 and *S*-CE-123 increased the concentrations of DA in the microdialysates. Specifically, the administration of 10 mg/kg of *rac*-CE-123 and *S*-CE-123 caused a moderate increase in DA concentrations for at least 20 min and 40 min, respectively, and returned to baseline levels within the subsequent 40–60 min (**Figure 5B**). The high dose (100 mg/kg) of both *rac*-CE-123 and of *S*-CE-123 caused a maximum increase in the DA concentration within 40 min ($p < 0.01$) after injections and remained elevated throughout the whole sampling period (**Figure 5C**).

DISCUSSION

The present study was designed to investigate the impact of recently synthesized and potentially cognitive enhancing drugs on retroactive and proactive interference of SRM. For this purpose, mice injected sc with either *rac*-CE-123 or *S*-CE-123 were tested in the social discrimination task. Treatment with *rac*-CE-123 blocked the otherwise seen retroactive interference at 3 h after the 1st sampling (**Figure 2**). However, if the S_i between both samplings was 6 h, both drugs failed to affect retroactive interference (**Figure 2**). Surprisingly, when treated with a vehicle at a S_i = 6 h during the choice session, experimental subjects explored the novel stimulus animal significantly longer than the 1st sampled one (**Figure 2B**). At first glance, this may indicate that the vehicle treatment has blocked interference. The investigation duration during the 2nd sampling was similar to the 1st sampling (**Table 1**) with the ANOVA detecting an increased latency to investigate an increased aggression (**Table 2**). A similar effect of vehicle treatment on investigation durations during choice, however, is not observed in the other groups tested in the present study (**Figures 2**, **3**) and numberous own unpublished observations with other drugs including modafinil analogues applied under otherwise identical conditions and solvent. Thus, we propose to consider this as an extraordinary outliner, which, nevertheless, will be the focus of further investigations.

With respect to the observed blockage of retroactive interference, administration of *rac*-CE-123 seems to affect the

TABLE 2 | Experimental subject's latency to investigate and duration of aggressive behavior (means in seconds + SEM) towards the presented stimulus animal measured during the 1st and 2nd sampling, respectively.

S_i	Parameter	1st sampling		2nd sampling		ANOVA
		Vehicle	Drug	Vehicle	Drug	
rac-CE-123 (retroactive)						
3 h	Latency	5.50 + 2.08	3.45 + 0.85	28.13 + 11.07	18.16 + 11.21	$F_{(3,64)} = 2.11$; $p = 0.11$
	Aggression	3.25 + 0.71	3.80 + 1.14	2.19 + 1.00	5.14 + 1.42	$F_{(3,64)} = 1.25$; $p = 0.30$
6 h	Latency	8.62 + 2.38	2.46 + 0.44	21.90 + 7.39	10.41 + 1.79	$F_{(3,72)} = 4.49$; $P < 0.01$
	Aggression	2.59 + 1.04	1.62 + 0.50	1.90 + 0.78	1.01 + 0.35	$F_{(3,72)} = 0.90$; $p = 0.44$
S-CE-123 (retroactive)						
3 h	Latency	2.62 + 0.34	3.13 + 0.55	4.47 + 1.24	6.44 + 1.79	$F_{(3,80)} = 2.25$; $p = 0.09$
	Aggression	2.05 + 0.40	1.57 + 0.42	2.57 + 0.73	1.90 + 0.47	$F_{(3,80)} = 0.63$; $p = 0.63$
6 h	Latency	3.38 + 1.16	3.85 + 0.70	4.66 + 0.98	2.53 + 0.49	$F_{(3,80)} = 1.05$; $p = 0.38$
	Aggression	1.65 + 0.37	3.79 + 1.25	4.81 + 1.10	5.19 + 1.38	$F_{(3,80)} = 2.10$; $p = 0.11$
S-CE-123 (proactive)						
3 h	Latency	4.05 + 1.07	5.03 + 1.85	3.48 + 0.71	4.60 + 1.23	$F_{(3,80)} = 0.27$; $p = 0.84$
	Aggression	2.30 + 0.72	1.96 + 0.54	4.32 + 0.69	4.96 + 1.13	$F_{(3,80)} = 2.92$; $p = 0.03$

Experimental subjects were treated with either rac-CE-123 or S-CE-123 30 min before the 1st sampling (when testing retroactive interference) or the 2nd sampling (when testing proactive interference) and subsequently tested as illustrated in ***Figures 1A,B****). The ANOVA results refer to the values of the sample line.*

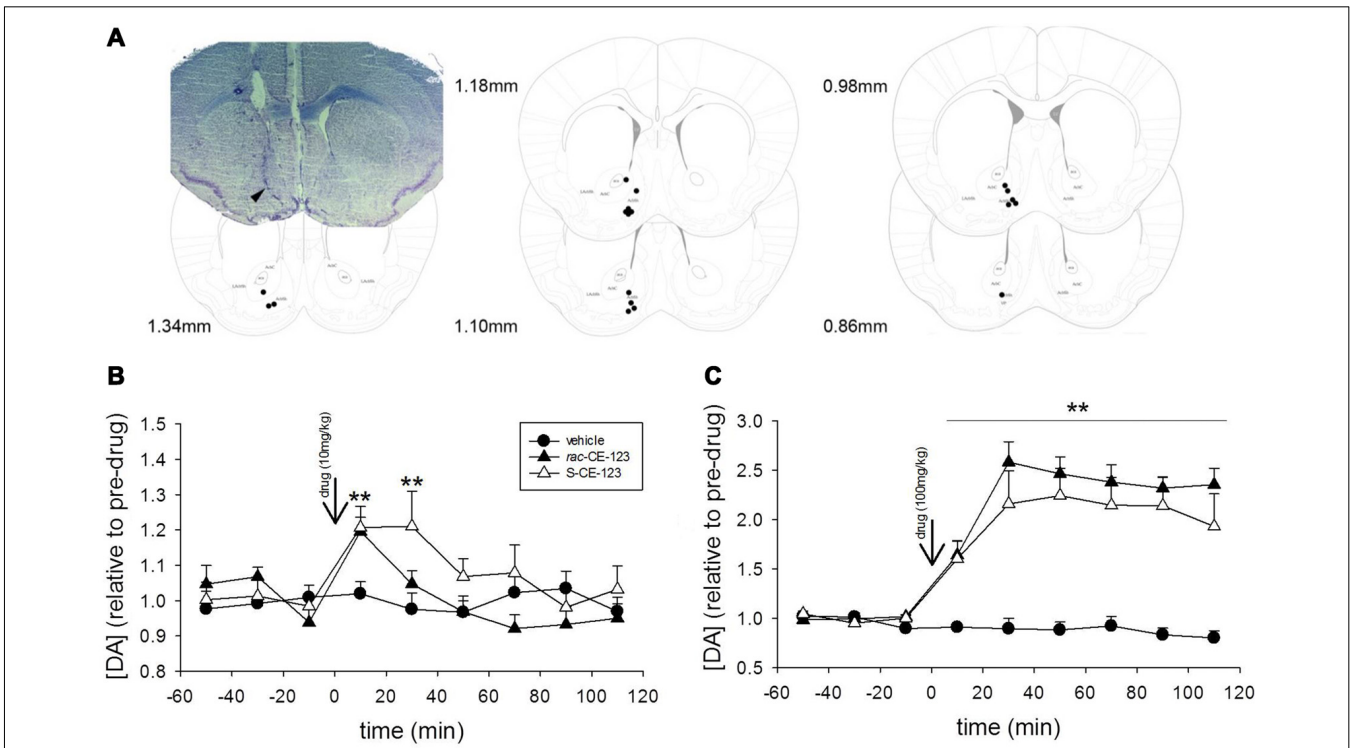

FIGURE 5 | Microdialysis experiments. Coronal section diagrams modified from Franklin and Paxinos (2007) and a representative photomicrograph illustrating the reconstructed tip of the probe placement and the track left by the microdialysis probe in the brain tissue, respectively in the nucleus accumbens **(A)**. Effect of subcutaneous injection of vehicle or a low dose (10 mg/kg, **B**) and high dose (100 mg/kg, **C**) of *rac*-CE-123 or *S*-CE-123 on DA levels in dialysates from the nucleus accumbens of mice. Dopamine values in microdialysates are shown as changes in basal DA values, calculated as the mean of three consecutive samples immediately preceding the drug or vehicle injection. Data are expressed as mean ± SEM. $n = 6$ per group; $^{**}p < 0.01$ vs. respective value in the vehicle-treated controls (two-way ANOVA followed by Fisher's *post hoc* test).

information processing linked to the 1st sampling in a way that it becomes insensitive to a potential interference impact within <6 h after learning. It is well known that blocking the DAT activity by psychostimulants prevents the re-uptake of DA and increases the extra-synaptic concentration of DA in the brain (Kuhar et al., 1991; Li et al., 1996). Indeed, our microdialysis experiments revealed a significant increase of extracellular DA levels in dialysates collected from the nucleus

accumbens after sc administration of both *rac*-CE-123 and *S*-CE-123. Both drugs increased the DA concentration in the mcirodialysates in a dose- and time-dependent manner. Notably, at the concentration of 10 mg/kg the maximal response occurred within the first 20 min followed by a gradual decrease within the next 1–2 h (**Figure 5B**). The drug-induced release profiles suggest that increased DA levels are to be expected for at least 30–40 min after the sc injection and thus during sampling in the social discrimination task. The data demonstrating a rapid and long-lasting stimulatory effects of novel modafinil analogues *rac*-CE-123 and *S*-CE-123 on extracellular DA levels in distinct brain areas including the nucleus accumbens are in line with previous microdialysis studies using modafinil and/or related analogues (Loland et al., 2012; Mereu et al., 2017; Keighron et al., 2019) acting as potential DAT inhibitors. Followed by DAT inhibition, DA D1 receptors seem to be the key mediators in the downstream signaling process (Kalaba et al., 2017) involved in SRM. It is of note that, similar to previous findings, we failed to observe additional effects of the drug treatment on defined behavioral parameters. This speaks in favor of a specific action on memory and not on other behaviorally relevant central nervous processes.

Previous studies have shown that 30 min after a peripheral injection of *rac*-CE-123, elevated DAT and DA receptor 1 protein levels in CA1 and CA3 was produced in the hippocampus (Kristofova et al., 2018). Based on these data, it is plausible to state that *rac*-CE-123 temporarily (i.e., <6 h) protect the memory trace against retroactive interference by manipulating DA signaling in the brain. The action of the drugs cannot easily be explained by an alteration of the general social behavior of the experimental subjects (e.g., reduced interest in the 2nd sampled juvenile or increased aggressive behavior that may have covered reduced investigation) as the behavioral parameters analyzed here failed to differ between vehicle and drug treatment (**Table 2**). Research studies in rodents revealed that consolidation of long-term SRM is supported by information processing within defined brain areas including the olfactory bulb, anterior olfactory nucleus, medial prefrontal cortex, medial amygdala, basolateral amygdala, and different sub-regions of the hippocampus (Richter et al., 2005; Hitti and Siegelbaum, 2014; Noack et al., 2015; Tanimizu et al., 2017; Lin et al., 2018). Systemic and direct infusion of DA D1 receptor agonists either into the frontal cortex or into the nucleus accumbens improved short-term SRM in rats (Di Cara et al., 2007). Intra-insular cortex administration of agonists for DA D1/D5 receptors, β-adrenergic and serotonergic 5-HT_{1A} receptors improved the consolidation of SRM in rats (Cavalcante et al., 2017). Further, the potentiation of CA3-CA1 hippocampal synapses facilitates the consolidation of object recognition memory (Clarke et al., 2010). Due to the route of administration used in our study and the analysis of the DA levels in the nucleus accumbens only, we cannot relate the interference blocking effect of the tested drugs to an action within defined brain areas. Inspired by the fact that we could—at least as a potential target—identify the nucleus accumbens, further studies are in progress in which we will analyze the impact of our drugs on distinct areas in which the processing of information for SRM takes place in more detail. Different lines of investigation suggested a contribution of the dopaminergic system in distinct brain regions beyond the nucleus accumbens to the generation of short-term and long-term SRM of laboratory rats. Among them, the hippocampus and striatum are likely to be interesting brain areas (Garrido Zinn et al., 2016; Cavalcante et al., 2017) in which an increased DA signaling might contribute to a "stabilization" of the "SRM trace" and thereby making it resistant against interference.

The enantiomer *S*-CE-123 was able to block retroactive interference at a S_i of 6 h (**Figure 3B**), but not at a S_i of 3 h (**Figure 3A**). This indicates that *S*-CE-123 and *rac*-CE-123 administered *via* the same route and dose may affect differently the dopaminergic signaling relevant for SRM. This could be due to a different profile of washin and washout of the drugs targeting the brain tissue. Unpublished data show that compared to *rac*-CE-123, *S*-CE-123 is detectable in a ∼5–10 times higher concentration in both liquor and brain tissue after intraperitoneal administration in adult male rats. The impression of a different duration of action of *S*- vs. *rac*-CE-123 is—to some aspect—supported by the microdialysis data. The release profile of the racemate at a dosage of 10 mg/kg differs from that of the *S*-enatiomer by showing elevated DA levels at the sample collected 20–40 min after treatment when *rac*-CE-123 is already indistinguishable from baseline (**Figure 5B**). In addition, a distinct action of the two drugs on different phases of SRM consolidation might be hypothesized: previous studies demonstrated two separate phases of sensitivity within the first 24 h after learning in paradigms testing SRM using the protein synthesis blocker anisomycin. This resulted in the hypothesis that the consolidation of long-term SRM requires two stages of protein synthesis with a gap of sensitivity to anisomycin at ∼3 h after learning (Richter et al., 2005; Wanisch et al., 2008). Thus, DA signaling might be involved in the consolidation of SRM at both stages of anisomycin sensitivity. In this context, the effects of *S*-CE-123 differ to that of *rac*-CE123 in blocking the retroactive interference induced at 3 h vs. 6 h after the 1st sampling. Upon first view, this could result from a counter regulatory mechanism of the *S*-enantiomer in the administered racemate. However, such conclusions would be too premature without further studies investigating possible differences in the effects between the two CE-123 treatments including the analysis of molecular mechanisms involved.

The results of the experiment in which we administered *S*-CE-123 in the context with the induction of proactive interference failed to provide a protective effect of this enantiomer for a memory of the 2nd sampled stimulus animals (**Figure 4**). This speaks in favor of a specific effect of this substance on retroactive, but not proactive interference, and indicates distinct neuronal procedures underlying both phenomena. Previous studies suggested a time-depending interaction of two subsequently initiated memory traces in SRM (Engelmann, 2009), the present data suggest that DA signaling might be involved differently in the generation of retroactive and proactive interference.

Taken together, the results of the present study, show for the first time, that modafinil-derived drugs increasing

extracellular DA in the nucleus accumbens and acting at both the DAT and DA D1 receptor are able to make SRM resistant against retroactive interference. The drug- and time-dependent action suggests distinct action profiles of the different drugs and provides insight into the mechanisms underlying the consolidation of SRM which requires further investigations. Further studies will focus on the cellular mechanisms *via* which the modafinil analogues tested here affect SRM. The molecular signatures linked to the blockade of interference are likely to provide further insight into the neurobiological basis of this type of learning and memory in mammals.

AUTHOR CONTRIBUTIONS

JC-P performed the animal experiments, treated the animals, performed a part of the statistical analysis of the behavioral data and contributed to the manuscript draft. PK was involved in the design and synthesis of the drugs; he also contributed to the design of the study. HV analyzed part of the behavioral data and provided the first draft of the manuscript. NA was involved in the synthesis of *rac*-CE-125, VD in that of *S*-CE-123. ME designed and supervised the behavioral experiments and contributed to the writing of the manuscript. GL initiated the project and contributed to the study design and to the writing of the manuscript. NS, SS and KE designed and performed the microdialysis experiments including statistical analysis and wrote the draft of the respective parts in the manuscript.

FUNDING

This study has been partly supported by the Austrian Science Fund (FWF): I2433-B26, SFB F4410 and private funding.

ACKNOWLEDGMENTS

We wish to thank Rita Murau (Magdeburg) for support in behavioral testing and Dr. Andrew Kaus (Brisbane, QLD, Australia) for polishing the English.

REFERENCES

Battleday, R. M., and Brem, A. K. (2015). Modafinil for cognitive neuroenhancement in healthy non-sleep-deprived subjects: a systematic review. *Eur. Neuropsychopharmacol.* 25, 1865–1881. doi: 10.1016/j.euroneuro.2015.07.028

Camats Perna, J., and Engelmann, M. (2017). Recognizing others: rodent's social memories. *Curr. Top. Behav. Neurosci.* 30, 25–45. doi: 10.1007/7854_2015_413

Cavalcante, L. E. S., Zinn, C. G., Schmidt, S. D., Saenger, B. F., Ferreira, F. F., Furini, C. R. G., et al. (2017). Modulation of the storage of social recognition memory by neurotransmitter systems in the insular cortex. *Behav. Brain Res.* 334, 129–134. doi: 10.1016/j.bbr.2017.07.044

Clarke, J. R., Cammarota, M., Gruart, A., Izquierdo, I., and Delgado-Garcia, J. M. (2010). Plastic modifications induced by object recognition memory processing. *Proc. Natl. Acad. Sci. U S A* 107, 2652–2657. doi: 10.1073/pnas.0915059107

Dantzer, R., Bluthe, R. M., Koob, G. F., and Le Moal, M. (1987). Modulation of social memory in male rats by neurohypophyseal peptides. *Psychopharmacology* 91, 363–368. doi: 10.1007/bf00518192

Di Cara, B., Panayi, F., Gobert, A., Dekeyne, A., Sicard, D., De Groote, L., et al. (2007). Activation of DA D1 receptors enhances cholinergic transmission and social cognition: a parallel dialysis and behavioural study in rats. *Int. J. Neuropsychopharmacol.* 10, 383–399. doi: 10.1017/s1461145706007103

Engelmann, M. (2009). Competition between two memory traces for long-term recognition memory. *Neurobiol. Learn. Mem.* 91, 58–65. doi: 10.1016/j.nlm.2008.08.009

Engelmann, M., Hädicke, J., and Noack, J. (2011). Testing declarative memory in laboratory rats and mice using the non-conditioned social discrimination procedure. *Nat. Protoc.* 6, 1152–1162. doi: 10.1038/nprot.2011.353

Franklin, K. B. J., and Paxinos, G. (2007). *The Mouse Brain in Stereotaxic Coordinates.* San Diego, CA: Academic Press.

Garrido Zinn, C., Clairis, N., Silva Cavalcante, L. E., Furini, C. R., De Carvalho Myskiw, J., and Izquierdo, I. (2016). Major neurotransmitter systems in dorsal hippocampus and basolateral amygdala control social recognition memory. *Proc. Natl. Acad. Sci. U S A* 113, E4914–4919. doi: 10.1073/pnas.1609883113

Hitti, F. L., and Siegelbaum, S. A. (2014). The hippocampal CA2 region is essential for social memory. *Nature* 508, 88–92. doi: 10.1038/nature13028

Kalaba, P., Aher, N. Y., Ilic, M., Dragacevic, V., Wieder, M., Miklosi, A. G., et al. (2017). Heterocyclic analogues of modafinil as novel, atypical DA transporter inhibitors. *J. Med. Chem.* 60, 9330–9348. doi: 10.1021/acs.jmedchem.7b01313

Keighron, J. D., Quarterman, J. C., Cao, J., Demarco, E. M., Coggiano, M. A., Gleaves, A., et al. (2019). Effects of (R)-modafinil and modafinil analogues on DA dynamics assessed by voltammetry and microdialysis in the mouse nucleus accumbens shell. *ACS Chem. Neurosci.* doi: 10.1021/acschemneuro.8b00340 [Epub ahead of print].

Kristofova, M., Aher, Y. D., Ilic, M., Radoman, B., Kalaba, P., Dragacevic, V., et al. (2018). A daily single dose of a novel modafinil analogue CE-123 improves memory acquisition and memory retrieval. *Behav. Brain Res.* 343, 83–94. doi: 10.1016/j.bbr.2018.01.032

Kuhar, M. J., Ritz, M. C., and Boja, J. W. (1991). The DA hypothesis of the reinforcing properties of cocaine. *Trends Neurosci.* 14, 299–302. doi: 10.1016/0166-2236(91)90141-g

Li, M. Y., Yan, Q. S., Coffey, L. L., and Reith, M. E. (1996). Extracellular DA, norepinephrine and serotonin in the nucleus accumbens of freely moving rats during intracerebral dialysis with cocaine and other monoamine uptake blockers. *J. Neurochem.* 66, 559–568. doi: 10.1046/j.1471-4159.1996.66020559.x

Lin, Y.-T., Hsieh, T.-Y., Tsai, T.-C., Chen, C.-C., Huang, C.-C., and Hsu, K.-S. (2018). Conditional deletion of hippocampal CA2/CA3a oxytocin receptors impairs the persistence of long-term social recognition memory in mice. *J. Neurosci.* 38, 1218–1231. doi: 10.1523/JNEUROSCI.1896-17.2017

Loland, C. J., Mereu, M., Okunola, O. M., Cao, J., Prisinzano, T. E., Mazier, S., et al. (2012). R-modafinil (armodafinil): a unique DA uptake inhibitor and potential medication for psychostimulant abuse. *Biol. Psychiatry* 72, 405–413. doi: 10.1016/j.biopsych.2012.03.022

Mereu, M., Chun, L. E., Prisinzano, T. E., Newman, A. H., Katz, J. L., and Tanda, G. (2017). The unique psychostimulant profile of (±)-modafinil: investigation of behavioral and neurochemical effects in mice. *Eur. J. Neurosci.* 45, 167–174. doi: 10.1111/ejn.13376

Müller, G. E., and Pilzecker, A. (1900). "Experimentelle Beiträge zur Lehre vom Gedächtnis," in *Zeitschrift für Psychologie und Physiologie der Sinnesorgane* EB, 1–300.

Nikiforuk, A., Kalaba, P., Ilic, M., Korz, V., Dragacevic, V., Wackerlig, J., et al. (2017). A novel DA transporter inhibitor CE-123 improves cognitive flexibility and maintains impulsivity in healthy male rats. *Front. Behav. Neurosci.* 11:222. doi: 10.3389/fnbeh.2017.00222

Noack, J., Murau, R., and Engelmann, M. (2015). Consequences of temporary inhibition of the medial amygdala on social recognition memory performance in mice. *Front. Neurosci.* 9:152. doi: 10.3389/fnins.2015.00152

Noack, J., Richter, K., Laube, G., Haghgoo, H. A., Veh, R. W., and Engelmann, M. (2010). Different importance of the volatile and non-volatile fractions of an olfactory signature for individual social recognition in rats versus mice and

short-term versus long-term memory. *Neurobiol. Learn. Mem.* 94, 568–575. doi: 10.1016/j.nlm.2010.09.013

Perna, J. C., Wotjak, C. T., Stork, O., and Engelmann, M. (2015). Timing of presentation and nature of stimuli determine retroactive interference with social recognition memory in mice. *Physiol. Behav.* 143, 10–14. doi: 10.1016/j.physbeh.2015.02.029

Ploeger, G. E., Willemen, A. P., and Cools, A. R. (1991). Role of the nucleus accumbens in social memory in rats. *Brain Res. Bull.* 26, 23–27. doi: 10.1016/0361-9230(91)90187-O

Richter, K., Wolf, G., and Engelmann, M. (2005). Social recognition memory requires two stages of protein synthesis in mice. *Learn. Mem.* 12, 407–413. doi: 10.1101/lm.97505

Steckler, T., Drinkenburg, W. H., Sahgal, A., and Aggleton, J. P. (1998). Recognition memory in rats–I. Concepts and classification. *Prog. Neurobiol.* 54, 289–311.

Tanimizu, T., Kenney, J. W., Okano, E., Kadoma, K., Frankland, P. W., and Kida, S. (2017). Functional connectivity of multiple brain regions required for the consolidation of social recognition memory. *J. Neurosci.* 37, 4103–4116. doi: 10.1523/jneurosci.3451-16.2017

Thor, D. H., and Holloway, W. R. (1982). Social memory of the male laboratory rat. *J. Comp. Physiol. Psychol.* 96, 1000–1006. doi: 10.1037/0735-7036.96.6.1000

Wanisch, K., Wotjak, C. T., and Engelmann, M. (2008). Long-lasting second stage of recognition memory consolidation in mice. *Behav. Brain Res.* 186, 191–196. doi: 10.1016/j.bbr.2007.08.008

Conflict of Interest Statement: 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 Ecology and Evolution

ORIGINAL RESEARCH
published: 21 May 2019
doi: 10.3389/fevo.2019.00177

Individual and Colony Level Foraging Decisions of Bumble Bees and Honey Bees in Relation to Balancing of Nutrient Needs

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Foraging decisions of social animals occur in the context of social groups, and thus may be subject to considerations of not only an individual's nutritional state and nutrient input, but those of the social group in which they live. In eusocial insects, which live in colonies containing workers that forage for food that is mostly consumed by others, foraging decisions that reflect colony needs may also be considered at both the colony and individual level. If colony energy balance is perturbed, is the counteracting response occurring on the group level (a change in division of labor) or on the individual level (a change in individual foraging choices)? To address this, colony and individual level foraging behaviors were observed in two species of eusocial bees: the highly social honey bee *Apis mellifera* and the primitively eusocial bumble bee *Bombus terrestris*. After manipulations of protein (P) and carbohydrate (C) stores in colonies of both species, there were changes in multiple different behavioral responses including colony level (number of foragers, allocation to nectar and pollen foraging, nutrient mass foraged) and individual level (P and C concentration preference and loading during foraging). These results suggest both honey bee and bumble bee colonies balance nutrient needs through a combination of both colony level shifts in foraging allocation, as well as slight modulation of individual nutrient preferences. This study also uncovered colony level differences between the two bee species; honey bees balanced P intake while bumble bees balanced C intake. These patterns may reflect differences in life history traits such as perenniality and hoarding, traits that are developed in more highly social species. Overall, this study highlights the importance of considering both group and individual level behavioral responses in foraging decisions in social animals.

Keywords: ***Apis mellifera, Bombus terrestris*, intake target, nutritional homeostasis, p:c ratio, pollen, nectar, foraging**

OPEN ACCESS

Edited by:
James P. Curley, University of Texas at Austin, United States

Reviewed by:
Brian R. Johnson, University of California, Davis, United States
Jose A. Masero, University of Extremadura, Spain

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Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *09 January 2019*
Accepted: *02 May 2019*
Published: *21 May 2019*

Citation:
Hendriksma HP, Toth AL and Shafir S (2019) Individual and Colony Level Foraging Decisions of Bumble Bees and Honey Bees in Relation to Balancing of Nutrient Needs. Front. Ecol. Evol. 7:177. doi: 10.3389/fevo.2019.00177

INTRODUCTION

What to eat? Where to forage, and for how long? Decision making in the context of food-searching behavior can be a complex process, involving multiple sensory modalities, specific forms of learning and memory, and integration of multiple different sources of external environmental and internal physiological information. Optimal foraging theory (OFT) makes predictions about the types of

decisions individual animals make in order to minimize energy expenditure and maximize their own nutrient intake, some of which have been supported by studies in a variety of animal taxa (Pyke, 1984; Sih and Christensen, 2001). However, OFT has been heavily criticized for ignoring life history traits, because foraging may occur in contexts outside of satisfying the immediate needs of individuals, e.g., parental care and food hoarding (Pierce and Ollason, 1987).

Foraging decisions take on an even greater level of complexity in considering social animals, especially highly social species in which inclusive fitness and group benefits are important considerations (Galef and Giraldeau, 2001; Kay, 2002). Eusocial insects, with a reproductive division of labor that includes a non-reproducing worker caste such as ants and honey bees, represent an extreme case in which foragers are often highly specialized on food-collecting behavior (Wilson, 1971). However, this food collection is often divorced from their own individual nutritional needs, and collected food is often not directly consumed, but instead shared to serve the nutritional needs of the colony. An extreme example is the *Myrmecocystus* honey ant, in which a specialized caste of individuals serves as living food receptacles (Burgett and Young, 1974), storing huge amounts of sweet plant secretions in their crops, but not consuming or digesting the vast majority of this food to fuel their own physiological needs.

Eusocial insects evolved from solitary species in which individuals needed to respond to their own internal physiological cues in order to feed themselves to fuel personal reproductive opportunities (Ament et al., 2010). During the evolution of sociality, there must have also been a transition from foraging decision making based on individual needs to decisions based on needs of the social group (Behmer, 2009b; Boomsma and Gawne, 2018). Across the diversity of extant insect species found today, there are highly eusocial species, in which colonies are very large and individual workers rarely reproduce, as well as primitively eusocial species, in which colonies are smaller and individual workers retain some reproductive capacity (Wilson, 1971). Thus, it may be possible to uncover differences in how social insects with different forms of sociality balance individual vs. colony level foraging decisions. If colony energy balance is perturbed, is the counteracting response occurring on the group level (a change in division of labor) or on the individual level (a change in individual foraging choices)? And do these responses differ in species with different levels of sociality? We aimed to address this knowledge gap by studying behavioral responses of two bee species with different forms of social organization.

Within the well-studied bee subfamily Apinae, *Apis mellifera* honey bees are a classic example of a highly eusocial species with task-specialized workers (Johnson, 2010), while *Bombus terrestris* bumble bees have smaller colonies with more behaviorally flexible workers (Crall et al., 2018). *A. mellifera* live in perennial, food-hoarding colonies consisting of tens of thousands of female workers, most of which will never lay an egg during the course of a normal colony cycle (Winston, 1991). Honey bee foragers are known to be highly specialized on collecting pollen, nectar, or non-food substances such as water or propolis (Page and Fondrk, 1995). On the contrary, *B. terrestris* colonies are annual, with small pollen and nectar stores, and as colonies decline in autumn, workers engage in dominance contests and several will lay unfertilized eggs (Goulson, 2010). Although larger workers tend to forage, while smaller workers tend to care for brood, individual foragers are not highly specialized as in honey bees (Goulson et al., 2002; Crall et al., 2018). Furthermore, bumble bees have only a relatively simple foraging communication system compared to the honey bees' sophisticated dance language (Dornhaus and Chittka, 2001). Colony-level decision making, such as colony allocation of foragers for pollen or for nectar, have also been extensively studied in both honey bees and bumble bees (Cartar, 1992; Seeley, 1995; Hagbery and Nieh, 2012; Leonhardt and Blüthgen, 2012; Konzmann and Lunau, 2014; Vaudo et al., 2014; Ruedenauer et al., 2015). Individual decision making during foraging trips has also been extensively studied in honey bees and bumble bees, providing important tests of OFT predictions (Heinrich, 1983; Hodges, 1985; Wells and Wells, 1986; Lihoreau et al., 2012; Katz and Naug, 2015). However, to our knowledge, few studies have considered both individual and colony level responses concurrently (Schulz et al., 1998).

As a guiding framework for this study, we utilized the geometric framework for nutritional balancing of protein (P) and carbohydrate (C) needs, as developed from studies in solitary insects (Lee et al., 2002; Behmer, 2009a; Raubenheimer et al., 2009). The geometric framework predicts that food choices for key macronutrients such as P and C will scale geometrically along an optimal ratio that supports individual reproductive fitness. The geometric framework can also be extended to whole colonies (Behmer, 2009b; Dussutour and Simpson, 2009; Lihoreau et al., 2014). This study extends findings of P to C balancing of isolated caged cohorts of honey bees (Altaye et al., 2010; Pirk et al., 2010; Paoli et al., 2014a,b) and bumble bees (Stabler et al., 2015) to the whole colony level.

We altered P and C inputs into colonies of both bee species and examined how this affected (1) colony allocation of foragers to P and C biased feeding stations, and (2) individual preferences (as inferred by choice of and food loading at different feeding stations) given a range of P and of C concentrations. We hypothesized that both honey bees and bumble bees would adjust colony division of labor and allocate more foragers to P and/or C depending on colony needs. Individual preferences were predicted to be skewed toward higher P and C concentrations for foragers from colonies more severely deprived of P or C, respectively, in order to address short-term colony needs rather than long-term optimal foraging. We further predicted, due to differences in level of sociality and food hoarding, that bumble bees would show stronger individual foraging responses to nutrient balance, whereas individual honey bee would be more fixed in response to colony nutrient shifts.

MATERIALS AND METHODS

Honey Bee Colony Preparations

Twenty-four honey bee colonies (*A. mellifera ligustica*) were standardized in nucleus hive boxes with ∼5,000 workers, a brood nest in 3 center frames and honey stores in 2 side frames (i.e., 5 frames total). This colony size represented an active growth phase of the colony life cycle (i.e., not senescing or reproducing)

TABLE 1 | Experimental manipulations applied to colonies.

Colony (*N*)	Protein manipulation	Carbohydrate manipulation	Colony signal
HB (8)	Adding a pollen frame (P+)	Removing a honey frame (C–)	P surplus
HB (8)	No manipulation (P)	No manipulation (C)	Baseline control
HB (8)	Sealing off pollen stores and adding an empty frame (P–)	Removing a honey frame (C–)	P deficit
BB (8)	Adding pollen patty (P+)	No hive sucrose feeder (C)	P surplus
BB (8)	No pollen patty (P)	No hive sucrose feeder (C)	Baseline control
BB (8)	No pollen patty (P)	Adding hive sucrose feeder (C+)	C surplus

Forage opportunities for both honey bees (HB) and bumble bees (BB) included a protein (P) gradient and a carbohydrate (C) gradient.

(Johnson, 2010). After standardization (October 9, 2014), all colonies were relocated for a period of 3 weeks to a location 4.2 km away, to avoid bees drifting back to former hive locations and restore balance between workers, brood, and stores.

One day preceding honey bee experiments, each colony was randomly assigned one of three pollen treatments: pollen deficit (P–), pollen control (P), or pollen surplus (P+). Eight colonies were given an empty comb in the middle of the brood nest, while any pollen cells in those colonies were sealed off using molten wax (treatment P–). Sealing away pollen is alike a natural sealing behavior by honey bees, called capping or entombing (vanEngelsdorp et al., 2009). Eight colonies were controls, which underwent no manipulation (treatment P). Eight other colonies were manipulated by introducing a comb fully filled with pollen in the middle of the brood nest (treatment P+). These pollen combs had been made by filling pre-weighed empty drawn combs with mixed pollen pellets. The pellets were compacted into the cells by worker bees in a large colony for 1 day. Subsequently each comb was reweighed (mean 581 g added pellets $\pm$ 29.7 SE; $n = 8$), and kept refrigerated until use.

In order to introduce either an empty comb (P–) or full comb (P+) in the brood nest, one of the side combs with honey was taken out. The honey removal was regarded as a manipulation of the level of carbohydrate nutrition (treatment C–). Thus, the design allowed assessing in-hive manipulation of both P (levels: less, normal, and more) and C (levels: less and normal) stores (**Table 1**).

Bumble Bee Colony Preparations

Twenty-four bumble bee colonies of European buff-tailed bumble bees (*B. terrestris)* were obtained from a commercial breeder (Biobee, Sde Eliyahu, Israel). They were prepared on December 1st, and delivered December 3rd, 2014. This colony size represented an active growth phase of the colony life cycle, as no drones or gynes were being reared yet. The colonies were killed after the experiment to minimize the chance of pathogens spreading into native bee populations (Aizen et al., 2018). Subsequent metrics were collected on colony workers, brood and food (pollen and nectar) storage (**Supplement 1**).

Standard in-hive sucrose feeders (2 L; 50% w/v sucrose solution) and in-hive pollen patties (50 g; 90% pollen with 10% sucrose solution) were removed from eight bumble bee colony boxes. These colonies were baseline control colonies that needed to forage for both protein (P; pollen diet) and carbohydrate (C; sucrose solution) according to a normal need (P:C ratio). Eight additional colonies were left with their supplied in-hive pollen patties (P+), but not sucrose feeders (C). An additional eight colonies retained the in-hive sucrose feeders (C+) but not the pollen patties (P). Thus, the design allowed for colony manipulation of both P diet (levels: normal and more) and C diet (levels: normal and more) (**Table 1**).

Honey Bee and Bumble Bee Colony Manipulation Differences

Because the experiment involved two different bee taxa with innate differences in pollen and nectar storage characteristics, exactly parallel colony manipulations were not possible. Honey bee colonies store a multi-day buffer of pollen within combs to safeguard nurse bee provisioning. Nurse bees digest pollen to produce a jelly to feed larvae (Wright et al., 2018). In contrast, bumble bees collect pollen for prompt brood cell provisioning as their larvae eat the pollen directly (Stabler et al., 2015). This disparity is reflected by experimental manipulations to include treatment levels of less, normal, and more in-hive P as treatments for honey bees, whereas bumble bees only included treatment levels normal and more in-hive P, because removing pollen from bumble bee colonies was not feasible. Secondly, honey bee colonies store large quantities of honey while bumble bees maintain a relatively small number of honey pots. As compared to honey bee combs, bumble bee honey pots are more difficult to manipulate. Therefore, carbohydrate stores were manipulated in honey bee colonies by removing a honey comb (C–) but in bumble bee colonies by adding a sucrose feeder (C+), while a baseline colony state (C) was present for both species (**Table 1**). Colony treatments can be summarized as P+C–, PC, and P–C– for honey bees, and P+C, PC, and PC+ for bumble bees.

Experimental Site and Setup

Experiments were conducted at the Joseph Marguleas Experimental Farm of the Faculty of Agriculture, Food and Environment at the Hebrew University of Jerusalem in Rehovot (31°54′16.02″N, 34°47′50.09″E). Twenty-four tunnel cages of 2 × 3 × 2 m (length × width × height) with a gauze cover of 1.3 mm mesh were used. Every colony had access to a water bucket with cork floats and a feeding arena consisting of two platforms covered with 50 × 50 cm white paper sheets, to present each colony five pollen and five sucrose feeders. Spatial bias on bee choice (e.g., due to the distance to the hive entrance or the

direction of the sun) was countered using different permutations of diet placement. In addition, spatial bias effects were mitigated by turning feeding arenas 90°, after recording bee visitation every hour (see section Data on Bee Visits, Collected Diet Weights and Overall Colony Activity).

Data Collection Days

Honey bee colonies were placed into the tents on November 4, after sunset. After 4 days the experiment was ended, with a total exposure to diet feeders per colony of 19:15 h, a mean 4:45 h per day (November 5, 6, 7, and 8). The bumble bee colonies were tested a month later, placed into the tents December 4, at sunset. The bumble bee experiment was ended after 20 of 24 bumble bee colonies had showed foraging activity at the feeding arena ($\geq$1 bee). The bumble bee colonies showed negligible activity over the first 5 days (excluded), which included 2 days with rain and cold temperatures. Exposure to diet feeders per bumble bee colony during the following days was 26:00 h, a mean 5:15 h per day (December 10, 11, 12, 13, and 14). The diurnal average temperature was 2.8°C warmer during the honey bee experiment, as compared to the bumble bee experiment (**Supplement 1**; $t = 2.60$, $p = 0.04$*), yet the day-time high temperatures were favorable for colonies to forage, and were not found to differ between the honey bee and bumble bee trial (24.2°C on average; $t = 2.02$, $df = 7$, $p = 0.08$).

Sugar Solution Feeders

Colonies were housed in tunnels and were provided with feeders (external to colonies) containing sugar solutions in five 1 L square plastic containers, holding 400 g solution per feeder. Each solution had a square bubble-wrap float on top as a platform for bees to land and drink. Solutions were kept *ad libitum* and refreshed every day or second day (closing feeders with lids overnight). By dissolving sugar in water, five solutions in a concentration gradient of 16.5, 18.5, 20.5, 22.5, 24.5% (w/w) sucrose were made, verified by refractometry. This range represented nectar concentrations at our apiary during the month of November, being 20.7 % ± 1.6 SEM ($n = 22$ sampled nectar foragers) (Tamar Drezner-Levy, unpublished data, 2003).

Pollen Powder Feeders

Colonies were provided with feeders (external to colonies) containing 100 g pollen diet batches, offered in five 13 cm Ø petri dishes (20 g diet per dish). These amounts were refreshed every day or second day (closing the dishes overnight), provided *ad libitum* to assure there was no limitation on choice options. Each diet contained 70% pulverized bee-collected pollen pellets mix with a 30% mix of different tapioca to casein ratios (10:0, 9:1, 8:2, 7:3, or 6:4). Casein use is common for manipulating protein levels in test diets (Pirk et al., 2010; Corby-Harris et al., 2018). Tapioca flour was used as a filler to create dilutions of the casein protein (Hendriksma and Shafir, 2016).

The three diet components (tapioca, casein, and pollen) were mixed in a table top blender and the resulting diets were passed through a honey-sieve and kept refrigerated until use. The tapioca was purchased as alimentary flour (Duchan, Gan Shmuel, Israel). The casein (C7078, Sigma, Missouri, USA) was first pulverized in a ball grinder (Pulverisette 6, Fritsch GmbH, Idar-Oberstein, Germany), applying 7 balls in 2 cycles of 3 min at 300 rpm for batches of 80 g casein. The pollen had been collected using traps on local honey bee hives during the year and kept frozen at −20°C. These mixed floral pollen pellets (5.9 kg) had been homogenized with an industrial blender.

Protein Analyses of All Pollen Diets and Dietary Components

To study colony macro-nutrient intake in the geometric framework of nutrition, the absolute foraged amounts of protein (P) and carbohydrate (C) were quantified. Dietary protein concentrations were assessed by Kjeldahl nitrogen analysis (Foss Tecator Kjeltec Auto Sampler System 1035 Analyzer, Hillerød, Denmark). Samples of 200 mg were oxidized by H_2SO_4 with catalysts Se and $CuSO_4$ at 400°C. N-amines that had been converted into $(NH_4)_2SO_4$ were hereafter titrated with HCl to indicate the quantity of nitrogen. The % crude protein (w/w) was calculated as %N multiplied with a conversion factor of 6.25 (Roulston and Cane, 2000), or 6.38 for casein (Sigma product specification sheet).

Separate diet batches were made for the honey bee and bumble bee trials, using the same pollen pellets mix, but using different casein and tapioca batches, which may have accounted for the slight differences (1.9% on average) in protein content. Final pollen diets contained 17.3, 18.1, 19.9, 22.3, and 24.6% protein in the honey bee trial, and 14.5 16.4, 19.0, 22.2, and 21.8% protein in the bumble bee trial. All samples were measured in duplicate.

Data on Bee Visits, Collected Diet Weights, and Overall Colony Activity

Bees collecting pollen or sucrose were counted by means of digital photography at approximate hourly intervals. The amount of collected diet was measured to the nearest 0.01 g pollen and 1 g sucrose solution. All feeders were weighed before and after colony exposure. Data from non-foraging colonies (0–2 total recorded visits) indicated a daily mean 1.5% desiccation of pollen diet and 2.8% evaporation of sucrose solutions, for which forage weight data at active colonies were corrected.

Absence of foraging was considered a proxy for colony inactivity. Colonies were considered inactive if over the full course of the experiment $\leq$7 foragers were counted on the feeding arena. Colonies that were inactive for sucrose collection ($N = 12$) had a mean 0.02 ± 0.01 SE sucrose foragers counted per observation, and collected a mean 0.00 gh^{-1} ± 0.16 SE sucrose solution, i.e., not significantly different from zero (one-sample *t*-test; $t = 0.0$, $p = 1.0$). Colonies inactive in pollen collection ($N = 28$) had a mean 0.09 ± 0.02 SE pollen foragers, and collected mean 0.01 gh^{-1} ± 0.02 SE pollen diet, also not significantly different from zero (one-sample *t*-test; $t = 0.69$, $p = 0.49$).

For all active colonies, relative collection of diet weight was calculated by dividing the sum of collected weights per concentration level, on the total weight collected. In addition, an index of foraging load was calculated per concentration level, by dividing the weight of diet collected per hour by the average count of bees on the diet (unit: $gh^{-1}{\cdot}bee^{-1}$). Assuming that

the length of time a bee spent on a feeder is proportional to the amount of food she collected and the probability of her being captured in the photograph census, the loading indexes are proxies to diet concentration-specific nectar crop loads and corbicular pollen loads, respectively. Individual bee identities were not recorded, however our data can still provide insights into individual foraging decisions. Foragers could choose to differentially spend their foraging time across different feeders, thus their choice, the relative amounts of food removed, and also the food loads may serve as proxies for individual bee motivation regarding the different nutrient concentrations in the offered diets.

P:C Ratio of Diet Uptake by Active Colonies

P:C ratios of colonies were calculated by dividing grams of P uptake by C uptake. Inactive colonies were excluded ($\leq$7 foragers or $\leq$0 g diet collected). P intake was the sum of collected pollen diet weights times the %P. The C intake of colonies was the sum of collected pollen diet weights times the %C in the pollen diet, plus the collected sucrose solution weights times %C in the solution. We assumed pollen pellets to contain 50% w/w C (Nicolson, 2011), which includes pollen starch (0–22%; Roulston and Buchmann, 2000), pollen sugars (9.6%; Speranza et al., 1997), and the nectar sugars added by foragers while pelleting the pollen (Bertoncelj et al., 2018). Casein (product specification sheet, Sigma) and tapioca flour (food label, Duchan) were considered to contain 0 and 99.8%C, respectively. The five pollen diets were thus calculated to contain 64.2, 61.3, 58.4, 55.5, and 52.6%C, respectively.

Statistics

Analyses were performed with JMP 13.1 Pro statistical software.

Colony Activity

The number of active vs. inactive honey bee and bumble bee colonies was compared by Fisher Exact Tests regarding pollen foraging, sucrose foraging, and overall foraging activity (**Table 1**).

Bee Counts

Generalized Linear Models (GLM) were fitted by maximum likelihood with an exponential distribution and reciprocal link function, to analyze treatment effects on forager counts. Pollen forager counts were analyzed on protein treatment effects (3 levels P–/P/P+ for honey bees; 2 levels P/P+ for bumble bees), and nectar forager counts on carbohydrate treatment effects (2 levels C–/C for honey bees; 2 levels C/C+ for bumble bees). Model fit was assessed by a Pearson χ^2 test for overdispersion. Final p-value approximations were based on Wald tests.

Allocation of Foragers Among Diets, Treatments, and Bee Types

The percentage pollen foragers on the total number of foragers was taken as measure of relative forager allocation. Allocation (arcsine square root transformed % data) as response variable was analyzed by 1-way ANOVA, with treatment as independent variable (3 levels), for honey bees and bumble bees separately. *Post-hoc* comparisons were performed by Tukey honest significant difference (HSD) tests. Relative forager allocations of honey bee and bumble bee colonies were compared under the PC treatment (i.e., in the baseline colonies) with bee type as independent variable (2 levels).

P:C Ratio

The intake of protein and carbohydrate by colonies was described, per treatment, as a P to C ratio. For the baseline colonies (PC treatment), P:C ratios per colony were compared among bee types (2 levels) with a 1-way ANOVA on ranks (Kruskal–Wallis test). The non-baseline colony treatments (2 levels) were compared for honey bees and bumble bees separately.

Dose Response Effects on Diet Collection, and Carrying Loads

Relative collected diet weights and forage load indices were compared by ANCOVA, for honey bee and bumble bee separately, with independent variables diet concentration (continuous variable) and treatment (3 levels). Significant interactions would indicate that concentration dependent choices of bees differed due to colony manipulations. For the baseline colonies (PC treatment), relative weights and load indices were compared with the independent variables diet concentration (continuous variable), and bee type (2 levels). Here an interaction would show different choice responses for honey bees and bumble bees, regarding dietary P or C concentrations. Non-significant interactions ($p \geq 0.05$) were removed from models. As proportionalized data are on average identical between groups ($p = 1.0$), the covariable (i.e., treatment or bee type) was also removed from the model in cases in which the interaction was not significant.

RESULTS

Honey Bee Colony Level Balancing the Uptake of P and C

A total 5,149 honey bee visits to diets were recorded. Altogether, honey bee colonies ($N = 24$) collected 493 g pollen diet and 15.7 Kg sucrose solution (ratio 1:32). The colonies were significantly more active with respect to sucrose collection as compared to pollen collection (Fisher Exact $p < 0.001$). Counts of pollen foragers indicated a difference by protein manipulation (**Figure 1A**; $N = 12$, $\chi^2 = 13.1$, $df = 2$, $p = 0.001$). Pollen foragers were significantly more active under the P- treatment, as compared to the treatments P ($\chi^2 = 5.27$, $df = 1$, $p = 0.02$) and P+ ($\chi^2 = 10.6$, $df = 1$, $p = 0.001$). Counts of pollen foragers did not differ between the P and P+ treated colonies ($\chi^2 = 2.53$, $df = 1$, $p = 0.11$). Nectar forager counts did not differ between colonies depending on C treatment (comparing C vs. C–, **Figure 1A**; $\chi^2 = 2.55$, $df = 1$, $p = 0.11$).

Under conditions of pollen excess and carbohydrate reduction (P+C–), honey bees shifted toward a C biased foraging division of labor, with higher numbers of foragers for sucrose (**Table 2**). Also, the relative forager allocation comparison indicated treatment effects [**Figure 1B**; $N = 24$, $F_{(2,21)} = 4.33$, $p = 0.027$], with the lowest pollen forage counts in the P+ treatment. These colonies showed a relatively high sucrose foraging allocation

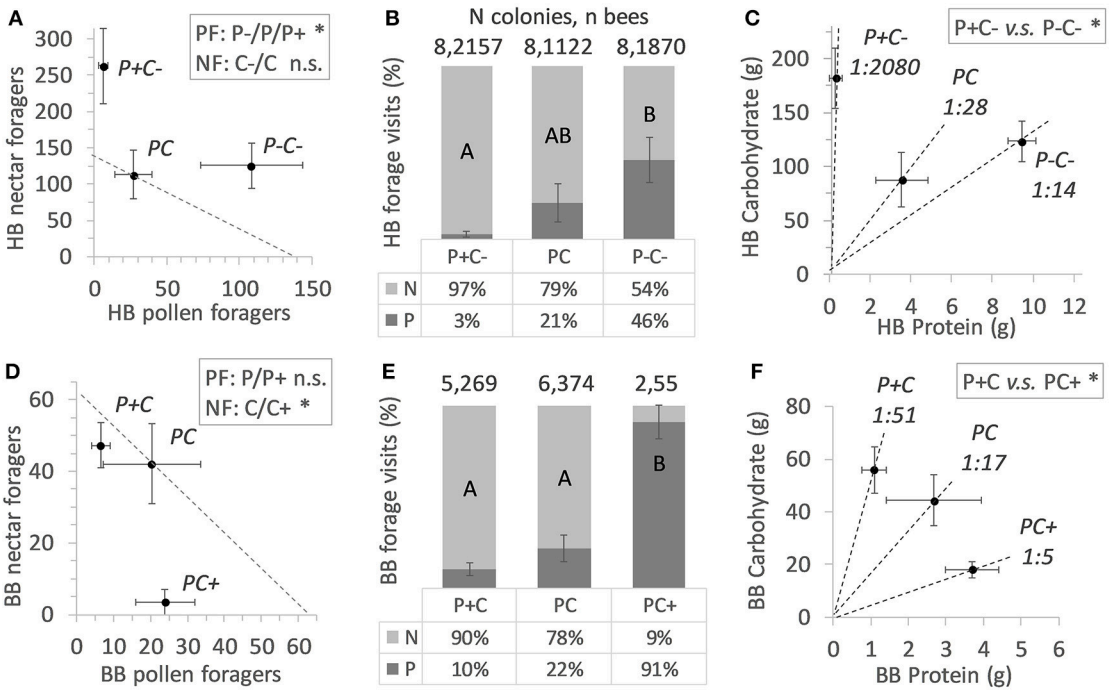

FIGURE 1 | Colony level effects of in-hive nutrient manipulations. Nectar forager (NF) counts and pollen forager (PF) counts are shown in **(A)** for honey bees (HB) and in **(D)** for bumble bees (BB), under conditions of increased or decreased protein (P) or carbohydrate (C) food stores. Mean active colony values of forager counts ± SE error bars are shown, with dotted diagonals indicating the mean forager number counted at baseline colonies. Statistical significance is indicated with an asterisk (∗). Foraging allocation to nectar (N) and pollen (P) is shown in **(B,E)** (mean % values ± SE error bars) with significant treatment effects indicated by different lettering (*post hoc* Tukey HSD). Colony level P:C ratios are indicated in **(C,F)** with dotted lines illustrating the mean Protein to Carbohydrate intake ± SE error bars per treatment. *Post hoc* statistical significances are indicated with an asterisk (∗).

TABLE 2 | Active vs. inactive colony numbers.

Bee	Treatment	*N*	*N* inactive/active Overall	*N* inactive/active Pollen diet (*n*; gh^{-1})	*N* inactive/active Sucrose diet (*n*; gh^{-1})
HB	P+C–	8	0/8	6/2 (1.5; 0.05)	0/8 (22.0; 55.6)
HB	PC	8	0/8	4/4 (4.4; 1.63)	0/8 (9.4; 22.4)
HB	P–C–	8	0/8	2/6 (12.0; 3.27)	0/8 (10.5; 26.3)
HB		**24**	**0/24**	**12/12**	**0/24**
BB	P+C	8	3/5	5/3 (0.5; 0.33)	3/5 (2.1; 9.1)
BB	PC	8	2/6	5/3 (1.7; 0.90)	2/6 (1.9; 5.0)
BB	PC+	8	6/2	6/2 (1.1; 0.75)	7/1 (0.3; 1.4)
BB		**24**	**11/13**	**16/8**	**12/12**

For active honey bee (HB) and bumble bee (BB) colonies, mean numbers of foragers per observation (n) and the collected diet weight in gram per h (gh^{-1}) are given per treatment. Treatment acronyms as defined in ***Table 1.*** *Sums per species are shown in bold.*

(**Figure 1B**; 97% nectar vs. 3% pollen foraging bouts, illustrated by their remarkable 1/2080 P:C intake ratio (**Figure 1C**). For the reverse treatment of pollen deprivation and carbohydrate reduction (P–C–), honey bees shifted their task allocation to collecting pollen (**Table 2**). Almost half the colony foraged for pollen (**Figure 1B**; 46% pollen vs. 56% sucrose) with an overall P to C intake shifted to 1:14 (**Figure 1C**). The P:C ratios of the non-baseline treated honey bee colonies differed significantly [P+C– vs. P–C+; $F_{(1,14)} = 8.09$, $p = 0.013$].

Bumble Bee Colony Level Balancing the Uptake of P and C

A total 714 bumble bee visits to diets were recorded. Altogether, bumble bee colonies ($N = 24$) collected a total of 155 g pollen diet and 2.0 Kg sucrose solution (ratio 1:13). For bumble bee colonies, the activities for collecting sucrose and pollen did not significantly differ (**Table 2**; $N = 12$ vs. $N = 8$, Fisher Exact $p = 0.38$) (**Table 2**). Pollen forager counts indicated no difference between the P and P+ manipulation (**Figure 1D**; $N = 8$, $\chi^2 = 3.56$, $df = 1$, $p = 0.06$), yet nectar forager counts were significantly lower in the C+ treatment compared to the C treatment (**Figure 1D**; $N = 12$, $\chi^2 = 8.32$, $df = 1$, $p = 0.004$).

Bumble bee colonies ($N = 13$) showed significant treatment effects on foraging allocation [**Figure 1E**; $F_{(2,10)} = 14.2$, $p = 0.001$]. Notably, the PC+ treated colonies showed significantly lower allocation of workers to the collection of sucrose, compared to the other 2 treatments (**Figure 1E**; 9% foraging for sucrose vs. 91% for pollen). The P+C colonies had the lowest P:C ratio, i.e., 1:51, which contrasted the reverse treatment P+C, where colonies had an intake of 1:5 PC+ ratio (**Figure 1F**). The P:C ratios of the non-baseline colonies differed significantly from one another [P+C vs. PC+; $F_{(1,5)} = 675.8$, $p < 0.001$].

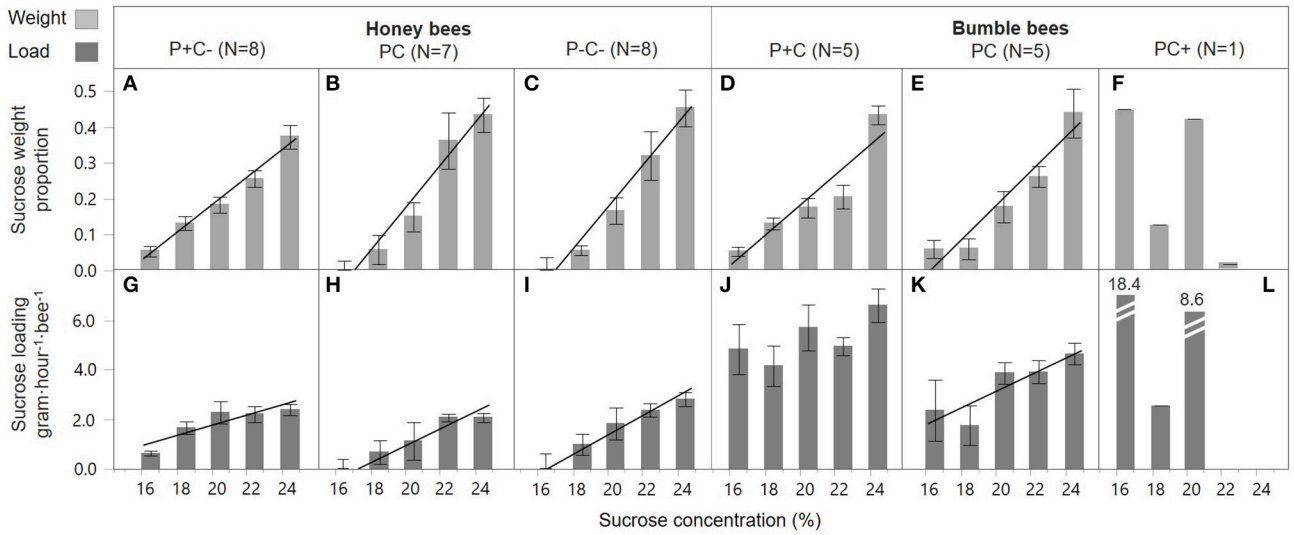

FIGURE 2 | Honey bee and bumble bee preference during sucrose collection. With the sucrose diet carbohydrate concentration in percent on the x-axis, relative sucrose collection by individual bees is shown as weight proportions on the y-axis **(A–F)**, and sucrose loading on the y-axis **(G–L)**. Trendlines indicate dose-response significance per treatment, involving protein (P) and carbohydrate (C) manipulations, within honey bee colonies (left side) and bumble bee colonies (right side).

Colony Level Differences Between Bee Species

Large differences between and within bee species were observed regarding colony activity (**Table 2**). Honey bee colonies were more likely to be active than bumble bee colonies (overall foraging 24 > 13 colonies, respectively, Fisher Exact $p < 0.001$; sucrose foraging 24 > 12 colonies, respectively, Fisher Exact $p < 0.001$; pollen foraging 12 ≈ 8 colonies, respectively, Fisher Exact $p = 0.38$). At the colony level, honey bees but not bumble bees balanced their pollen foraging in response to colony needs and in contrast, bumble bees but not honey bees balanced their sucrose foraging (**Figures 1A,D**). PC treatment (baseline colonies) revealed that the allocation of 21.1% pollen foragers for honey bee colonies ($N = 8$) did not differ from the 21.9% pollen forager allocation for bumble bee colonies ($N = 6$) [central bars **Figures 1B,E**; $F_{(1,12)} = 0.05$, $p = 0.83$]. Both bee species baseline colonies did not differ in their protein to carbohydrate intake ratio, with P:C ratios of 1:28 vs. 1:17, respectively [**Figures 1C,F**; $F_{(1,11)} = 0.09$, $p = 0.77$].

Individual Honey Bee Responses to P and C Nutrient Needs

Overall, honey bees collected 23 times more sucrose of the highest concentration as compared to the lowest concentration (42.0 vs. 1.8%, respectively), demonstrating a highly significant preference for higher C concentrations [**Figures 2A–C**, concentration; $F_{(1,109)} = 205.5$, $p < 0.001$]. Bees in the pollen supplemented hives (P+C–) showed a less pronounced preference compared to the other two treatments, namely 7 times more sucrose solution collection of the highest concentration as compared to the lowest concentration, with this difference illustrated by a shallower slope [**Figure 2A** vs. **Figure 2B,C**; slope interaction $F_{(2,109)} = 3.69$, $p = 0.028$]. The sucrose forage loads were also significantly higher when bees were visiting higher C feeders [concentration; $F_{(1,111)} = 46.1$, $p < 0.001$] (**Figures 2G–I**). Foragers did not show a concentration dependent effect on loading in response to colony P and C manipulations, i.e., similar slopes in the three treatment groups [**Figures 2G–I**; slope interaction $F_{(2,109)} = 2.94$, $p = 0.057$]. The loading of sucrose solution was not affected by colony manipulations [treatment; $F_{(2,111)} = 1.61$, $p = 0.20$].

P concentration preference by honey bee foragers differed by colony treatment [slope interaction; $F_{(2,49)} = 6.31$, $p = 0.004$]. In the PC and P–C– treated colonies, foragers preferred collecting pollen diets with lower P (casein) content [**Figures 3B,C**; $F_{(1,18)} = 64.2$, $p < 0.001$ and $F_{(1,23)} = 77.47$, $p < 0.001$, respectively]. This negative P preference was significantly stronger in the PC treatment as compared to the P–C– treatment [slope interaction; $F_{(1,41)} = 18.1$, $p < 0.001$]. There was no indication of a negative P preference in the P+C– colonies [**Figure 3A**; $F_{(1,8)} = 0.17$, $p = 0.69$]. In contrast, there was no effect of treatment on pollen loading under different concentrations [**Figures 3G–I**; slope interaction $F_{(2,47)} = 0.50$, $p = 0.61$]. In general, pollen loading by honey bees was not affected by protein concentration [$F_{(1,49)} = 0.14$, $p = 0.71$]. Pollen load did differ per P+C– , PC, and P–C– treatment [$F_{(2,49)} = 7.56$, $p = 0.001$], with baseline colony foragers (PC **Figure 3H**) showing a higher loading as compared to pollen supplemented colony foragers (P+C– **Figure 3G**).

Individual Bumble Bee Responses to P and C Nutrient Needs

Bumble bee foragers in the PC+ treatment did not favor high C concentrations [**Figure 2F**; $F_{(1,3)} = 3.55$, $p = 0.16$]. This was in significant contrast [slope interaction; $F_{(2,49)} = 19.5$, $p < 0.001$] with foragers of P+C and PC treated colonies, which did

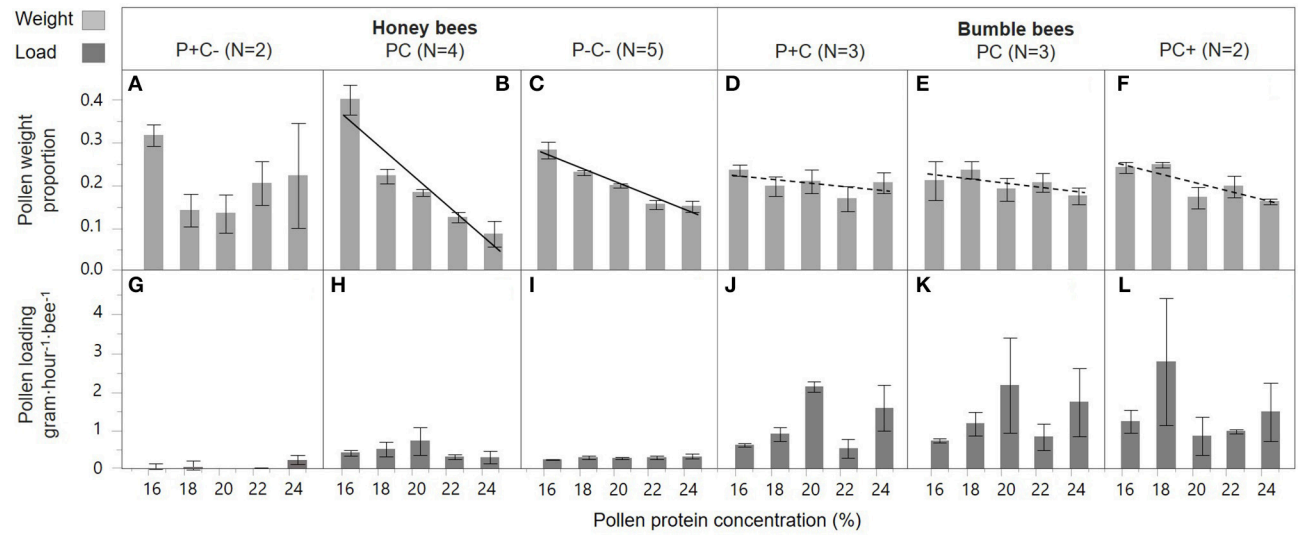

FIGURE 3 | Honey bee and bumble bee preference during pollen collection. With the pollen diet protein concentration in percent on the x-axis, relative pollen collection by individual bees is shown as weight proportions on the y-axis **(A–F)**, and pollen loading on the y-axis **(G–L)**. Trendlines indicate dose-response significance per treatment, involving protein (P) and carbohydrate (C) manipulations, within honey bee colonies (left side) and bumble bee colonies (right side). The striped trend lines indicate a significant dose-response for bumble bees in general, albeit this effect was insignificant for the treatment groups individually.

show a significant preference for higher sucrose concentrations [**Figure 2D,E**; $F_{(1,23)} = 68.6$, $p < 0.001$ and $F_{(1,23)} = 48.3$, $p < 0.001$, respectively] and collected proportionally 8 times more sucrose of the highest concentration as compared to the lowest concentration. Bumble bee foraging loads differed in C dose-responses [slope interaction $F_{(2,45)} = 15.6$, $p < 0.001$]. Under the PC treatment, foragers increased their crop loading with higher C concentration [**Figure 2K**; $F_{(1,21)} = 7.56$, $p = 0.012$], yet no such pattern was present at the P+C and PC+ treated colonies [**Figures 2J,L**; $F_{(1,22)} = 3.18$, $p = 0.09$ and $F_{(1,2)} = 2.87$, $p = 0.23$, respectively]. Individuals collected lighter loads under treatment PC, as compared to treatments P+C and PC+ [**Figures 2J–L**; $F_{(2,45)} = 7.40$, $p = 0.002$, *post-hoc* A B A]. These data suggest the addition of pollen patties motivated the bees to collect greater sucrose loads (**Figure 2J**), which may relate to increased brood production activity, motivating a higher fuel intake (**Supplement 1**). Notably, when giving sucrose solution to bumble bees within their hive, foragers loaded mostly at the least concentrated solution (i.e., 18.4 gh^{-1}·$visit^{-1}$), suggesting the foragers may have shifted to water collection behavior (**Figure 2L**).

Bumble bees, over all treatments considered, showed a significant preference for collecting diets with lower protein concentrations [**Figures 3D–F**; $F_{(1,31)} = 7.93$, $p = 0.008$]. This preference, however, was not affected by colony P or C manipulations [slope interaction; $F_{(2,34)} = 0.62$, $p = 0.54$ and treatment $F_{(2,34)} = 0.00$, $p = 1.00$]. There was also no effect of treatment on forager pollen loads [**Figures 3J–L**, slope interaction; $F_{(2,31)} = 0.59$, $p = 0.56$]. Bumble bee pollen loading was not affected by protein concentration [$F_{(1,33)} = 0.60$, $p = 0.44$], and forage loads did not differ in response to colony P and C treatment [**Figures 3J–L**; $F_{(2,33)} = 0.25$, $p = 0.78$].

Individual Response Differences Between Bee Species

A direct comparison was made for honey bee and bumble bee colonies only for the single treatment in which they experienced the same condition, that of baseline treatment PC (**Table 2**). Both species preferred to collect more sucrose solution of higher C concentrations [**Figures 2B,E**; $F_{(1,58)} = 104.6$, $p < 0.001$], and this effect was similar between the species [slope interaction; $F_{(1,56)} = 1.07$, $p = 0.31$, and bee type $F_{(2,34)} = 0.00$, $p = 1.00$]. Honey bee and bumble bee individuals increased loading with higher sucrose concentrations [$F_{(1,55)} = 21.2$, $p < 0.001$], with no difference between species in their concentration response [slope interaction; $F_{(1,54)} = 0.00$, $p = 0.96$]. Bumble bee foragers showed 3x higher sucrose loading as compared to honey bee foragers [bee type; $F_{(1,55)} = 27.2$, $p < 0.001$].

Honey bee and bumble bee foragers both preferred collecting pollen diets with lower protein concentration [**Figures 3B,E**; $F_{(1,31)} = 41.9$, $p < 0.001$], yet this preference was stronger for honey bees as compared to bumble bees [**Figures 3B,E**, slope interaction; $F_{(1,31)} = 24.0$, $p < 0.001$]. There was also 3x higher pollen loading by bumble bee foragers as compared to honey bee foragers [bee type; $F_{(1,32)} = 8.24$, $p = 0.007$]. A difference in protein dose-response on loading was not found between the species [slope interaction; $F_{(1,31)} = 0.96$, p=0.33], and loading was not found affected by protein concentrations [$F_{(1,32)} = 0.28$, $p = 0.60$].

DISCUSSION

In this study, two very different eusocial bee species were investigated (*A. mellifera* honey bees and *B. terrestris* bumble bees) in responses to shifts in their colony carbohydrate and

TABLE 3 | Summary of effects by treatment, diet concentration, bee species, and interactions.

Level	Response	Honey bees (HB)	Bumble bees (BB)	HB vs. BB
Colony	Activity	Sucrose > Pollen	n.s.[1]	Overall[1], Sucrose[1]
	Bee counts	P manipulation[2]	C manipulation[3]	N.A.
	Allocation	P+C– vs. P–C–[4]	P+C and PC vs. PC+[5]	n.s.[6]
	P:C ratio	P+C– vs. P–C–[7]	P+C vs. PC+[8]	n.s.[9]
Individual	C choice	C concentration[10], Treatment interaction[11]	C concentration[12], Treatment interaction[13]	C concentration[14]
	C load	C concentration[15]	C concentration[16], Treatment[17], Interaction[18]	C concentration[19], Species[20]
	P choice	P concentration[21], Treatment interaction[22]	P concentration[23]	P concentration[24], Species interaction[25]
	P load	Treatment[26]	n.s.[27]	Species[28]

*[1]**Table 2**, [2]**Figure 1A**, [3]**Figure 1D**, [4]**Figure 1B**, [5]**Figure 1E**, [6]**Figures 1B,E**, [7]**Figure 1C**, [8]**Figure 1F**, [9]**Figures 1C,F**, [10]**Figures 2A,B,C**, [11]**Figures 2A<B,C**, [12]**Figures 2D,E**, [13]**Figures 2D,E>F**, [14]**Figure 2B,E**, [15]**Figures 2G,H,I**, [16]**Figure 2K**, [17]**Figures 2J,L>K**, [18]**Figures 2K>J,L**, [19]**Figures 2H,K**, [20]**Figures 2H<K**, [21]**Figures 3B,C**, [22]**Figures 3A<B,C**, [23]**Figures 3D,E,F**, [24]**Figures 3B,E**, [25]**Figures 3B>E**, [26]**Figures 3G<H**, [27]**Figures 3J,K,L**, [28]**Figures 3H<K**. Significant effects are listed with numbers in superscript referring to specific illustrations. Non-significant effects are indicated with "n.s.," and "N.A." indicates when comparisons were not possible. Treatment acronyms as defined in **Table 1**.*

protein stores. In the baseline P to C treatment, the ratio of P to C foragers and P:C forage collected were strikingly similar between the two species, with close to 80% C foraging allocation (**Figures 1B,E**) and roughly comparable P:C ratios collected (**Figures 1C,F**). Overall, the results support the original hypothesis that both bee species would shift the division of labor to counteract shifts in colony nutrient balance.

For example, both honey bee and bumble bee colonies with perturbed P:C balance made adaptive shifts in the P:C ratio of the collected forage (**Figures 1C,F**). Thus, the division of labor shifts for both species had an impact on the actual mass nutrients taken in by foragers. Overall, these data reveal that both species have the ability to shift colony level foraging efforts to balance protein and carbohydrate nutrient intake.

There were also some key differences between species in their specific responses to colony energy balance (summarized in **Table 3**). Both honey bee and bumble bee colonies shifted the number of foragers and percent of foraging visits (**Figures 1A,B,D,E**) in response to nutrient manipulations. However, the shifts were significant only for P shifted treatments in honey bees, and significant only for C shifted treatments in bumble bees. These data suggest honey bees are more responsive to P needs, whereas bumble bees are more responsive to C needs. This is in agreement with the known high sensitivity of honey bee colonies to colony pollen needs (Camazine, 1993), and also in agreement with previously published carbohydrate responses in three other bumble bee species (Cartar, 1992). The different dynamics between bee species are consistent with their different life histories. Perennial honey bee colonies, which are keenly tuned toward hoarding large amounts of honey needed for overwintering survival, are expected to always allocate foragers to collect nectar, while more finely modulating pollen collection in response to colony stores and current brood needs (Keller et al., 2005). Annual bumble bee colonies, which do not store large amounts of honey for overwintering, should reduce allocation of nectar foragers once sufficient carbohydrate stores have been reached, but always allocate foragers to collect pollen to support maximum brood production (Cartar, 1992).

In general, individual bee preferences were predicted to be skewed toward higher nutrient concentrations during foraging. In line with this prediction, foragers of both species generally preferred the higher sucrose concentration feeders (**Figure 2**, top row). An underlying key question was if foragers changed in concentration preference under conditions of colony deprivation as compared to satiation. Both bee species were found to shift individual responses when colony nutrient levels were manipulated. For honey bees, sucrose preference was dampened in colonies supplemented with pollen. This shift toward acceptance of lower concentrations may have been caused by a sensory modulation depending on the nutritional state of the individual bee (**Figure 2A**). However, a shift in pollen vs. nectar forager allocation could also be the cause. It is likely that among the observed 97% nectar collectors there were some bees with a predisposition to collect pollen (**Figure 1B**). Pollen foragers have lower sucrose sensitivity thresholds (Pankiw and Page, 2000; Scheiner et al., 2001; Pankiw, 2003; Drezner-Levy et al., 2009). Hence, the nutritional state of colonies could have shifted forager allocation, and with that the average sensory modality for sucrose preference.

Bumble bee foragers were also found to prefer higher sucrose concentrations, with the exception of the sucrose supplemented treatment (**Figure 2F**). Here, foragers showed a remarkable shift in preference toward low sucrose concentrations. With an in-hive feeder containing 50% w/v sucrose, foragers may have shifted toward collecting forage with high water content, even in the presence of supplemental water feeders at the experimental arena (**Figure S2**).

Foragers of both species generally loaded with more sucrose from higher concentration feeders (**Figure 2**, bottom row). Load increase with sucrose concentration is consistent with the findings of Núñez (1966) and the descriptive model of Varjú and Núñez (1991), in which sucrose concentration is a main determinant of crop loads. However, for honey bees, patterns of loading in response to sucrose concentration did not differ across colony nutrient balance treatments (**Figures 2G–I**). This suggests that, despite changing colony needs, individual bees maintain behaviors that optimize C energy intake (Schmid-Hempel et al.,

1985; Kacelnik et al., 1986; Schmid-Hempel, 1987; Varjú and Núñez, 1991; Afik and Shafir, 2007). In contrast to the honey bee finding, bumble bees showed significant colony nutrient treatment effects on mean sucrose loads (**Figures 2J–L**). Unlike honey bee foragers which are all the same size, bumble bees vary greatly in size within a colony (**Figure S2**), with foragers being on average larger than nest bees (Goulson et al., 2002). One explanation for why mean bumble bee crop loads were significantly lower in the PC treatment (**Figure 2K**), as compared to the other treatments, is that the total number of foragers was greatest in the PC treatment. Because of a high forage demand for both pollen and nectar (**Figure 1D**), this may have induced additional recruitment of foragers, including the physically smaller bumble bees. Another explanation is that nutritional depletion caused mortality of forager bees (**Supplement 1**), which may have (again) induced the recruitment of smaller individuals with lower crop loads (Hagbery and Nieh, 2012). In fact, whereas bumble bee foragers are biased toward the larger individuals, when foragers are depleted by induced mortality, the probability of nest bees to switch to foraging is independent of their size (Crall et al., 2018).

Individual level responses to protein forage differed drastically from those of carbohydrate forage. Although individual forager preferences were predicted to be skewed toward higher P nutrient concentrations, instead foragers showed an aversion to high protein concentrations (**Figure 3**; top row). Why did bees avoid higher protein concentration? The pollen content (70%) was constant for all diets, thus the effect can be attributed to the casein or tapioca in the diet. Either bees found casein aversive, or bees were attracted to tapioca. When tapioca is imbibed it may evoke preference because the starch is hydrolyzed into sugar by α-amylase, an enzyme produced in honey bee salivary glands (Wright et al., 2018). The sweetness may subsequently act as a reward and evoke preference. Fat content may be another explanation why bees may have an aversion for casein. Technical grade casein from bovine milk has some fat content (C7078, Sigma) and Hendriksma and Shafir (2016; supplement within) found higher dietary fat contents to negatively affect diet preference of bees.

Despite the aversive nature of high protein concentrations, honey bee foragers showed modulation of individual responsiveness to protein based on colony nutrient balance. In baseline colonies foragers showed the most pronounced negative concentration preference, while foragers of pollen supplemented colonies were relatively indiscriminate. This suggests that satiated honey bees lost nutrient discrimination and/or preference (**Figure 3A**).

Pollen loads (**Figure 3** bottom row) were heavier for the overall larger bumble bees relative to honey bees, as also found under natural conditions (Minahan and Brunet, 2018). In honey bees, there was a significant treatment effect, with pollen supplemented colonies showing reduced loading compared to baseline. Thus, honey bees do appear to modulate their P foraging responses in response to colony energy balance shifts. Bumble bees showed less pronounced aversion for pollen diet protein content, and there were no significant treatment effects (**Figures 3D–F**), and the same was true for pollen loading (**Figures 3J,K,L**). This suggests that bumble bees are less responsive to dietary protein concentration, and/or that they forage with a hardwired preference baseline which is not affected by differential colony needs.

It is important to note that observed shifts in individual-level responses (**Table 3**) may not truly represent individuals altering behavior; rather they may represent alterations in the allocation of foragers that differ in size and/or in threshold sensitivity to nutrient stimuli. Future experiments following individual bees before and after colony nutrient shifts are necessary to disentangle whether the observed differences represent direct individual shifts vs. shifts in colony allocation of individuals with differing nutrient preferences. In addition, future study into individual and colony responses for colonies in different phases of the colony life cycle would complement current results, as forager responses may alter when colonies prepare for reproduction or reach the end of the season (Pankiw and Page, 1999; Johnson, 2010).

Returning to the original hypotheses, this study presents evidence to support the hypothesis that both highly eusocial and primitively eusocial bees balance colony nutrient needs by shifting division of labor for foraging. Although bumble bees are not as highly social as honey bees, the fact that they have passed the "superorganism threshold" by some definitions (because they have permanent morphological castes; Boomsma and Gawne, 2018), indicates that colony-level decision making is well developed even in "simpler" social insect societies. Based on differences in the level of sociality between honey bees and bumble bees, we also predicted stronger individual level responses in bumble bees compared to honey bees. Contrary to this prediction, we found evidence for shifts in individual level nutrient responsiveness (especially with respect to carbohydrate preference and loading) for both species. Instead of being related to differences in sociality, differences between species in their specific responses (summarized in **Table 3**) instead may be related to differences in colony life history traits (such as level of food hoarding, form of brood provisioning, etc.). Thus, we urge a stronger consideration of life history traits such as parental care, hoarding, and seasonality, in considerations of foraging decisions and in the context of OFT.

AUTHOR CONTRIBUTIONS

The study design, data collection and data analyses were performed by HH. The manuscript was written by HH, AT, and SS.

ACKNOWLEDGMENTS

We are thankful to the Biotechnology & Biological Sciences Research Council (BBSRC), Natural Environment Research Council (NERC), the Wellcome Trust, Department for Environment, Food & Rural Affairs (Defra), and the Scottish Government under the Insect Pollinators Initiative (grant no: BB/ I000968/1), and the United States Department of

Agriculture - National Institute of Food and Agriculture (grant no: 2016-07965) for funding, and to Prof. Dr. Amos Nussinovitch and Zahi Rabinovitz for casein preparation, Sameer Mabjeesh for protein analyses, and Haim Kalev for colony placement in his pomegranate orchard.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00177/full#supplementary-material

REFERENCES

Afik, O., and Shafir, S. (2007). Effect of ambient temperature on crop loading in the honey bee, *Apis mellifera* (Hymenoptera: *Apidae*). *Entomol. Gen.* 29, 135–148. doi: 10.1127/entom.gen/29/2007/135

Aizen, M. A., Smith-Ramírez, C., Morales, C. L., Vieli, L., Sáez, A., Barahona-Segovia, R. M., et al. (2018). Coordinated global species-importation policies are needed to reduce serious invasions globally: the case of alien bumble bees in South America. *J. Appl. Ecol.* 56, 100–106. doi: 10.1111/1365-2664.13121

Altaye, S. Z., Pirk, C. W., Crewe, R. M., and Nicolson, S. W. (2010). Convergence of carbohydrate-biased intake targets in caged worker honeybees fed different protein sources. *J. Exp. Biol.* 213, 3311–3318. doi: 10.1242/jeb.046953

Ament, S. A., Wang, Y., and Robinson, G. E. (2010). Nutritional regulation of division of labor in honey bees: toward a systems biology perspective. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 2, 566–576. doi: 10.1002/wsbm.73

Behmer, S. T. (2009a). Insect herbivore nutrient regulation. *Annu. Rev. Entomol.* 54, 165–187. doi: 10.1146/annurev.ento.54.110807.090537

Behmer, S. T. (2009b). Animal behaviour: feeding the superorganism. *Curr. Biol.* 19, R366–R368. doi: 10.1016/j.cub.2009.03.033

Bertoncelj, J., Polak, T., Pucihar, T., Lilek, N., Kandolf Borovšak, A., and Korošec, M. (2018). Carbohydrate composition of Slovenian bee pollens. *Int. J. Food Sci. Technol.* 53, 1880–1888. doi: 10.1111/ijfs.13773

Boomsma, J. J., and Gawne, R. (2018). Superorganismality and caste differentiation as points of no return: how the major evolutionary transitions were lost in translation. *Biol. Rev.* 93, 28–54. doi: 10.1111/brv.12330

Burgett, D. M., and Young, R. G. (1974). Lipid storage by honey ant repletes. *Ann. Entomol. Soc. Am.* 67, 743–744. doi: 10.1093/aesa/67.5.743

Camazine, S. (1993). The regulation of pollen foraging by honey bees - how foragers assess the colony need for pollen. *Behav. Ecol. Sociobiol.* 32, 265–272. doi: 10.1007/BF00166516

Cartar, R. V. (1992). Adjustment of foraging effort and task switching in energy-manipulated wild bumblebee colonies. *Anim. Behav.* 44, 75–87. doi: 10.1016/S0003-3472(05)80757-2

Corby-Harris, V., Snyder, L., Meador, C., and Ayotte, T. (2018). Honey bee (*Apis mellifera*) nurses do not consume pollens based on their nutritional quality. *PLoS ONE* 13:e0191050. doi: 10.1371/journal.pone.0191050

Crall, J. D., Gravish, N., Mountcastle, A. M., Kocher, S. D., Oppenheimer, R. L., Pierce, N. E., et al. (2018). Spatial fidelity of workers predicts collective response to disturbance in a social insect. *Nat. Commun.* 9:1201. doi: 10.1038/s41467-018-03561-w

Dornhaus, A., and Chittka, L. (2001). Food alert in bumblebees (*Bombus terrestris*): possible mechanisms and evolutionary implications. *Behav. Ecol. Sociobiol.* 50, 570–576. doi: 10.1007/s002650100395

Drezner-Levy, T., Smith, B., and Shafir, S. (2009). The effect of foraging specialization on various learning tasks in the honey bee (*Apis mellifera*). *Behav. Ecol. Sociobiol.* 64, 135–148. doi: 10.1007/s00265-009-0829-z

Dussutour, A., and Simpson, S. J. (2009). Communal nutrition in ants. *Curr. Biol.* 19, 740–744. doi: 10.1016/j.cub.2009.03.015

Galef, B. G. Jr., and Giraldeau, L.A. (2001). Social influences on foraging in vertebrates: causal mechanisms and adaptive functions. *Anim. Behav.* 61, 3–15. doi: 10.1006/anbe.2000.1557

Goulson, D. (2010). *Bumblebees: Behaviour, Ecology, and Conservation*. New York, NY: Oxford University Press.

Goulson, D., Peat, J., Stout, J. C., Tucker, J., Darvill, B., Derwent, L. C., et al. (2002). Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? *Anim. Behav.* 64, 123–130. doi: 10.1006/anbe.2002.3041

Hagbery, J., and Nieh, J. C. (2012). Individual lifetime pollen and nectar foraging preferences in bumble bees. *Naturwissenschaften* 99, 821–832. doi: 10.1007/s00114-012-0964-7

Heinrich, B. (1983). Do bumblebees forage optimally, and does it matter? *Am. Zool.* 23, 273–281. doi: 10.1093/icb/23.2.273

Hendriksma, H. P., and Shafir, S. (2016). Honey bee foragers balance colony nutritional deficiencies. *Behav. Ecol. Sociobiol.* 70, 509–517. doi: 10.1007/s00265-016-2067-5

Hodges, C. M. (1985). Bumble bee foraging: the threshold departure rule. *Ecology* 66, 179–187. doi: 10.2307/1941318

Johnson, B. R. (2010). Division of labor in honeybees: form, function, and proximate mechanisms. *Behav. Ecol. Sociobiol.* 64, 305–316. doi: 10.1007/s00265-009-0874-7

Kacelnik, A., Houston, A. I., and Schmid-Hempel, P. (1986). Central-place foraging in honey bees: the effect of travel time and nectar flow on crop filling. *Behav. Ecol. Sociobiol.* 19, 19–24. doi: 10.1007/BF00303838

Katz, K., and Naug, D. (2015). Energetic state regulates the exploration-exploitation trade-off in honeybees. *Behav. Ecol.* 26, 1045–1050. doi: 10.1093/beheco/arv045

Kay, A. (2002). Applying optimal foraging theory to assess nutrient availability ratios for ants. *Ecology* 83, 1935–1944. doi: 10.1890/0012-9658(2002)083[1935:AOFTTA]2.0.CO;2

Keller, I., Fluri, P., and Imdorf, A. (2005). Pollen nutrition and colony development in honey bees—Part II. *Bee World* 86, 27–34. doi: 10.1080/0005772X.2005.11099650

Konzmann, S., and Lunau, K. (2014). Divergent rules for pollen and nectar foraging bumblebees – a laboratory study with artificial flowers offering diluted nectar substitute and pollen surrogate. *PLoS ONE* 9:e91900. doi: 10.1371/journal.pone.0091900

Lee, K. P., Behmer, S. T., Simpson, S. J., and Raubenheimer, D. (2002). A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval). *J. Insect Physiol.* 48, 655–665. doi: 10.1016/S0022-1910(02)00088-4

Leonhardt, S., and Blüthgen, N. (2012). The same, but different, pollen foraging in honeybee and bumblebee colonies. *Apidologie* 43, 449–464. doi: 10.1007/s13592-011-0112-y

Lihoreau, M., Buhl, J., Charleston, M. A., Sword, G. A., Raubenheimer, D., and Simpson, S. J. (2014). Modelling nutrition across organizational levels, from individuals to superorganisms. *J. Insect Physiol.* 69, 2–11. doi: 10.1016/j.jinsphys.2014.03.004

Lihoreau, M., Chittka, L., Le Comber, S. C., and Raine, N. E. (2012). Bees do not use nearest-neighbour rules for optimization of multi-location routes. *Biol. Lett.* 8, 13–16. doi: 10.1098/rsbl.2011.0661

Minahan, D. F., and Brunet, J. (2018). Strong interspecific differences in foraging activity observed between honey bees and bumble bees using miniaturized radio frequency identification (RFID). *Front. Ecol. Evol.* 6:156. doi: 10.3389/fevo.2018.00156

Nicolson, S. W. (2011). Bee food: the chemistry and nutritional value of nectar, pollen and mixtures of the two. *Afr. Zool.* 46, 197–204. doi: 10.1080/15627020.2011.11407495

Núñez, J. A. (1966). Quantitative Beziehungen zwischen den Eigenschaften von Futterquellen und Verhalten von Sammelbienen. *Z. Vergl. Physiol.* 53, 142–164.

Page, R. E. Jr., and Fondrk, M. K. (1995). The effects of colony-level selection on the social organization of honey bee (*Apis mellifera L.*) colonies: colony-level components of pollen hoarding. *Behav. Ecol. Sociobiol.* 36, 135–144. doi: 10.1007/BF00170718

Pankiw, T. (2003). Directional change in a suite of foraging behaviors in tropical and temperate evolved honey bees (*Apis mellifera L.*). *Behav. Ecol. Sociobiol.* 54, 458–464. doi: 10.1007/s00265-003-0640-1

Pankiw, T., and Page, R. E. Jr. (1999). The effect of genotype, age, sex, and caste on response thresholds to sucrose and foraging behavior of honey bees (*Apis mellifera L.*). *J. Comp. Physiol. A* 185, 207–213. doi: 10.1007/s003590050379

Pankiw, T., and Page, R. E. Jr. (2000). Response thresholds to sucrose predict foraging division of labor in honeybees. *Behav. Ecol. Sociobiol.* 47, 265–267. doi: 10.1007/s002650050664

Paoli, P. P., Donley, D., Stabler, D., Saseendranath, A., Nicolson, S. W., Simpson, S. J., et al. (2014a). Nutritional balance of essential amino acids and carbohydrates of the adult worker honeybee depends on age. *Amino Acids* 46, 1449–1458. doi: 10.1007/s00726-014-1706-2

Paoli, P. P., Wakeling, L. A., Wright, G. A., and Ford, D. (2014b). The dietary proportion of essential amino acids and Sir2 influence lifespan in the honeybee. *Age* 36:9649. doi: 10.1007/s11357-014-9649-9

Pierce, G. J., and Ollason, J. G. (1987). Eight reasons why optimal foraging theory is a complete waste of time. *Oikos* 49, 111–118. doi: 10.2307/3565560

Pirk, C. W. W., Boodhoo, C., Human, H., and Nicolson, S. (2010). The importance of protein type and protein to carbohydrate ratio for survival and ovarian activation of caged honeybees (*Apis mellifera scutellata*). *Apidologie* 41, 62–72. doi: 10.1051/apido/2009055

Pyke, G. H. (1984). Optimal foraging theory: a critical review. *Annu. Rev. Ecol. Syst.* 15, 523–575. doi: 10.1146/annurev.es.15.110184.002515

Raubenheimer, D., Simpson, S. J., and Mayntz, D. (2009). Nutrition, ecology and nutritional ecology: toward an integrated framework. *Funct. Ecol.* 23, 4–16. doi: 10.1111/j.1365-2435.2009.01522.x

Roulston, T. H., and Buchmann, S. L. (2000). A phylogenetic reconsideration of the pollen starch - 676 pollination correlation. *Evol. Ecol. Res.* 2, 627–643. Available online at: http://www.evolutionary-ecology.com/issues/v02n05/ggar1186.pdf

Roulston, T. H., and Cane, J. H. (2000). Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* 222, 187–209. doi: 10.1007/978-3-7091-6306-1_10

Ruedenauer, F. A., Spaethe, J., and Leonhardt, S. D. (2015). How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J. Exp. Biol.* 218, 2233–2240. doi: 10.1242/jeb.118554

Scheiner, R., Page, R. E. Jr., and Erber, J. (2001). The effects of genotype, foraging role, and sucrose responsiveness on the tactile learning performance of honey bees (*Apis mellifera*). *Neurobiol. Learn. Mem.* 76, 138–150. doi: 10.1006/nlme.2000.3996

Schmid-Hempel, P. (1987). Efficient nectar-collecting by honeybees I. Economic models. *J. Anim. Ecol.* 56, 209–218. doi: 10.2307/4810

Schmid-Hempel, P., Kacelnik, A., and Houston, A. I. (1985). Honeybees maximize efficiency by not filling their crop. *Behav. Ecol. Sociobiol.* 17, 61–66. doi: 10.1007/BF00299430

Schulz, D. J., Huang, Z. Y., and Robinson, G. E. (1998). Effects of colony food shortage on behavioral development in honey bees. *Behav. Ecol. Sociobiol.* 42, 295–303. doi: 10.1007/s002650050442

Seeley, T. D. (1995). *The Wisdom of the Hive: the Social Physiology of Honey Bee Colonies*. Cambridge, MA: Harvard University Press.

Sih, A., and Christensen, B. (2001). Optimal diet theory: when does it work, and when and why does it fail? *Anim. Behav.* 61, 379–390. doi: 10.1006/anbe.2000.1592

Speranza, A., Calzoni, G. L., and Pacini, E. (1997). Occurrence of mono- or disaccharides and polysaccharide reservesin mature pollen grains. *Sex. Plant Reprod.* 10, 110–115. doi: 10.1007/s0049700 50076

Stabler, D., Paoli, P. P., Nicolson, S. W., and Wright, G. A. (2015). Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *J. Exp. Biol.* 218, 793–802. doi: 10.1242/jeb.114249

vanEngelsdorp, D., Evans, J. D., Donovall, L., Mullin, C., Frazier, M., Frazier, J., et al. (2009). "Entombed pollen": a new condition in honey bee colonies associated with increased risk of colony mortality. *J. Invertebr. Pathol.* 101, 147–149. doi: 10.1016/j.jip.2009.03.008

Varjú, D., and Núñez, J. (1991). What do foraging honeybees optimize? *J. Comp. Physiol. A* 169, 729–736. doi: 10.1007/BF00194901

Vaudo, A. D., Patch, H. M., Mortensen, D. A., Grozinger, C. M., and Tooker, J. F. (2014). Bumble bees exhibit daily behavioral patterns in pollen foraging. *Arthropod. Plant Interact.* 8, 273–283. doi: 10.1007/s11829-014-9312-5

Wells, H., and Wells, P. H. (1986). Optimal diet, minimal uncertainty and individual constancy in the foraging of honey bees, *Apis mellifera*. *J. Anim. Ecol.* 55, 881–891. doi: 10.2307/4422

Wilson, E. O. (1971). *The Insect Societies*. Cambridge, MA: Harvard University Press.

Winston, M. L. (1991). *The Biology of the Honey Bee*. Cambridge, MA: Harvard University Press.

Wright, G. A., Nicolson, S. W., and Shafir, S. (2018). Nutritional physiology and ecology of honey bees. *Annu. Rev. Entomol.* 63, 327–344. doi: 10.1146/annurev-ento-020117-043423

Conflict of Interest Statement: 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: 13 September 2019
doi: 10.3389/fnbeh.2019.00206

The Impact of Early Postnatal and Juvenile Social Environments on the Effects of Chronic Intranasal Oxytocin in the Prairie Vole

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Interactions between social experiences at different stages of development (e.g., with parents as juveniles and peers as subadults) can profoundly shape the expression of social behavior. Rarely are the influences of more than one stage of developmental sensitivity to social environment investigated simultaneously. Furthermore, oxytocin (OT) has an extraordinary effect on a breadth of social behaviors, activationally or organizationally. The use of intranasal OT (IN-OT) has become increasingly common therapeutically in humans and scientifically in non-human experiments, however very little attention has been paid to the potential developmental consequences on social behavior that might result. We investigated the effects of early-life social environments and the impact of chronic IN-OT on social behavior at different stages of development in male prairie voles (*Microtus ochrogaster*). We raised animals under two conditions: "socially enriched" (in which they were biparentally reared and then weaned into group housing as subadults), or "socially limited" (in which they were reared by a single-mother, and that were then weaned into social isolation). Males raised under each condition were either administered daily doses of IN-OT or a saline control for 21 days from postnatal day (PND) 21–42. During this time, we assessed the prosocial behavior subjects demonstrated by evaluating juvenile affiliation (as subadults), alloparental care (as adults no longer being exposed to IN-OT), and partner preference tests to assess tendencies to form adult monogamous pairbonds. We found that "socially limited" males, exhibited increased social contact in juvenile affiliation tests at PND 35 and 42. These males were also more likely to form a partner preference than "socially enriched" males and formed stronger partner preferences overall. IN-OT did not alter these behavioral effects. We also found that "socially limited" males exhibited a distinct response to chronic IN-OT treatment. When compared to all other treatment groups, "socially limited" males that received IN-OT exhibited a greater amount of huddling behavior in the alloparental care test. This effect was, in part, explained by an absence of attack behavior, found only in these males. This study contributes to understanding the complex interactions between the developmental social environment, oxytocin, and social behavior.

Keywords: intranasal oxytocin, early-life social experience, alloparental care, partner preference, prairie voles, *Microtus ochrogaster*

OPEN ACCESS

Edited by:
Rui F. Oliveira, Higher Institute of Applied Psychology (ISPA), Portugal

Reviewed by:
Valery Grinevich, German Cancer Research Center (DKFZ), Germany
Zhimin Song, Emory University, United States

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Specialty section:
This article was submitted to Behavioral Endocrinology, a section of the journal Frontiers in Behavioral Neuroscience

Received: *31 May 2019*
Accepted: *29 August 2019*
Published: *13 September 2019*

Citation:
Prounis GS and Ophir AG (2019) The Impact of Early Postnatal and Juvenile Social Environments on the Effects of Chronic Intranasal Oxytocin in the Prairie Vole. Front. Behav. Neurosci. 13:206. doi: 10.3389/fnbeh.2019.00206

INTRODUCTION

Social environments can vary tremendously across stages of development and profoundly shape the social behavior of an individual (Tzanoulinou and Sandi, 2017). Oxytocin (OT) is often implicated as a major regulator of social behavior in human and non-human species, and as a mechanism that shapes social development (Neumann, 2008; Rilling and Young, 2014; Feldman, 2015a,b; Walum and Young, 2018). Therefore, one way in which early-life social experience can have long-term consequences on social behavior is through developmental effects on the OT system. However, it remains unclear how social experiences at different stages of development impact adult behavior, and if and how social experience at different developmental stages might interact. Furthermore, the therapeutic use of OT for some children is becoming increasingly common and it remains unclear how the exogenous delivery of OT might further alter the complex nature of social development. In the current study, we briefly discuss each of these points and ask to what extent juvenile (i.e., subadult) and adult social behavior are altered by: (i) social environments experienced during two stages of development; (ii) by the non-invasive treatment of OT; and (iii) the potential interactions therein.

Specific features of social environments during critical developmental periods of life, such as interactions with parents or peers, could induce natural changes to the oxytocin system by altering OT synthesis or release, and/or OT receptor (OTR) density. Indeed, rats that receive higher amounts of maternal care develop increased densities of OTR in important parts of the brain that regulate social behavior, and they exhibit higher maternal care as adults (Francis et al., 2000, 2002; Champagne et al., 2001). On the other hand, rats experiencing routine maternal separation express similar brain region-specific increases or decreases of OTR and exhibit increases in anxiety and aggression (Kalinichev et al., 2002; Veenema, 2009; Lukas et al., 2010). In bi parental species, such as the mandarin vole and prairie vole, removal of fathers from the family unit alters OTR development and impairs social cognition (Cao et al., 2014; Prounis et al., 2015), and this can also reduce alloparental care and retard establishment of partner preferences (Ahern and Young, 2009). Interestingly, evidence has indicated that male and female prairie voles equally contribute to offspring care (Thomas and Birney, 1979) and single mothers do not appear to modify the amount of licking and grooming directed towards pups if fathers are removed (Ahern and Young, 2009). Together, this suggests that some of the OT-mediated behavioral effects on developing offspring just discussed could be attributable to the total reduction of care they received.

Social factors beyond the natal environment can also shape the OT system and behavior during juvenile and adolescent development. For example, male prairie voles that live in a socially and spatially enriched environment after weaning develop higher densities of OTR in many regions of the forebrain (Prounis et al., 2018). Similarly, mice that were exposed to high levels of early postnatal peer interactions later showed enhanced adult affiliative behavior and greater OTR density within the amygdala (Branchi et al., 2013). On the other hand, social isolation as subadults (the life-stage between weaning and adulthood) alters OTR receptor density (Prounis et al., 2015), and promotes anxiety (Pan et al., 2009) and depressive-like behaviors (Grippo et al., 2007b) in prairie voles.

Importantly, the impact of social environments during perinatal and subadult stages of development can interact. The quality of maternal care behavior received during perinatal development and environmental enrichment during subadult development interactively shape OTR expression and maternal behavior in rats (Champagne and Meaney, 2007). Furthermore, male prairie voles that are reared by a single-mother and then later experience isolated housing, demonstrate increases in lateral septum (LS) OTR and an impairment to social recognition (Prounis et al., 2015). Taken together, the aforementioned examples demonstrate that oxytocin is not only a key regulator of social behavior, but it is highly sensitive to socio-environmental influences, laying the groundwork to develop a deeper understanding of how variable social environments impact the development of social behavior.

Chronic exposure to chemical factors over development can also impact social behavior in a developing animal (e.g., Trezza et al., 2014; Davis et al., 2019). For instance, it is well-established that administration of exogenous chemicals (even those with endogenous sources) can compensatorily reduce ligand and/or receptor expression, in turn impacting neural function and behavior. OTRs are no different; persistently agonist-stimulated OTRs will desensitize, internalize and downregulate (Gimpl and Fahrenholz, 2001). Such phenomena raise important questions about how chronic early-life administration of OT could affect the neural development of animals, especially considering the common practices of administering therapeutic drugs, including OT, to children. Not surprisingly, exogenous manipulations of OT during early development affect social behavior later in life (Bales and Perkeybile, 2012). For example, intraperitoneal (i.p.) exposure to OT on postnatal day (PND) 1 facilitates adult male partner preference formation, whereas exposure to an OT antagonist reduces alloparenting in males (Bales and Carter, 2003; Bales et al., 2004). Moreover, i.p. injections of OT in neonatal prairie voles lead to dose-specific changes in alloparental care and attachment behaviors (Bales et al., 2007b). Oxytocin i.p. injections reverse the effect of social isolation on depressive-like behaviors (e.g., helplessness and anhedonia; Grippo et al., 2009), but not anxiety (Grippo et al., 2012).

A recent body of research suggests that intranasal oxytocin spray (IN-OT) provides a non-invasive exogenous means to alter central levels of extracellular oxytocin in rodents (Neumann et al., 2013). The development of this spray as a pharmacologic treatment for an array of social disorders has generated great excitement because OT effectively modulates social behavior (DeMayo et al., 2017; but see Leng and Ludwig, 2016). Behavioral studies in rodents suggest that IN-OT treatment can alter social behavior. In prairie voles, for example, chronic IN-OT treatment increases social contact with sibling cage-mates (Bales et al., 2013), whereas medium and low doses (but not large doses) of IN-OT impair partner preference behavior in males (Bales et al., 2013), and such changes in behavior may be mediated by the effects of IN-OT on OTR density (Guoynes et al., 2018).

Although, the acute use of IN-OT appears to be quite safe (DeMayo et al., 2017), few studies have investigated the potential long-term developmental impacts of IN-OT on social behavior (Bales and Perkeybile, 2012; but see Bales et al., 2013).

The circular nature of the potential for social environment to impact the OT system and for the OT system to impact social behavior (and thus the social environment) raises fundamental questions about the developmental mechanisms that shape adaptive behavioral repertories in adulthood. Moreover, because the social environment during development is complex and varies over time, interactions at different stages of development might create combinatorial interactions on behavioral and brain outcomes. We explored the dynamic interaction between early post-natal social environment, subadult social environment, and chronic dosing of extracellular OT *via* intranasal application on the social behavior of prairie voles at various stages of development. Several characteristics of prairie voles make the species particularly useful for the study of interactions between early social environments, social behavior, and the administration of OT. First, prairie voles in nature experience a wide variety of social experiences early in life, including single-mother and bi-parental rearing, and communal rearing in which older siblings alloparentally contribute to the care of younger siblings (Getz et al., 1981). As discussed above, simulating these social environments in the laboratory by removing fathers, and/or housing post-weaned animals in isolation or in groups alters the behavior in offspring (Grippo et al., 2007b; Ahern and Young, 2009; Pan et al., 2009; Prounis et al., 2015). Second, juvenile prairie voles in the lab readily engage in spontaneous alloparental care (care behavior towards novel unrelated pups; Roberts et al., 1998), and adult prairie voles form social attachments to opposite sex conspecifics (i.e., "partner preference behavior"; Getz et al., 1981; Williams et al., 1992). Last, these social behaviors are causally linked to OT function in the brain (Liu and Wang, 2003; Olazábal and Young, 2006a; Walum and Young, 2018).

In the current study, we hypothesized that the interaction between perinatal social environments (single-mother reared or bi parentally reared) and subadult social environments (isolated housing or group housing) would result in distinct expression of prosocial behavior during adolescence and adulthood. Furthermore, we predicted that IN-OT would increase the prosocial behavior of "socially limited" prairie voles (i.e., reared by single-mothers, followed by social isolation) to a greater extent than voles experiencing standard (and relatively enriched) rearing. This prediction was based on a previous study that showed "socially limited" voles exhibited higher densities of OTR in regions of the brain implicated in prosocial behavior, including the LS, prefrontal cortex (PFC), and basolateral amygdala (BLA; Prounis et al., 2015).

MATERIALS AND METHODS

Animals and Early Life Manipulations

All subjects came from the first litter of breeding pairs created specifically for this experiment. The breeders originated from our colony of prairie voles, which were originally trapped in Champaign County, IL, USA. Animals were housed in standard polycarbonate rodent cages (29 × 18 × 13 cm) lined with Sani-chip bedding and provided nesting material, and kept on a 14:10 light-dark cycle. Animals were provided rodent chow (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and water *ad libitum*. Ambient temperature was maintained at 20 ± 2°C. All procedures were approved by the Institutional Care and Use Committee of Cornell University (protocol number 2013-0102).

All breeding units created for this experiment had litters culled to 3–5 pups. Male subjects were assigned to groups at birth that exposed them to one of two different social experiences across pre-weaning and post-weaning development. One group of "socially enriched" males was first reared by both a mother and father (i.e., a biparental family unit), and then group-housed with a male sibling at weaning (PND 21; **Figure 1**). The second group of "socially limited" males was reared by a single-mother after the father was removed on PND 0 and then housed in isolation at weaning (**Figure 1**). We use the terms "socially enriched" and "socially limited" to label the treatments in an overly simplistic way to convey that the social experiences we created incorporated social opportunities with more or fewer individuals, relative to each other. We do not intend for these terms to convey preconceived notions of one condition being "better" or more adaptive than the other. We have previously shown that these manipulations during both pre-weaning and post-weaning produce group differences in OTR expression and social cognition in male prairie voles (Prounis et al., 2015).

Intranasal Oxytocin Treatments

Between PND 21 and 42, subjects received daily intranasal treatments of either saline or oxytocin (0.8 IU/kg) between 08:00 h and 12:00 h (**Figure 1**). This dose of chronic IN-OT treatment impacts social behavior of male prairie voles and is closely equivalent to a weight-adjusted dose commonly used in human studies (Bales et al., 2013). We applied a total of 25 μl of saline or OT from a pipette tip around the nasal cavity while the subject was scruffed and held belly-up, alternating sides so that 12.5 μl was applied to each nostril and resulting in inhalation of the solution. In total, four groups were created corresponding with both early-life manipulation and intranasal treatment: "socially enriched" + Saline, "socially enriched" + OT, "socially limited" + Saline, "socially limited" + OT (**Figure 1**). Subjects were weighed every week to determine the effects of the different conditions on body size and growth and to factor any mass differences into performance on behavioral tests.

Behavioral Testing

Subjects performed a series of behavioral tests during and after the period of intranasal treatment. These included four juvenile affiliation tests, two alloparental care tests, and a partner preference test (see below).

Juvenile Affiliation Test

Immediately after intranasal treatment on PND 22, 28, 35, and 42, subjects were placed in a standard-sized cage (29 × 18 × 13 cm) for 30 min of acclimation prior to testing.

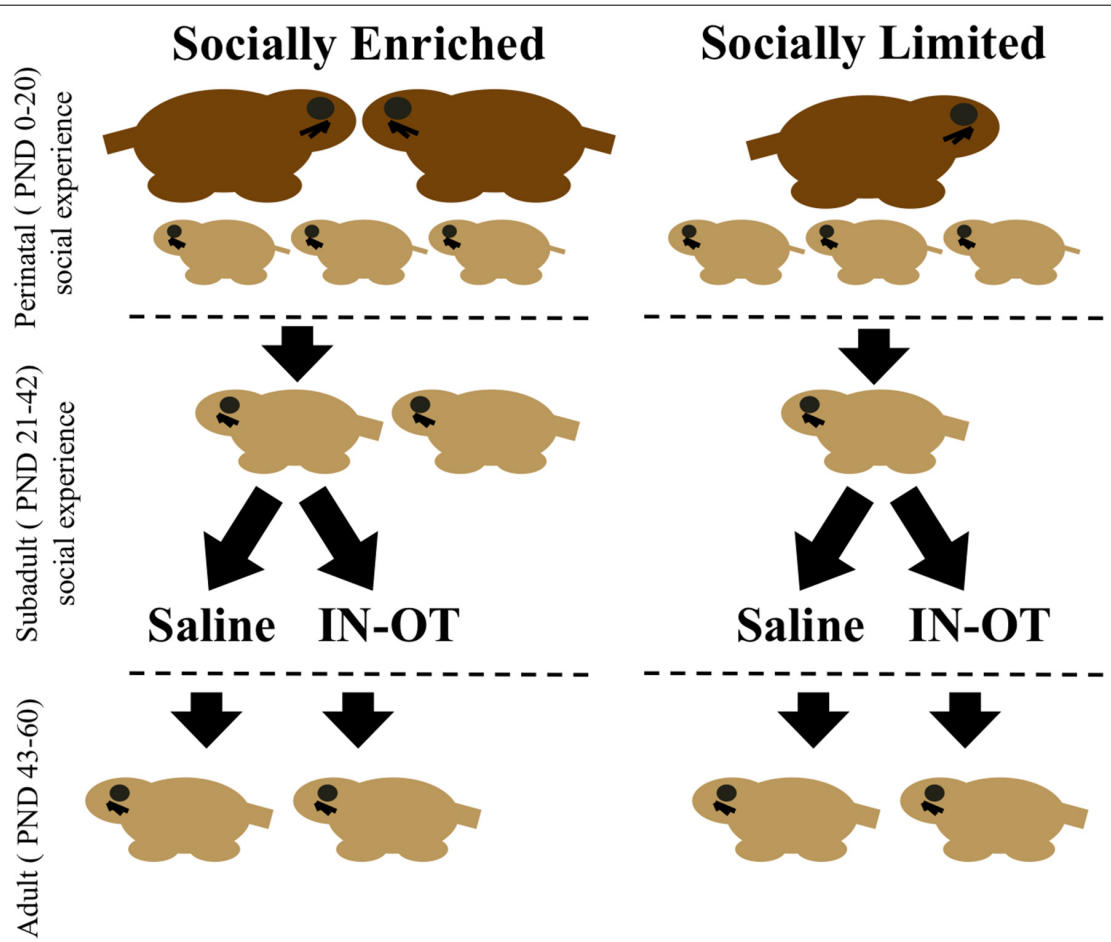

FIGURE 1 | Timeline of social manipulations and intranasal treatment. Perinatal social manipulations occurred between postnatal day (PND) 0–20 and consisted of presence or absence of the father. Subadult social manipulations occurred between PND 21 (weaning) through PND 42 and consisted of being group-housed with a same-sex sibling or housed in isolation. During this period, intranasal saline, or intranasal oxytocin (IN-OT) was administered daily. Juvenile affiliation tests were conducted during this period. Adulthood was defined as beginning at PND 43 and lasted through the end of behavioral testing (PND 60), during which time alloparental care tests and partner preference tests were conducted.

After acclimation, unrelated and novel juvenile voles (between PND 15 and 21) were placed on the opposite end of the cage from the subject. We assessed the social contact time (defined as all non-agonistic physical contact) between subjects and the juvenile voles during the 10-min test. The timing of these tests correspond with expected release of oxytocin in the brain after intranasal treatment (Neumann et al., 2013). Thus, the weekly juvenile affiliation tests were intended to examine the immediate effects of IN-OT on prosocial behavior with a non-threatening stimulus animal, while also testing for changes in social responses over the weeks of chronic administration. The final sample sizes analyzed for all four juvenile affiliation tests were: "socially enriched" + Saline, $N = 11$; "socially enriched" + OT, $N = 10$; "socially limited" + Saline, $N = 12$; "socially limited" + OT, $N = 12$.

Alloparental Care Tests

On the day after the last intranasal treatment (PND 43), subjects performed the first of two alloparental care tests. Subjects were placed in a novel standard-sized cage (29 × 18 × 13 cm) to acclimate for 30 min. After acclimation, two unrelated neonates (between PND 2 and 5) were placed at the opposite end of the cage from the subject. The amount of huddling behavior and aggression was quantified during the 10-min test. Huddling behavior was scored as the total time the subject was stationary and completely covering at least one of the two stimulus pups in the test. Aggression was scored as any lunges and biting behavior. An experimenter watched the social interaction from a close distance but out of sight of the voles to ensure that if the subject behaved aggressively to the stimulus pup, the trial could be terminated before the stimulus animal was harmed. A second alloparental care test was implemented on PND 58, as just described. This allowed us to determine behavioral effects both immediately after and weeks after the period of chronic intranasal treatment. This schedule also allowed us to examine the effects of treatment on behavior at an age associated with subadult peripubertal animals (∼PND 21–45) and adulthood (∼PND > 45). There were two instances where the subject

did not move from the corner of the cage at the start of the video (one during the PND 43 test, and one during the PND 58 test); these recordings were excluded from analysis. The final sample sizes analyzed for the PND 43 alloparental care test were: "socially enriched" + Saline, $N = 11$; "socially enriched" + OT, $N = 10$; "socially limited" + Saline, $N = 12$; "socially limited" + OT, $N = 11$. The final sample sizes analyzed for the PND 58 alloparental care test were: "socially enriched" + Saline, $N = 11$; "socially enriched" + OT, $N = 9$; "socially limited" + Saline, $N = 12$; "socially limited" + OT, $N = 12$.

Partner Preference Test

Last, on PND 60 subjects performed a partner preference test after 24 h of cohabitation with a sexually receptive female primed with dirty male bedding (Richmond and Stehn, 1976; Carter et al., 1980; Dluzen et al., 1981). To prime the females, urine-soaked bedding (taken from the cage of males that were unrelated to both the male subject and the female) was placed in the female's home cage a day before animal pairing. The 24 h cohabitation time is sufficient to form partner preference in male prairie voles (DeVries and Carter, 1999; Blocker and Ophir, 2016). On the day of testing, we evaluated animals in the "partner preference test" following Williams et al. (1992). Briefly, the female partner and the unfamiliar female were tethered to opposite compartments of a three-compartment apparatus. After 30 min of acclimation, the subject male was placed in the unoccupied middle compartment from which it could freely move between all three compartments of the apparatus. An observer blind to treatment scored the amount of time the subject spent in side-by-side contact with both the female partner and with an unfamiliar sexually primed adult female over a 180-min test period. These times were compared to determine the degree of preference to cohabitate with the partner. Two recordings were unable to be analyzed due to technical issues. The final sample sizes analyzed for the partner preference test were: "socially enriched" + Saline, $N = 11$; "socially enriched" + OT, $N = 9$; "socially limited" + Saline, $N = 11$; "socially limited" + OT, $N = 12$.

Data Analysis

All behavioral data were collected using Noldus Observer XT 14.0 (Noldus Information Technology Inc., Leesburg, VA, USA). For the juvenile affiliation tests, we performed a two-factor ANOVA to compare social contact time between groups according to social manipulation and intranasal treatment, and we used Tukey HSD tests to determine significant *post hoc* comparisons between groups. The findings from our initial two-factor ANOVA motivated us to perform a mixed factorial repeated measures ANOVA to compare changes in social contact time over the four timepoints between socially enriched and socially limited males. Due to non-normal distribution of data for proportion of huddling time in the alloparental care tests, we performed a Kruskal–Wallis test to detect significant group differences, and we used Dunn's test for *post hoc* comparisons between groups. We performed a Pearson's chi-square test for independence to compare the incidence of attack behavior in the alloparental care tests. We included subjects that attacked pups in the analysis of huddling time because we believe the attack behavior fairly represents an absence of care behavior and is critical to understanding group differences. Excluding subjects that attacked the pups from analysis of the alloparental care test would also severely reduce our sample size and result in a drastic loss of statistical power. Paired *t*-tests compared the side-by-side contact time with a partner female vs. an unfamiliar female for individual groups in the partner preference test. A two-factor ANOVA compared the preference score (contact with partner—contact with unfamiliar female) according to social manipulation and intranasal treatment, and Tukey HSD determined significant *post hoc* comparisons between groups. We performed a Pearson's chi-square test for independence to compare the proportion of subjects displaying a partner preference in the partner preference test. Both chi-square tests (for the alloparental care test and the partner preference test) were limited to analysis of main effects (comparison based on social housing, or on intranasal treatment) due to sample size constraints when comparing all four groups. We considered an alpha ≤ 0.05 to be statistically significant for all tests. We report all F and p-values rounded to the nearest one-hundredth decimal place, except where $p < 0.01$, in which case we report them as such.

RESULTS

Physical Development

No weight differences were found among males at PND 21 (ANOVA: $F_{(1,41)} = 1.00$, $p = 0.32$), but "socially limited" males weighed less than "socially enriched" males at PND 28 ($F_{(1,41)} = 4.21$, $p = 0.05$), PND 35 ($F_{(1,41)} = 4.82$, $p = 0.03$), and PND 42 ($F_{(1,41)} = 4.73$, $p = 0.04$). Intranasal OT treatment did not affect body mass at any stage of development. *Post hoc* comparisons showed no significant difference in body mass between any combination of the four groups (Tukey HSD: all p's > 0.18). Body mass did not correlate with any behavioral test at the comparable age (see **Supplementary Table S1**).

Juvenile Affiliation Tests

Social rearing environment did not impact the amount of social contact time with juveniles at PND 22 (ANOVA: $F_{(1,41)} = 1.10$, $p = 0.30$) and PND 28 ($F_{(1,41)} = 1.77$, $p = 0.19$; **Figure 2A**). However, we found a main effect of social environment for tests performed on PND 35 ($F_{(1,41)} = 8.12$, $p < 0.01$) and PND 42 ($F_{(1,41)} = 5.86$, $p = 0.02$), with "socially limited" males having more social contact time than "socially enriched" males (**Figure 2A**). There was no effect of intranasal treatment on social contact with juveniles at any age (PND 22: $F_{(1,41)} < 0.01$, $p = 0.98$; PND 28: $F_{(1,41)} = 0.27$, $p = 0.60$; PND 35: $F_{(1,41)} = 0.51$, $p = 0.48$; PND 42: $F_{(1,41)} = 0.22$, $p = 0.64$). Likewise, there were no significant interactions between social environment and intranasal treatment at any age (PND 22: $F_{(1,41)} = 0.53$, $p = 0.47$; PND 28: $F_{(1,41)} = 0.20$, $p = 0.66$; PND 35: $F_{(1,41)} = 0.02$, $p = 0.90$; PND 42: $F_{(1,41)} = 0.01$, $p = 0.92$). *Post hoc* comparisons showed no differences between any of the four groups (Tukey HSD: all comparisons $p > 0.08$).

A mixed-effects ANOVA with a repeated measure for the age of testing revealed a significant interaction between

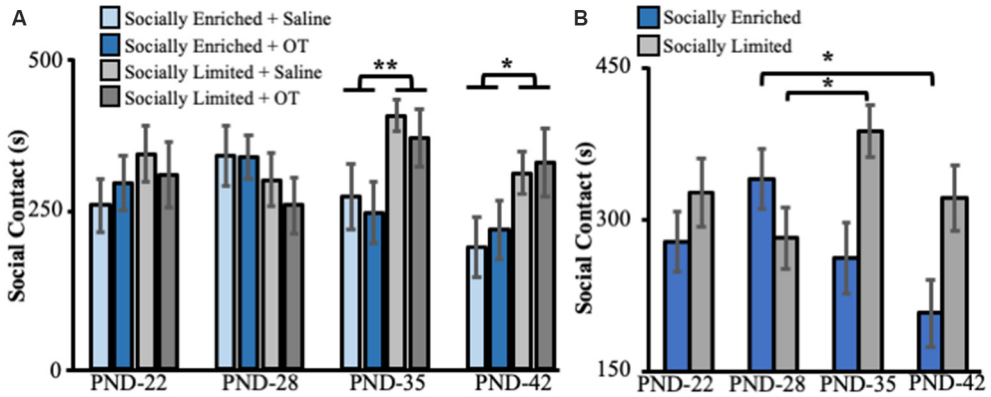

FIGURE 2 | (A) Mean (±SE) seconds (s) subjects spent in social contact with novel juvenile voles (aged PND 15–21) as a function of early-life social experience and intranasal treatment on juvenile affiliation behavior in subadults. **(B)** Mean (±SE) seconds (s) subjects spent in social contact with novel juvenile voles (aged PND 15–21) as a function of only early-life social experience on juvenile affiliation behavior in subadults. OT, intranasal oxytocin treated animals; Saline, intranasal saline-treated animals; PND, postnatal day. *Indicates $p < 0.05$; **indicates $p < 0.01$.

developmental time and social rearing environment on social contact with juveniles ($F_{(3,129)} = 4.43$, $p < 0.01$, **Figure 2B**). Pairwise comparisons (with Bonferroni correction for multiple comparisons) showed that "socially enriched" males significantly reduced their social contact time with juveniles between PND 28 and PND 45 ($p = 0.01$, **Figure 2B**), whereas "socially limited" males increased their contact time with juveniles between PND 28 and PND 35 ($p = 0.04$, **Figure 2B**) and displayed comparable levels of juvenile contact between PND 28 and 45 ($p = 0.99$, **Figure 2B**).

Alloparental Care Tests

We found group differences in the proportion of time subjects huddled with pups at PND 43 in the first alloparental care test (Kruskal–Wallis: $x^2_{(3)} = 8.91$, $p = 0.03$). *Post hoc* comparisons showed that "socially limited" + OT males spent more time huddling than "socially limited" + Saline males (Dunn's test: $p = 0.01$), "socially enriched" + Saline males ($p < 0.01$), and "socially enriched" + OT males ($p = 0.02$; **Figure 3**). We found nearly the same group difference in the PND 58 alloparental care test ($x^2_{(3)} = 10.60$, $p = 0.01$), with *post hoc* comparisons showing that "socially limited" + OT males spent more time huddling with pups than "socially enriched" + Saline ($p < 0.01$), and "socially enriched" + OT males ($p < 0.01$; **Figure 3**). A non-significant trend suggested "socially limited" + OT males might also spend more time huddling pups than "socially limited" + Saline males at PND 58 ($p = 0.06$; **Figure 3**).

At PND 43, immediate attack of pups during the test resulted in early termination of the test with zero proportion of time spent huddling scored for a subset of "socially enriched" + Saline males ($N = 5$, 45.5%), "socially enriched" + OT males ($N = 2$, 20%) and "socially limited" + Saline males ($N = 3$, 25%). Similar incidents of attack behavior were found at PND 58 ("socially enriched" + Saline: $N = 4$, 36.4%; "socially enriched" + OT: $N = 1$, 11.1%; "socially limited" + S: $N = 2$, 16.7%). At both PND 43 and PND 58, no "socially limited" + OT males attacked the pups. When comparing groups according to intranasal treatment, subjects receiving IN-OT ("socially limited" and "socially enriched" males combined) were less likely to attack than subjects receiving saline ("socially limited" and "socially enriched" males combined) at PND 43 ($x^2_{(1)} = 3.99$, $p = 0.05$; **Supplementary Table S2**) and PND 58 ($x^2_{(1)} = 3.73$, $p = 0.05$; **Supplementary Table S3**).

Partner Preference Test

Only "socially limited" + OT ($t_{(11)} = 3.89$, $p < 0.01$) and "socially limited" + Saline ($t_{(10)} = 2.53$, $p = 0.02$) males demonstrated a significant preference for the female partner based on side-by-side contact time with each female in the partner preference test (**Figure 4A**). Surprisingly, a preference for side-by-side contact with the partner was not found for "socially enriched" + Saline ($t_{(10)} = 0.48$, $p = 0.32$) and "socially enriched" + OT males ($t_{(8)} = 1.02$, $p = 0.17$). Comparison of a partner preference score (side-by-side time with partner − side-by-side time with unfamiliar female/total side-by-side time) revealed a main effect of social environment, with "socially limited" males having larger preference scores than "socially enriched" males (ANOVA: $F_{(1,39)} = 4.19$, $p = 0.05$; **Figure 4B**). We performed an additional analysis of group differences in partner preference behavior by comparing the proportion of individuals demonstrating a preference for the partner, defined as the subject spending over 60% of total side-by-side contact with the partner. According to this approach, "socially limited" subjects (+OT and +Saline males combined) were more likely to form a preference than "socially enriched" subjects (+OT and +Saline males combined; chi-square test of independence: $x^2_{(1)} = 3.74$, $p = 0.05$; **Supplementary Table S4**).

DISCUSSION

The combination of early social environment and IN-OT had variable effects depending on the developmental stage

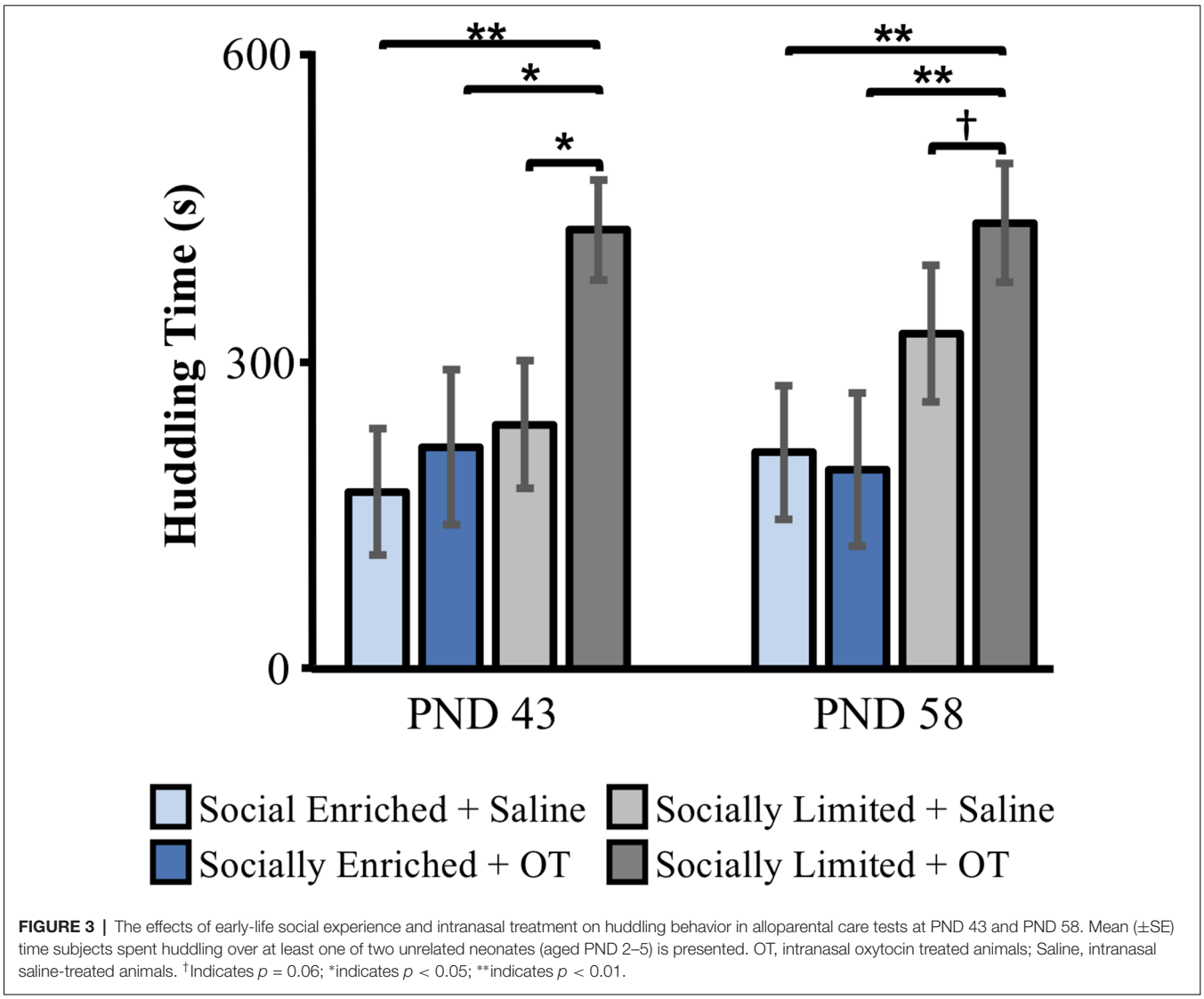

FIGURE 3 | The effects of early-life social experience and intranasal treatment on huddling behavior in alloparental care tests at PND 43 and PND 58. Mean (±SE) time subjects spent huddling over at least one of two unrelated neonates (aged PND 2–5) is presented. OT, intranasal oxytocin treated animals; Saline, intranasal saline-treated animals. †Indicates $p = 0.06$; *indicates $p < 0.05$; **indicates $p < 0.01$.

and the social behavior being tested. The social manipulation contrasted a relatively socially limited environment (no access to fathers before weaning or siblings after weaning) with a socially enriched environment (access to two parents and then a sibling). We found that males reared in "socially limited" environments engaged in more social contact with juveniles when they were subadults (PND 35 and PND 42) when compared to males reared in "socially enriched" environments. Furthermore, whereas "socially limited" males increased social contact with juveniles as they became older, males reared in "socially enriched" environments reduced social contact with juveniles as subadults. "Socially limited" males were also more likely to form a partner preference in adulthood when compared to "socially enriched" males. Lastly, chronic doses of IN-OT during post-weaning development led to a high degree of alloparental care behavior in "socially limited" males. This result suggests an additive or synergistic effect wherein a two-hit social deprivation treatment during postnatal development and IN-OT treatment promotes pro-social behavior in male prairie voles (see Ebitz and Platt, 2014).

Our findings indicate that, regardless of intranasal OT treatment, the combination of single-mother rearing during perinatal stages and isolated housing during subadult stages produced male prairie voles that: (i) engaged in more social contact in peri-adolescence (**Figure 2**); and (ii) developed stronger partner preferences in adulthood (**Figure 4**). Our results contribute to the small but growing number of reports that address the effects of perinatal and subadult social environments on prairie vole development. For example, prairie vole pups weigh more, open their eyes and grow hair sooner, and begin eating solid food and exploring outside the nest more rapidly when fathers were present (Wang and Novak, 1992, 1994), indicating that paternal care accelerates physical development of prairie vole offspring in the lab. Indeed, our data were consistent with this interpretation, showing that

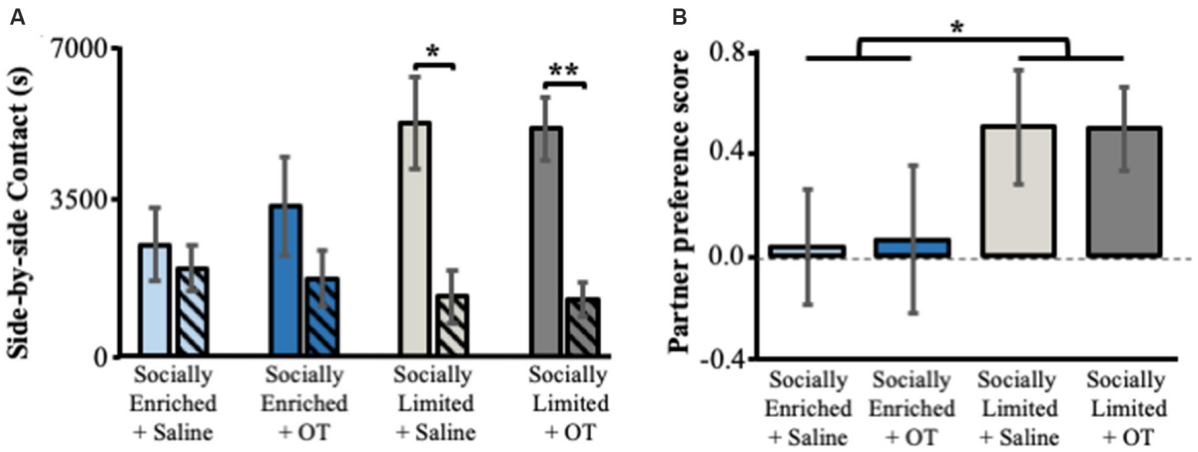

FIGURE 4 | The effects of early-life social experience and intranasal treatment on partner preference behavior. **(A)** Mean (±SE) side-by-side contact in seconds (s) with the female partner (solid bars) and unfamiliar female (hatched bars). **(B)** Mean (±SE) partner preference score (time in seconds subjects spent in side-by-side contact with partner—time spent in side-by-side contact with the unfamiliar female/total time in side-by-side contact). Color scheme follows **Figures 2**, **3**. OT, intranasal oxytocin treated animals; Saline, intranasal saline-treated animals. *Indicates $p < 0.05$; **indicates $p < 0.01$.

"socially enriched" males weighed more than "socially limited" males shortly after weaning and throughout subadulthood. Ahern and Young (2009) demonstrated that single-mother reared males did not form partner preferences after 1 day of cohabitation, instead, requiring a week of cohabitation before a preference was formed. This study also found no effect of rearing condition on alloparental care behavior in males, although females without fathers were less alloparental (Ahern and Young, 2009). Our results replicate this, finding no main effect of single-mother rearing on alloparental care in males (**Figure 3**). Surprisingly, our results in the partner preference test were inconsistent with Ahern and Young (2009), showing that single-mother reared males were more likely to form a partner preference than bi-parentally reared males. Similarly, unlike our study, Wang and Novak (1994) reported that time engaged in allogrooming and play was lower in males reared without fathers compared to those reared with both parents.

We suspect that these inconsistencies between our study and others could be attributable to elements of our experimental design. These differences could include the impact of social isolation at the subadult life stage (which was not investigated in these other studies), the unintended consequences of the necessary handling associated with the delivery of intranasal OT or Saline, or the combination of manipulating social experience at two important stages of development (see below).

First, several studies have found that pre-adult social isolation leads to an increase in social interaction (Wongwitdecha and Marsden, 1996; Pan et al., 2009; Gilles and Polston, 2017). For example, compared to group-housed males, male prairie voles that experience post-weaning social isolation prefer spending time in a cage containing a tethered novel male over an empty cage (Pan et al., 2009). Results such as these indicating that developmentally isolated animals become more social are consistent with our data in which "socially limited" animals increased social contact (alloparental care) with juveniles at PND 35 and PND 42.

Second, it is worth considering the ways in which the intranasal administration regimen might have impacted our animals. The handling that our animals experienced was extremely brief and they showed no observable evidence of discomfort or distress following each dosing. However, experimental scruffing of mothers increases maternal licking and grooming (Bales et al., 2007a) and could induce sub-threshold physiological reactions that cumulatively contribute to long-term effects on brain and behavior development. For example, we know that isolated male prairie voles have higher circulating concentrations of OT in their plasma, and higher activity of OT-reactive neurons in the paraventricular nucleus in response to experiencing a resident-intruder test (Grippo et al., 2007a), suggesting that social stress can induce endogenous release of OT in animals predisposed to be OT-reactive. It is worth noting that all of our animals experienced the same amount of handling for intranasal delivery during the subadult period (PND 21–45). However, if daily scruffing produced a subtler but similar reaction to resident-intruder tests and isolated males were more susceptible to this effect, then "socially limited" animals receiving IN-OT would have also experienced higher doses of OT than we had planned. Thus, the synergistic effects of experimental handling and early-life social experience, with the exogenous delivery of OT could have produced a heightened sensitivity to OT in "socially limited" + OT males in a way that none of the other groups in our study experienced. This might provide an explanation for the unique alloparental behavior we observed among "socially limited" + OT males. Interestingly, "socially limited" + OT males exhibited care towards pups in every individual trial. In contrast, approximately half of the control males ("socially enriched"

+ Saline) attacked the pups during the trial. Our data also show that IN-OT treatment reduced the likelihood of attack behavior during the alloparental care test (**Supplementary Tables S2, S3**). Thus, whether or not these effects are directly attributable to the handling the animals received, the interactions of complex early-life social experiences with OT exposure (endogenously released, exogenously released, or both) clearly produced an overarching prosocial (less-aggressive) phenotype in males.

Finally, it is also possible that group housing has protective or buffering effects on other developmental consequences resulting from being reared without fathers. Although the behavioral outcomes were quite different in how they interacted, we have reported results indicating that subadult housing conditions have the potential to alter the outcome of behavioral effects established by the absence of fathers in the perinatal nest (Prounis et al., 2015). Prounis et al. (2015) also found that "socially limited" reared males had greater expression of OTR in some forebrain areas (see below) suggesting that the sequence of single-mother rearing followed by social isolation produces a distinct social and neural phenotype. Indeed, social stress promotes prosocial behavior (e.g., adult social isolation, Perry et al., 2016), and this relationship is believed to be mediated by oxytocin (Taylor, 2006). We speculate that one outcome of reduced social opportunities during both perinatal and subadult stages might be increased motivation to seek social interaction when opportunities arise, which might buffer against the stress of social isolation. This unique combination of early social experiences might also facilitate prosocial behaviors in adulthood that resulted in the increased likelihood of pairbond formation in "socially limited" males. These possibilities highlight the importance of considering how social experiences at different life stages might interact to alter and, in some cases, rescue or even reverse behavioral phenotypes.

Because prairie voles reared in standard conditions typically exhibit partner preferences, the relative lack of partner preferences in "socially enriched" voles was unexpected (with the issues discussed above notwithstanding). Interestingly, Bales et al. (2013) demonstrated that standard reared male prairie voles (i.e., "socially enriched") receiving IN-OT at the same dose that we used, also failed to form bonds. In this respect, our results complement and replicate this earlier result. And we note that our "socially enriched" + Saline and "socially enriched" + OT males also did not differ in juvenile affiliation or alloparental care behavior, just as was reported in Bales et al. (2013). However, this does not explain why our Saline treated "socially enriched" animals did not form bonds. "Typical" partner preference behavior in prairie voles has been shown to be highly variable (Vogel et al., 2018), and could be attributable to a number of factors including, for example, whether or not the pair produced fertilized embryos (Curtis, 2010). It is important to acknowledge that approximately half of our "socially enriched" subjects still exhibited a partner preference (**Supplementary Table S4**). Unfortunately, we did not directly examine the fertilization success of female-male pairs and it is possible that infertility contributed to the failure to find consistent pairbonding in this group.

Our study did not address the precise mechanism of action promoting alloparental care in "socially limited" + OT males, but previous research in prairie voles highlights some potential places to explore. OTR in the nucleus accumbens (NAc) is particularly high in juvenile prairie voles and is correlated with alloparental care (Olazábal and Young, 2006b), and mediates alloparental care (Olazábal and Young, 2006a). Notably, chronic IN-OT (at the same dose used in our study) has been reported to increase OTR in NAc in female, but not male, prairie voles (Guoynes et al., 2018). The effects of OT on OTR are notably dose-specific; the same study found no effect with lower or higher IN-OT doses (Guoynes et al., 2018). Furthermore, persistent application of OT agonists causes reduction of OTR *via* internalization (Gimpl and Fahrenholz, 2001). Therefore, it is possible that the "medium" chronic dose we used (and used by Bales et al., 2013; Guoynes et al., 2018) exerted a classic inverted U effect on behaviors that was high enough to alter behavior, but low enough to avoid inducing OTR internalization, in which case behavioral effects might have disappeared. Interestingly, the prosocial effect on social affiliation (i.e., huddling) was observed after IN-OT treatment had stopped, and this effect persisted for approximately 2 weeks (PND 43 and 58). This long-lasting effect of chronic IN-OT into adulthood may be due to the combination of limited access to social interaction during juvenile and subadult development, and the organizational effects resulting from administration of exogenous OT during subadult development. Ebitz and Platt (2014) provide a compelling argument for why the effects of exogenous OT might reduce the typical prioritization of social information at the expense of other information or goals. This can occasionally lead to counter intuitive but adaptive ways that prosociality might, or might not, be expressed, and can be attributable to how OT regulates the way the neural circuitry responsible for social behavior accesses information about the social environment. The administration of exogenous OT and its interactions with early-life social experiences certainly could have caused long-lasting organizational effects on the endogenous OT system, observable later in adulthood after IN-OT administration had stopped but modified and enhanced prosocial behavior persisted. Such effects could express themselves in the endogenous release patterns or functioning of OT or other aspects of the neurochemistry in the brain, leading to an altered prioritization of alloparental care, pairbonding or both. Indeed, earlier studies demonstrate the complex relationship between exogenous OT and behavior, showing highly variable outcomes of alloparental care behavior and partner preference behavior according to four different doses of OT given i.p. shortly after birth in female prairie voles (Bales et al., 2007b).

We previously showed that animals experiencing the same rearing conditions as those in our "socially limited" group exhibited significant increases of OTR in the LS, PFC, septohippocampal nucleus (SHi), and BLA when compared to control subjects ("socially enriched"; Prounis et al., 2015). With the exception of the SHi, all of these structures are important

nodes of the Social-Decision-Making Network (SDMN; see O'Connell and Hofmann, 2011)—a network of structures that integrates processing of social information with reward circuitry to facilitate an animal's ability to respond to and prioritize social information in their natural world. Indeed, OTR and vasopressin receptor expression, particularly within the SDMN, are highly plastic and sensitive to environmental and developmental forces (Prounis et al., 2018). Chronic IN-OT may have further developmentally impacted mechanisms that alter the social decision-making process. In this light, our multifaceted manipulations of early-life social experience and IN-OT exposure might have altered the way in which the SDMN, and potentially OT's function therein, processed social behavior. Developmental effects on OTR or other factors that assert functional influence over the SDMN could have altered function of the SDMN globally, in effect, shifting animals' valanced prioritization of the social value of pups. This hypothesis could explain why "socially limited" males reduced attack responses towards pups and instead demonstrated greater huddling with them. This could have been even further exaggerated as a result of chronic IN-OT. For example, IN-OT doubles extracellular levels of OT in the amygdala and hippocampus of rats (Neumann et al., 2013). This potential combination of increased exogenous OT and probable developmentally induced OTR upregulation in the SDMN of "socially limited" + OT males provides a plausible mechanism that could facilitate behavioral differences in this group. We believe that a greater understanding of complex brain phenotypes and the behaviors they generate can be found in a deeper appreciation of the SDMN—a network of structures that collectively regulate emergent properties of social behavior.

Social behavior is dynamically shaped by interactions between the early social environment and developing nonapeptide systems. We altered both the social environment and the OT system in developing prairie voles, and in doing so we identified that responses to intranasally administered oxytocin can be mediated by the degree of social opportunities experienced in perinatal and subadult development. Our results have translational application to exploring clinical use of IN-OT in children, adolescent, and adult humans. The effects of IN-OT on human behavior are remarkably inconsistent (Bartz et al., 2011; Keech et al., 2018). This generates controversy ranging from people who fear negative long-term effects of chronic IN-OT application, to people who doubt there are any effects at all (Young, 2013; Leng and Ludwig, 2016). We offer a potential source of variable responses to IN-OT, highlighting the shaping force of early-life social environments. Importantly, future studies must continue to explore how early life social environments across life stages interactively shape phenotypes. As this foundation of knowledge grows we can begin to form predictions about how physiological and neural systems adaptively tune to these early environments. Future research should test predictions of adaptive tuning by combining early-life manipulations in the lab with fitness outcomes in ecologically relevant and complex environments. Such efforts are likely to reveal that, in the proper context, phenotypic outcomes of socially limited contexts sometimes described as deprivation or even pathological can have adaptive or positive outcomes.

DATA AVAILABILITY

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

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Care and Use Committee of Cornell University (protocol number 2013-0102).

AUTHOR CONTRIBUTIONS

GP conceptualized the experiment, developed the experimental design, analyzed data, and wrote the manuscript. AO conceptualized the experiment, and wrote the manuscript.

FUNDING

This work would not be possible without the generous funding from the National Institutes of Health (Eunice Kennedy Shriver National Institute of Child Health and Human Development HD079573 to AO).

ACKNOWLEDGMENTS

We thank Stephen Parry at the Cornell Statistical Consulting Unit for statistical input regarding our analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00206/full#supplementary-material

REFERENCES

Ahern, T. H., and Young, L. J. (2009). The impact of early life family structure on adult social attachment, alloparental behavior, and the neuropeptide systems regulating affiliative behaviors in the monogamous prairie vole (*Microtus ochrogaster*). *Front. Behav. Neurosci.* 3:17. doi: 10.3389/neuro.08.017.2009

Bales, K. L., and Carter, C. S. (2003). Developmental exposure to oxytocin facilitates partner preferences in male prairie voles (*Microtus ochrogaster*). *Behav. Neurosci.* 117, 854–859. doi: 10.1037/0735-7044.117.4.854

Bales, K. L., Lewis-Reese, A. D., Pfeifer, L. A., Kramer, K. M., and Carter, C. S. (2007a). Early experience affects the traits of monogamy in a sexually dimorphic manner. *Dev. Psychobiol.* 49, 335–342. doi: 10.1002/dev.20216

Bales, K. L., van Westerhuyzen, J. A., Lewis-Reese, A. D., Grotte, N. D., Lanter, J. A., and Carter, C. S. (2007b). Oxytocin has dose-dependent developmental effects on pair-bonding and alloparental care in female

prairie voles. *Horm. Behav.* 52, 274–279. doi: 10.1016/j.yhbeh.2007.05.004

Bales, K. L., and Perkeybile, A. M. (2012). Developmental experiences and the oxytocin receptor system. *Horm. Behav.* 61, 313–319. doi: 10.1016/j.yhbeh.2011.12.013

Bales, K. L., Perkeybile, A. M., Conley, O. G., Lee, M. H., Guoynes, C. D., Downing, G. M., et al. (2013). Chronic intranasal oxytocin causes long-term impairments in partner preference formation in male prairie voles. *Biol. Psychiatry* 74, 180–188. doi: 10.1016/j.biopsych.2012.08.025

Bales, K. L., Pfeifer, L. A., and Carter, C. S. (2004). Sex differences and effects of manipulations of oxytocin on alloparenting and anxiety in prairie voles. *Dev. Psychobiol.* 44, 123–131. doi: 10.1002/dev.10165

Bartz, J. A., Zaki, J., Bolger, N., and Ochsner, K. N. (2011). Social effects of oxytocin in humans: context and person matter. *Trends Cogn. Sci.* 15, 301–309. doi: 10.1016/j.tics.2011.05.002

Blocker, T. D., and Ophir, A. G. (2016). A preference to bond? Male prairie voles form pair bonds even in the presence of multiple receptive females. *Anim. Behav.* 122, 89–97. doi: 10.1016/j.anbehav.2016.10.007

Branchi, I., Curley, J. P., D'Andrea, I., Cirulli, F., Champagne, F. A., and Alleva, E. (2013). Early interactions with mother and peers independently build adult social skills and shape BDNF and oxytocin receptor brain levels. *Psychoneuroendocrinology* 38, 522–532. doi: 10.1016/j.psyneuen.2012.07.010

Cao, Y., Wu, R. Y., Tai, F. D., Zhang, X., Yu, P., An, X. L., et al. (2014). Neonatal paternal deprivation impairs social recognition and alters levels of oxytocin and estrogen receptor α mRNA expression in the MeA and NAcc and serum oxytocin in mandarin voles. *Horm. Behav.* 65, 57–65. doi: 10.1016/j.yhbeh.2013.11.005

Carter, C. S., Getz, L. L., Gavish, L., McDermott, J. L., and Arnold, P. (1980). Male related pheromones and the activation of female reproduction in the prairie vole (*Microtus ochrogaster*). *Biol. Reprod.* 23, 1038–1045. doi: 10.1095/biolreprod23.5.1038

Champagne, F., Diorio, J., Sharma, S., and Meaney, M. J. (2001). Naturally occurring variations in maternal behavior in the rat are associated with differences in estrogen-inducible central oxytocin receptors. *Proc. Natl. Acad. Sci. U S A* 98, 12736–12741. doi: 10.1073/pnas.221224598

Champagne, F. A., and Meaney, M. J. (2007). Transgenerational effects of social environment on variations in maternal care and behavioral response to novelty. *Behav. Neurosci.* 121, 1353–1363. doi: 10.1037/0735-7044.121.6.1353

Curtis, T. J. (2010). Does fertility trump monogamy? *Anim. Behav.* 80, 319–328. doi: 10.1016/j.anbehav.2010.05.014

Davis, A. N., Carlo, G., Gulseven, Z., Palermo, F., Lin, C. H., Nagel, S. C., et al. (2019). Exposure to environmental toxicants and young children's cognitive and social development. *Rev. Environ. Health* 34, 35–56. doi: 10.1515/reveh-2018-0045

DeMayo, M. M., Song, Y. J. C., Hickie, I. B., and Guastella, A. J. (2017). A review of the safety, efficacy and mechanisms of delivery of nasal oxytocin in children: therapeutic potential for autism and Prader-Willi syndrome and recommendations for future research. *Pediatr. Drugs* 19, 391–410. doi: 10.1007/s40272-017-0248-y

DeVries, A. C., and Carter, C. S. (1999) Sex differences in temporal parameters of partner preference in prairie voles (*Microtus ochrogaster*). *Can. J. Zool.* 77, 885–889. doi: 10.1139/z99-054

Dluzen, D. E., Ramirez, V. D., Carter, C. S., and Getz, L. L. (1981). Male vole urine changes luteinizing-hormone-releasing hormone and norepinephrine in female olfactory-bulb. *Science* 212, 573–575. doi: 10.1126/science.7010608

Ebitz, R. B., and Platt, M. L. (2014). An evolutionary perspective on the behavioral consequences of exogenous oxytocin application. *Front. Behav. Neurosci.* 7:225. doi: 10.3389/fnbeh.2013.00225

Feldman, R. (2015a). Sensitive periods in human social development: new insights from research on oxytocin, synchrony , high-risk parenting. *Dev. Psychopathol.* 27, 369–395. doi: 10.1017/s0954579415000048

Feldman, R. (2015b). The adaptive human parental brain: implications for children's social development. *Trends Neurosci.* 38, 387–399. doi: 10.1016/j.tins.2015.04.004

Francis, D. D., Champagne, F. C., and Meaney, M. J. (2000). Variations in maternal behaviour are associated with differences in oxytocin receptor levels in the rat. *J. Neuroendocrinol.* 12, 1145–1148. doi: 10.1046/j.1365-2826.2000.00599.x

Francis, D. D., Young, L. J., Meaney, M. J., and Insel, T. R. (2002). Naturally occurring differences in maternal care are associated with the expression of oxytocin and vasopressin (V1a) receptors: gender differences. *J. Neuroendocrinol.* 14, 349–353. doi: 10.1046/j.0007-1331.2002.00776.x

Getz, L. L., Carter, C. S., and Gavish, L. (1981). The mating system of the prairie vole, *Microtus ochrogaster*: field and laboratory evidence for pair-bonding. *Behav. Ecol. Sociobiol.* 8, 189–194. doi: 10.1007/bf00299829

Gilles, Y. D., and Polston, E. K. (2017). Effects of social deprivation on social and depressive-like behaviors and the numbers of oxytocin expressing neurons in rats. *Behav. Brain Res.* 328, 28–38. doi: 10.1016/j.bbr.2017.03.036

Gimpl, G., and Fahrenholz, F. (2001). The oxytocin receptor system: structure, function, and regulation. *Physiol. Rev.* 81, 629–683. doi: 10.1152/physrev.2001.81.2.629

Grippo, A. J., Cushing, B. S., and Carter, C. S. (2007a). Depression-like behavior and stressor-induced neuroendocrine activation in female prairie voles exposed to chronic social isolation. *Psychosom. Med.* 69, 149–157. doi: 10.1097/psy.0b013e31802f054b

Grippo, A. J., Gerena, D., Huang, J., Kumar, N., Shah, M., Ughreja, R., et al. (2007b). Social isolation induces behavioral and neuroendocrine disturbances relevant to depression in female and male prairie voles. *Psychoneuroendocrinology* 32, 966–980. doi: 10.1016/j.psyneuen.2007.07.004

Grippo, A. J., Pournajafi-Nazarloo, H., Sanzenbacher, L., Trahanas, D. M., McNeal, N., Clarke, D. A., et al. (2012). Peripheral oxytocin administration buffers autonomic but not behavioral responses to environmental stressors in isolated prairie voles. *Stress* 15, 149–161. doi: 10.3109/10253890.2011.605486

Grippo, A. J., Trahanas, D. M., Zimmerman, R. R. II., Porges, S. W., and Carter, C. S. (2009). Oxytocin protects against negative behavioral and autonomic consequences of long-term social isolation. *Psychoneuroendocrinology* 34, 1542–1553. doi: 10.1016/j.psyneuen.2009.05.017

Guoynes, C. D., Simmons, T. C., Downing, G. M., Jacob, S., Solomon, M., and Bales, K. L. (2018). Chronic intranasal oxytocin has dose-dependent effects on central oxytocin and vasopressin systems in prairie voles (*Microtus ochrogaster*). *Neuroscience* 369, 292–302. doi: 10.1016/j.neuroscience.2017.11.037

Kalinichev, M., Easterling, K. W., Plotsky, P. M., and Holtzman, S. G. (2002). Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. *Pharmacol. Biochem. Behav.* 73, 131–140. doi: 10.1016/s0091-3057(02)00781-5

Keech, B., Crowe, S., and Hocking, D. R. (2018). Intranasal oxytocin, social cognition and neurodevelopmental disorders: a meta-analysis. *Psychoneuroendocrinology* 87, 9–19. doi: 10.1016/j.psyneuen.2017.09.022

Leng, G., and Ludwig, M. (2016). Intranasal oxytocin: myths and delusions. *Biol. Psychiatry* 79, 243–250. doi: 10.1016/j.biopsych.2015.05.003

Liu, Y., and Wang, Z. X. (2003). Nucleus accumbens oxytocin and dopamine interact to regulate pair bond formation in female prairie voles. *Neuroscience* 121, 537–544. doi: 10.1016/s0306-4522(03)00555-4

Lukas, M., Bredewold, R., Neumann, I. D., and Veenema, A. H. (2010). Maternal separation interferes with developmental changes in brain vasopressin and oxytocin receptor binding in male rats. *Neuropharmacology* 58, 78–87. doi: 10.1016/j.neuropharm.2009.06.020

Neumann, I. D. (2008). Brain oxytocin: a key regulator of emotional and social behaviours in both females and males. *J. Neuroendocrinol.* 20, 858–865. doi: 10.1111/j.1365-2826.2008.01726.x

Neumann, I. D., Maloumby, R., Beiderbeck, D. I., Lukas, M., and Landgraf, R. (2013). Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. *Psychoneuroendocrinology* 38, 1985–1993. doi: 10.1016/j.psyneuen.2013.03.003

O'Connell, L. A., and Hofmann, H. A. (2011). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J. Comp. Neurol.* 519, 3599–3639. doi: 10.1002/cne.22735

Olazábal, D. E., and Young, L. J. (2006a). Oxytocin receptors in the nucleus accumbens facilitate "spontaneous" maternal behavior in adult female prairie voles. *Neuroscience* 141, 559–568. doi: 10.1016/j.neuroscience.2006.04.017

Olazábal, D. E., and Young, L. J. (2006b). Species and individual differences in juvenile female alloparental care are associated with oxytocin receptor density in the striatum and the lateral septum. *Horm. Behav.* 49, 681–687. doi: 10.1016/j.yhbeh.2005.12.010

Pan, Y. L., Liu, Y., Young, K. A., Zhang, Z. B., and Wang, Z. X. (2009). Post-weaning social isolation alters anxiety-related behavior and neurochemical gene expression in the brain of male prairie voles. *Neurosci. Lett.* 454, 67–71. doi: 10.1016/j.neulet.2009.02.064

Perry, A. N., Cater, C. S., and Cushing, B. S. (2016). Chronic social isolation enhances reproduction in the monogamous prairie vole (*Microtus ochrogaster*). *Psychoneuroendocrinology* 68, 20–28. doi: 10.1016/j.psyneuen.2016.02.016

Prounis, G. S., Foley, L., Rehman, A., and Ophir, A. G. (2015). Perinatal and juvenile social environments interact to shape cognitive behaviour and neural phenotype in prairie voles. *Proc. R. Soc. B Biol. Sci.* 282:20152236. doi: 10.1098/rspb.2015.2236

Prounis, G. S., Thomas, K., and Ophir, A. G. (2018). Developmental trajectories and influences of environmental complexity on oxytocin receptor and vasopressin 1A receptor expression in male and female prairie voles. *J. Comp. Neurol.* 526, 1820–1842. doi: 10.1002/cne.24450

Richmond, M. E., and Stehn, R. A. (1976). "Olfaction and reproductive behavior in microtine rodents," in *Mammalian Olfaction, Reproductive Processes and Behavior*, ed. R. L. Doty (New York, NY: Academic Press), 197–217.

Rilling, J. K., and Young, L. J. (2014). The biology of mammalian parenting and its effect on offspring social development. *Science* 345, 771–776. doi: 10.1126/science.1252723

Roberts, R. L., Miller, A. K., Taymans, S. E., and Carter, C. S. (1998). Role of social and endocrine factors in alloparental behavior of prairie voles (*Microtus ochrogaster*). *Can. J. Zool.* 76, 1862–1868. doi: 10.1139/cjz-76-10-1862

Taylor, S. E. (2006). Tend and befriend: biobehavioral bases of affiliation under stress. *Curr. Dir. Psychol. Sci.* 15, 273–277. doi: 10.1111/j.1467-8721.2006.00451.x

Thomas, J. A., and Birney, E. C. (1979). Parental care and mating system of the prairie vole, *Microtus ochrogaster*. *Behav. Ecol. Sociobiol.* 5, 171–186. doi: 10.1007/bf00293304

Trezza, V., Baarendse, P. J., and Vanderschuren, L. J. (2014). On the interaction between drugs of abuse and adolescent social behavior. *Psychopharmacology* 231, 1715–1729. doi: 10.1007/s00213-014-3471-z

Tzanoulinou, S., and Sandi, C. (2017). The programming of the social brain by stress during childhood and adolescence: from rodents to humans. *Curr. Top. Behav. Neurosci.* 30, 411–429. doi: 10.1007/7854_2015_430

Veenema, A. H. (2009). Early life stress, the development of aggression and neuroendocrine and neurobiological correlates: what can we learn from animal models? *Front. Neuroendocrinol.* 30, 497–518. doi: 10.1016/j.yfrne.2009.03.003

Vogel, A. R., Patisaul, H. B., Arambula, S. E., Tiezzi, F., and McGraw, L. A. (2018). Individual variation in social behaviours of male lab-reared prairie voles (*Microtus ochrogaster*) is non-heritable and weakly associated with V1aR density. *Sci. Rep.* 8:1396. doi: 10.1038/s41598-018-19737-9

Walum, H., and Young, L. J. (2018). The neural mechanisms and circuitry of the pair bond. *Nat. Rev. Neurosci.* 19, 643–654. doi: 10.1038/s41583-018-0072-6

Wang, Z. X., and Novak, M. A. (1992). Influence of the social-environment on parental behavior and pup development of meadow voles (*Microtus pennsylvannicus*) and prairie voles (*Microtus ochrogaster*). *J. Comp. Psychol.* 106, 163–171. doi: 10.1037/0735-7036.106.2.163

Wang, Z. X., and Novak, M. A. (1994). Alloparental care and the influence of father presence on juvenile prairie voles, *Microtus ochrogaster*. *Anim. Behav.* 47, 281–288. doi: 10.1006/anbe.1994.1040

Williams, J. R., Catania, K. C., and Carter, C. S. (1992). Development of partner preferences in female prairie voles (*Microtus ochrogaster*): the role of social and sexual experience. *Horm. Behav.* 26, 339–349. doi: 10.1016/0018-506x(92)90004-f

Wongwitdecha, N., and Marsden, C. A. (1996). Social isolation increases aggressive behaviour and alters the effects of diazepam in the rat social interaction test. *Behav. Brain Res.* 75, 27–32. doi: 10.1016/0166-4328(96)00181-7

Young, L. J. (2013). When too much of a good thing is bad: chronic oxytocin, development, and social impairments. *Biol. Psychiatry* 74, 160–161. doi: 10.1016/j.biopsych.2013.05.015

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

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ORIGINAL RESEARCH
published: 26 September 2019
doi: 10.3389/fnbeh.2019.00229

Fighting Assessment Triggers Rapid Changes in Activity of the Brain Social Decision-Making Network of Cichlid Fish

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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: *20 June 2019*
Accepted: *12 September 2019*
Published: *26 September 2019*

Citation:
Almeida O, Félix AS, Oliveira GA, Lopes JS and Oliveira RF (2019) Fighting Assessment Triggers Rapid Changes in Activity of the Brain Social Decision-Making Network of Cichlid Fish. Front. Behav. Neurosci. 13:229. doi: 10.3389/fnbeh.2019.00229

Social living animals have to adjust their behavior to rapid changes in the social environment. It has been hypothesized that the expression of social behavior is better explained by the activity pattern of a diffuse social decision-making network (SDMN) in the brain than by the activity of a single brain region. In this study, we tested the hypothesis that it is the assessment that individuals make of the outcome of the fights, rather than the expression of aggressive behavior *per se*, that triggers changes in the pattern of activation of the SDMN which are reflected in socially driven behavioral profiles (e.g., dominant vs. subordinate specific behaviors). For this purpose, we manipulated the perception of the outcome of an agonistic interaction in an African cichlid fish (*Oreochromis mossambicus*) and assessed if either the perception of outcome or fighting by itself was sufficient to trigger rapid changes in the activity of the SDMN. We have used the expression of immediate early genes (*c-fos* and *egr-1*) as a proxy to measure the neuronal activity in the brain. Fish fought their own image on a mirror for 15 min after which they were allocated to one of three conditions for the two last minutes of the trial: (1) they remained fighting the mirror image (no outcome treatment); (2) the mirror was lifted and a dominant male that had just won a fight was presented behind a transparent partition (perception of defeat treatment); and (3) the mirror was lifted and a subordinate male that had just lost a fight was presented behind a transparent partition (perception of victory treatment). Results show that these short-term social interactions elicit distinct patterns in the SDMN and that the perception of the outcome was not a necessary condition to trigger a SDMN response as evidenced in the second treatment (perception of defeat treatment). We suggest that the mutual assessment of relative fighting behavior drives these acute changes in the state of the SDMN.

Keywords: social decision making network, social competence, immediate early genes, androgens, challenge hypothesis

INTRODUCTION

Individuals from social species need to combine information about the social environment they live in with information about their internal state, such as previous social experience and organismal condition, in order to adaptively optimize their responses to changes in the social environment (Taborsky and Oliveira, 2012). This ability to rapidly and adaptively adjust behavior to daily social demands is known as social competence and is thought to be accomplished through rapid changes in the state of the neural network underlying social behavior (Oliveira, 2012). Accordingly, consistent changes in social behavior, such as adopting a dominant or subordinate behavioral profile, are associated with distinct behavioral states (that express different behavioral patterns) that are paralleled by specific states of the social-decision making network (SDMN) in the brain (Cardoso et al., 2015). The SDMN consists of an evolutionarily conserved set of core brain nuclei that together regulate the expression social behavior, such that the state of the network better explains the behavioral output rather than the activity of a single node *per se* (Goodson, 2005; Newman, 1999; O'Connell and Hofmann, 2011b, 2012). All of these brain nuclei are reciprocally interconnected with each other, such that differential activation of the nodes creates dynamic patterns responsible for multiple behaviors. Moreover, the nodes of the SDMN have an extensive expression of steroid neuropeptide and aminergic receptors, which allows this network to be modulated by these hormones, probably by altering the weight of its nodes or the strength of their connectivity (Goodson, 2005; O'Connell et al., 2011; O'Connell and Hofmann, 2011a; Oliveira, 2012). Thus, different behavioral states should result from divergent transcriptomes of the SDMN, and changes between states, such as acquiring or losing social status should be associated with rapid changes in patterns of gene expression in the SDMN. Given their fast and transient response to changes in extra- and intra-cellular environment and their effect as transcription factors, immediate early genes (e.g., *c-fos, egr-1*) play a key role in orchestrating transcriptomic responses to environmental changes. Thus, it has been hypothesized that immediate early genes can be the molecular first responders to perceived changes in the social environment that trigger subsequent changes in the neurogenomic state of the SDMN that allows the animal to adjust its behavioral state accordingly (Cardoso et al., 2015). Several studies have documented changes in immediate early gene (IEG) expression across the SDMN associated with changes in social behavior across different vertebrate taxa (e.g., Faykoo-Martinez et al., 2018; Kabelik et al., 2018; O'Connell and Hofmann, 2012), including teleost fish and also tilapia (e.g., Field and Maruska, 2017; Roleira et al., 2017; Teles et al., 2015). In particular, changes in social status (i.e., ascending or descending in a social hierarchy) have been associated with rapid changes in IEG expression in the SDMN paralleled by changes in social behavior (Maruska et al., 2013a,b; Teles et al., 2015; Williamson et al., 2019).

In this study, we sought to understand what are the key aspects of an agonistic interaction that trigger an IEG response across the SDMN and concomitantly a socially driven neuromolecular restructuring of this network. We reasoned that in order to be adaptive such network restructuring should match the post-fight social scenario anticipated by the individual in face of the information collected during the interaction. Therefore, the perception of the fight outcome rather than the expression of aggressive behavior *per se* should play a key role in triggering the SDMN IEG response to an aggressive interaction. Here, we have tested if the perception of the outcome of a single agonistic interaction in an African cichlid fish (*Mozambique tilapia*, *Oreochromis mossambicus*) is necessary to trigger an IEG response across the SDMN or if fighting itself is sufficient to trigger the response.

In order to manipulate the perception of fight outcome, we took advantage of the fact that male Tilapia do not recognize their own image in a mirror and fight aggressively towards it (e.g., Oliveira et al., 2005; Teles et al., 2013). Because in mirror fights the opponent's behavior (i.e., mirror image) always matches the behavior of the focal fish, there is no information available to the participant regarding the fight outcome. That is the males express aggressive behavior without experiencing either a win or a defeat. Thus, an IEG response triggered by a mirror fight would be driven by the experience of fighting and not by the perception of the interaction outcome (i.e., winning vs. losing). In this study, we have used three fighting treatments. After a mirror fighting phase that lasted 15 min focal males were allocated to one of three conditions for the last 2 min of the trial: (1) they remained fighting their mirror image (no outcome treatment, where the mirror image remained in both steps of the experiment; MM); (2) the mirror was lifted and a dominant male that had just won a fight was presented behind a transparent partition (opponent becoming dominant treatment, where the mirror image became dominant male; MD); and (3) the mirror was lifted and a subordinate male that had just lost a fight was presented behind a transparent partition (opponent becoming subordinate treatment, where the mirror image became a subordinate male; MS). Our prediction was that if the IEG response is challenge dependent, then all three treatments would trigger a similar IEG response; in contrast, if IEG responsiveness is dependent on perceiving a win or a defeat, divergent IEG responses across the SDMN are expected in the MD and MS treatments in relation to the mirror fights treatment (MM) where no information on outcome is available. Given that socially-driven changes in the SDMN are expected to produce integrated phenotypic responses, at the behavioral and physiological (hormonal) levels, to the social environment and that androgens have been described to respond to social challenges (challenge hypothesis, Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990), we have also characterized the response of the hypothalamic-pituitary-gonadal (HPG) axis to our experimental treatments by measuring the expression of gonadotrophin-releasing hormone (*gnrh1*) in the preoptic area and circulating androgen levels (testosterone, T, and 11-ketotestosterone, KT).

MATERIALS AND METHODS

Animals and Housing

The *Mozambique tilapia* is a freshwater fish with a lek-breeding system (Fryer and Iles, 1972). Males aggregate densely in mating territories, where they dig and defend spawning pits and compete for females (Oliveira and Almada, 1998). Males present two distinct phenotypes, which can rapidly reverse due to changes in the social environment (Oliveira and Almada, 1998). Dominant males are usually larger, dark-colored, establish territories and attract females. In contrast, subordinate males have a silver color pattern similar to that of females, and fail to establish territories.

O. mossambicus fish from a stock held at ISPA was used in this study. Fish were maintained in stable social groups of four males and five females per group, in glass tanks (120 × 40 × 50 cm, 240 L) with a fine gravel substrate. Tanks were supplied with a double filtering system (sand and external biofilter; Eheim) and constant aeration. Water quality was monitored on a weekly basis for nitrite (0.2–0.5 ppm), ammonia (<0.5 ppm; Pallintest kit) and pH (6.0–6.2). Fish were kept at a temperature of 26 ± 2°C, a 12L:12D photoperiod, and fed with commercial cichlid sticks. The social status of the males was monitored daily and territorial males were identified by dark body coloration and digging of a spawning pit on the substrate (Oliveira and Almada, 1996).

Experimental Procedure

The experimental setup consisted of two adjacent tanks (test and demo tank) with an opaque partition between them. Twenty territorial focal males (mean body mass ± SEM: 81.63 g ± 7.06 g) were used in this experiment. Each focal male was isolated for 7 days in the test tank (30 × 50 × 25 cm). On day 6, plasma was collected from the focal male to determine steroids baseline levels. On the same day, a male fish was introduced in the demo tank (30 ×70 × 40 cm), to allow it to adopt this tank as its territory. On the day of the experiment (day 7), an intruder male was introduced in the demo tank and both males were allowed to interact for 30 min. This agonistic interaction was accompanied by the experimenter and fight outcome was assessed by live observation. Accordingly, after fight resolution, winners continue to be aggressive and present a dark coloration while losers only display submissive behavior and present a light coloration. Thus, winners can be seen as clear/explicit dominant males (recently gaining social status) and losers as clear subordinate males (recently losing social status). Fifteen minutes after the beginning of the social interaction in the demo tank, a mirror was placed in the external wall of the test tank, adjacent to the demo tank. The interaction between the mirror and the focal male in the test tank was recorded for 15 min. At the end of the mirror interaction, males in the demo tank were separated by an opaque partition and the focal male in the test tank was allowed to see for 2 min one of the following stimuli: (i) its own image in the mirror (MM treatment, $N = 8$), or a real (opponent) male, either; (ii) the dominant male of the demo tank (Mirror becomes Dominant—MD treatment, $N = 6$); or (iii) the subordinate male of the demo tank (Mirror becomes Subordinate—MS treatment, $N = 6$; **Figure 1**). Fight outcome was manipulated by controlling the order of introduction of each fish in the demo tank and their size, so the male introduced first (in day 6) was always bigger than the intruder and won all staged fights. Using this procedure, we had no unsolved fights. Focal and opponent males were sized matched and were selected from different family tanks to control for familiarity effects. At the end of the experiment, an opaque partition was placed between the tanks to prevent the males from seeing each other and 20 min later a blood sample was collected from the caudal vein under anesthesia (MS-222, Pharmaq; 300–400 ppm). Blood sampling always took less than 4 min from the induction of anesthesia to prevent possible effects of handling stress on steroids levels (Foo and Lam, 1993). Blood samples were centrifuged (10 min, 600 *g*) and plasma was stored at −20°C until further processing. After blood sampling, the fish were returned to the anesthesia solution until muscular and opercular movements stopped completely and were then sacrificed by decapitation. The cranial fraction (brain and part of the cranial bones) was embedded in mounting media (OCT Compound, Tissue-Tek, Sakura) and frozen at −80°C during 15–30 min. Coronal sections were obtained at 150 μm thickness using a cryostat (Microm HM 500 M) and collected on previously cleaned slides (70% ethanol). Regions of interest were microdissected under a steromicroscope (VWR SZB350OH) and collected in 50 μl of Qiazol lysis buffer (RNeasy Lipid Tissue Mini Kit, Qiagen) with a modified 25G needle. Samples were stored at −80°C until RNA extraction. The following representative nodes of the SDMN (O'Connell and Hofmann, 2011b) were identified according to Teles et al. (2012): medial part of the ventral subdivision of the ventral telencephalon (VVm; putative homolog of the mammalian lateral septum), supracommissural part of the ventral telencephalon (Vs; putative homolog of the mammalian medial extended amygdala), anterior part of the periventricular preoptic nucleus (PPa), nucleus anterior tuberis (TA; putative homolog of the ventromedial hypothalamus) and central gray (GC).

Behavioral Observations

The behavior of the focal male, either towards the mirror or interacting with the opponent male, was analyzed using a computerized multi-event recorder software (Observer, Noldus technology, version 5). The behavior of the opponent male was also analyzed with the same software (see **Supplementary Figure S1** for the descriptive statistics of focal and opponent behavioral measures). The analysis was based on the ethogram repertoire provided by Baerends and Baerends-Van Roon (1950). Relevant behavioral patterns were identified to measure male aggressive behavior (i.e., bites, displays, attacks).

Gene Expression Analysis

Primers were designed using National Center for Biotechnology Information (NCBI) sequences for *c-fos* (accession #GR607679.1), *egr-1* (accession #AY493348.1), *gnrh1* (accession #AB101665.1) and the housekeeping gene *eef1A* (accession #AB075952.1). Primer3 software (Koressaar and Remm, 2007; Untergasser et al., 2012) was used to design the primers, which were commercially synthesized (Sigma-Aldrich, Hamburg, German). Primers were tested with a cDNA pool in a qRT-PCR, and PCR products were confirmed by sequencing.

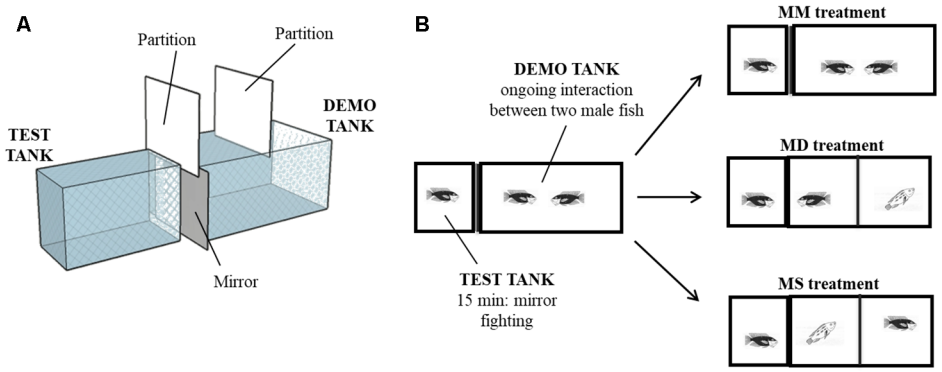

FIGURE 1 | Behavioral paradigm. **(A)** 3D diagram of the experimental setup. Test tank and demo tank were side-by-side and physically separated. **(B)** Schematic of the experimental treatments. Focal fish interacted with a mirror for 15 min while two males were fighting in the adjacent compartment. Then, focal fish were allowed to see for 2 min its own image in the mirror (MM treatment), a dominant male (Mirror becomes Dominant—MD treatment) or a subordinate male (Mirror becomes Subordinate—MS treatment).

Amplification products were 106 pb for *c-fos*, 135 pb for *egr-1*, 127 pb for *gnrh1* and 85 pb for *eef1A*. Primer dimer formation was controlled with FastPCR v5.4 software (Kalendar et al., 2017) and optimal annealing temperature was assessed for maximal fluorescence (**Supplementary Table S1**). qRT- PCR was performed using the Quantitative PCR System Stratagene MX3000P. The reaction mix included Sybr Green (Fermentas, #K0221), 400 nM of each primer and 1 μl of cDNA in a 25 μl reaction volume. Cycling parameters were: (i) denaturation: 5 min at 95°C; (ii) amplification and quantification: 40 cycles (30 s at 95°C, 30 s at primer-specific annealing temperature, 30 s at 72°C); and (iii) dissociation curve assessment (30 s at 95°C, 30 s at 55°C, 30 s at 95°C). The dissociation curve was performed to confirm a single melting curve proving the inexistence of primer-dimer formation and/or plate contamination. All samples were run in triplicate and controls with water instead of DNA templates showed no amplification. PCR Miner (Zhao and Fernald, 2005) was used to calculate reaction efficiencies (E) and cycle thresholds (CT), based on the kinetics of individual PCR reactions. *c-fos*, *egr-1* and *gnrh1* mRNA levels normalized for housekeeping (HK) gene *eef1A* were determined from the equation: $(1 + E_{HK})^{CT_{HK}}/(1 + E_{\text{gene}})^{CT_{\text{gene}}}$. Mean values for eef1A did not differ between treatments, thus confirming its suitability to be used as a reference gene in this study.

Quantification of Steroids Levels

Free steroids (testosterone, T; and 11-ketotestosterone, KT) were extracted from plasma samples by adding diethyl-ether to the samples, centrifuging the mix (800 *g*, 5 min, 4°C) and freezing it (15 min, −80°C) to separate the ether fraction (containing the free steroid). This process was repeated twice. The ether fraction was evaporated and the steroids were re-suspended in phosphate buffer. Steroid concentrations were measured by radioimmunoassay. The testosterone antibody was from Research Diagnostics Incorporation (#WLI-T3003, rabbit anti-testosterone) and the 11-ketotestosterone antibody was kindly donated by D. E. Kime (the specificity table was published in Kime and Manning, 1982). We used a testosterone reactive marker from Amersham Biosciences [(1, 2, 6, 7–3H) testosterone, #TRK402-250 μCi] and a titrated 11-ketotestosterone produced in-house from marked cortisol (Kime and Manning, 1982). Inter-assay variabilities were 4.1% and 8.9% for T and KT, respectively. Intra-assay variation coefficients were 2.4% and 2.0% for T and 4.1% and 4.0% for KT.

Data Analysis

Outlier observations were identified and replaced by missing values using a generalized extreme studentized deviate procedure (e.g., Jain, 2010) with a *p*-value of 0.05 and a maximum number of outliers set at 20% of the sample size. Behavioral variables and gene expression levels were logarithmically transformed [log10 (x + 1)] to meet parametric test assumptions. The behavioral variables (for frequency and latency) were reduced with Principal Component Analysis (PCA) using the variable principle normalization method. Two principal components (PC) were obtained that explain 86.3% of the variance and that seem to represent different aspects of aggressive behavior: "overt aggression" and "aggressive motivation" (see "Results" section). The component scores of each case on each of these PC were analyzed using separate Linear Mixed Models (LMM) with Treatment (MM, MD, MS) as a fixed effect and focal fish as a random effect. *Post hoc* tests were used to test for differences between experimental treatments, with *p*-values adjusted for the number of multiple comparisons (Benjamini and Hochberg, 1995).

Separate LMM were also used to check for differences between treatments in IEG (*c-fos, egr-1*) expression in each sampled brain area (GC, TA, Vs, VVm, PPa). *Post hoc* tests were used to test for differences between experimental treatments, with *p*-values adjusted for the number of multiple comparisons (Benjamini and Hochberg, 1995).

Pearson correlations between IEG expression of each brain area and between the behavioral principal component score were used to examine the association between aggressive behavior and gene expression. Pearson correlation matrices between each pair

of brain nuclei for each IEG were used as a measure of functional connectivity and tested using a Quadratic Assignment Procedure (QAP) with 5,000 permutations. Since the null-hypothesis for QAP states that there is a non-random association between the tested matrices, a QAP with a non-significant *p*-value indicates that there is no association between the treatment's IEG activational pattern. The *p*-values of the Pearson correlation matrices were adjusted (Benjamini and Hochberg, 1995). The brain patterns of IEG expression obtained for each experimental treatment were tested on a network perspective, by measuring density and centrality parameters (Makagon et al., 2012). Density was used as a measure of the network cohesion, given by the proportion of all possible connections that are present in the network (Makagon et al., 2012). Differences in network density between treatments were tested using a *t*-test (bootstrap set to 5,000 sub-samples). As a measure of node centrality we assessed eigenvector centrality, that takes into account not only how well a node is connected to other nodes in the network but also how well connected its relations are (Makagon et al., 2012).

Variation in hormone levels (KT, T) was computed as (Post-treatment levels) − (Baseline levels) for each individual. To test for differences between the treatments we performed unpaired *t*-tests. Pearson correlation analysis was used to examine the relationship between *gnrh1* gene expression and IEG expression in the PPa. Pearson correlation analysis was also used to examine the relationship between *gnrh1* gene expression in the PPa and androgen circulating levels. A LMM was used to test for differences between treatments in *gnrh1* in the PPa area. *Post hoc* tests were used to test for differences between experimental treatments, with *p*-values adjusted for the number of multiple comparisons (Benjamini and Hochberg, 1995).

Effect sizes were computed for *post hoc* tests (Cohen's *d*).

Statistical analysis was performed using IBM SPSS® statistics v.21, and R (R Core Team, 2015) with the following packages: nlme (LMM), dplyr (*t*-tests), multcomp (*post hoc* comparisons), Hmisc (correlations), ggplots (heatmaps). Characterization of the SDMN network was obtained with UCINET version 6.653 (Borgatti et al., 2002). Brain nuclei representations of the SDMN network were produced using a custom-made python script. Degrees of freedom may vary between the analyses due to missing values.

Ethics Statement

In this study, we have staged real opponent agonistic interactions to obtain winner and loser animals, since the use of video-playbacks in this species is inadequate (RO, personal observation). However, we have kept sample sizes to a minimum, and limited contests to a short duration. No signs of physical injuries were observed during any of the trials. Animal experimentation procedures were conducted in accordance with the European Communities Council Directive of 24 November 1986(86/609/EEC) and were approved by the Portuguese Veterinary Authority (Direcção Geral de Alimentação e Veterinária, Portugal; permit # 0421/000/000/2013).

TABLE 1 | Principal component analysis (PCA) of behavioral variables.

Behavioral variables	Component loading	
	PC1	PC2
Frequency of displays	0.793	−0.443
Frequency of bites	0.915	−0.161
Frequency of attacks	0.923	0.122
Latency to display	−0.595	0.717
Latency to bite	−0.887	−0.293
Latency to attack	−0.896	−0.287
Eigenvalue	4.262	0.919
% of variance explained	71.03	15.32

RESULTS

Behavior

A PCA of the behavioral variables resulted in two PC that together explained 86.3% of the variance in aggressive behavior (**Table 1**). PC1 had a high loading (>0.9) of frequency of bites and frequency of attacks, and hence it was interpreted as "overt aggression." The highest loading in PC2 was the latency to display, and hence its symmetric was interpreted as "aggressive motivation."

There was an effect of the experimental treatment in "overt aggression" (i.e., PC1 loadings; $F_{(2,17)} = 4.87$, $p = 0.02$), with focal fish assigned to the MS condition showing significantly less overt aggression than those in the MM and MD conditions (**Figure 2A**). In contrast there was no effect of experimental treatment on "aggressive motivation" (PC2 loadings; $F_{(2,17)} = 0.50$, $p = 0.62$; **Figure 2B**).

Immediate Early Gene Expression in the Social Decision-Making Network (SDMN)

Significant differences between treatments were only detected for *c-fos* in the TA area, specifically between the MM and the MS treatments (**Figure 3**; **Table 2**). No other significant main effect or *post hoc* comparison was detected for *c-fos* or *egr-1*.

No significant association between the correlation matrices for *c-fos* and *egr-1* expression in the brain areas of the SDMN was detected using QAP, suggesting that all treatments showed a distinct co-activation pattern for *c-fos* and *egr-1* (**Table 3**, **Supplementary Figure S2**). Thus, the pattern of functional connectivity across the SDMN is specific for each treatment. The density of the *egr-1* network was significantly higher for fish assigned to the MS treatment when compared to the MM and MD treatments (MM vs. MS: $t = 2.815$, $p = 0.005$; MD vs. MS: $t = 2.061$, $p = 0.037$; **Table 4**). The *egr-1* network density for MM and MD treatments was not significantly different (MM vs. MD: $t = 1.488$, $p = 0.137$). We have not detected significant differences between treatments for *c-fos* network density (MM vs. MD: $t = 1.861$, $p = 0.065$; MM vs. MS: $t = 0.461$, $p = 0.607$; MD vs. MS: $t = 1.588$, $p = 0.125$). The eigenvector centrality measures suggest that GC is a central node in the *c-fos* and *egr-1* networks for fish in the MM and MS treatments, but that it is a poorly connected node in the MD treatment (**Table 4**). The eigenvector centrality measures show that the MD and MS treatment networks are characterized by

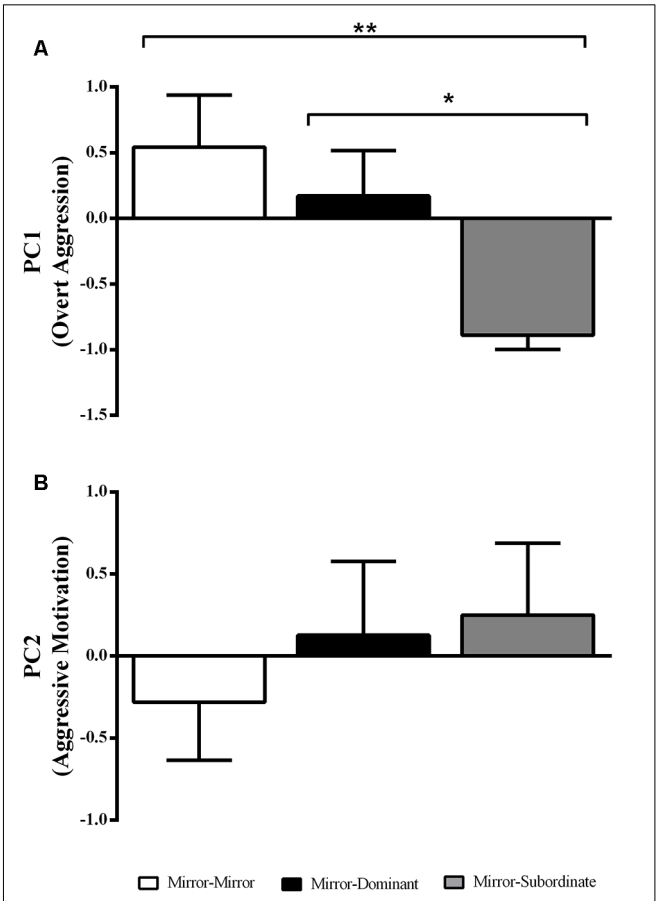

FIGURE 2 | Variation in the behavioral component scores obtained with the Principal Component Analysis (PCA) for each experimental treatment. **(A)** PC1 interpreted as "overt aggression"; and **(B)** PC2 interpreted as "aggressive motivation." *Significant difference for $p < 0.05$; **significant difference for $p < 0.01$. Results are expressed as mean ± standard error of the mean (SEM).

a high centrality of the PPa node for *egr-1* (**Table 4**). Centrality measures of the *egr-1* network for fish in the MM treatment show a high centrality for TA and a low centrality for PPa (**Table 4**).

There were no significant correlations between *c-fos* or *egr-1* expression in brain areas of the SDMN and aggressive behavior (**Figure 4**).

Activity of the Hypothalamic-Pituitary-Gonadal (HPG) Axis

There were no significant correlations between the neuronal activation of the PPa as measured by either *c-fos* or *egr-1* and the expression of gnrh1 in the PPa or circulating androgen levels (*c-fos*: $r = 0.170$, $p = 0.499$, $n = 18$; *egr-1*: $r = 0.107$, $p = 0.673$, $n = 18$). There were also no significant correlations between the expression of *gnrh1* in the PPa and circulating androgen levels (KT: $r = 0.276$, $p = 0.283$, $n = 17$; T: $r = 0.371$, $p = 0.143$, $n = 17$).

Furthermore, there were no differences between treatments either in *gnrh1* expression in the PPa ($F_{(2,16)} = 0.407$, $p = 0.672$; MM vs. MD: $t_{(16)} = 0.380$, $p = 0.704$, $d = 0.020$; MM vs. MS: $t_{(16)} = 0.903$, $p = 0.704$, $d = 0.053$; MD vs. MS: $t_{(16)} = 0.447$, $p = 0.704$, $d = 0.024$), or in the androgen response to the behavioral treatment (KT: MM vs. MD: $t_{(12)} = -0.644$, $p = 0.532$, $d = 0.041$; MM vs. MS: $t_{(12)} = -0.905$, $p = 0.383$, $d = 0.034$; MD vs. MS: $t_{(10)} = -0.441$, $p = 0.669$, $d = 0.006$; T: MM vs. MD: $t_{(10)} = -0.984$, $p = 0.348$, $d = 0.306$; MM vs. MS: $t_{(11)} = -0.377$, $p = 0.714$, $d = 0.034$; MD vs. MS: $t_{(9)} = 0.978$, $p = 0.353$, $d = 0.006$; **Figure 5**).

DISCUSSION

Contrary to our predictions, fish assigned to the MM and the MD treatments showed similar behavioral patterns, that is, they equally fought aggressively their opponents, suggesting that the focal fish of the MD condition did not interpret a recently winning male as having a higher social status than itself, i.e., fish did not perceive the MD interaction as a defeat. In this context, it seems plausible that the visual signal presented was insufficient *per se* to communicate higher status, originating an agonistic interaction that, like the MM, was also unsolved, either because of the short interaction time allowed (only 2 min) or because of the symmetry of the fight. A study in another cichlid fish has shown that males previously interacting with a mirror have a higher probability to win a fight than non-mirror stimulated control individuals, probably because of an enhanced aggressive motivation (Dijkstra et al., 2012). On the other hand, the opponent fish had just won a fight, which is known to induce motivational changes that lead to the winner effect (Oliveira et al., 2009). Thus, it seems plausible that the behavior of the MD opponent was paralleled by that of the focal fish due to the heightened motivation of both contestants. In the case of the MS treatment, the losing experience of the opponent leads to a decrease in the willingness to engage in another contest (Hsu et al., 2006). So, it is plausible that the focal fish interpreted the interaction outcome as a win since they performed aggressive displays towards the subordinate opponent male first, which replied much later. Thus, due to a lack of an aggressive motivation by the opponent the focal fish did not further escalate its aggressive behavior (no attacks or bites), hence avoiding extra energetic costs (Hsu et al., 2011). Thus, at least for the MD condition, the experimental treatment may not have effectively altered the focal fish's perception of the outcome, yet fish seem to constantly monitor the social interaction and adjust their behavior according to their internal state and to the behavior of their opponent. The ability of fish to compare their behavior with the one of the opponents and assess their competitive ability (mutual assessment) has few support in the literature (Hsu et al., 2011) but our data suggest its involvement. Of course, future experiments are necessary to fully uncover the underlying cognitive mechanisms.

In the present study, we showed that the pattern of expression of immediate early genes across the SDMN responds

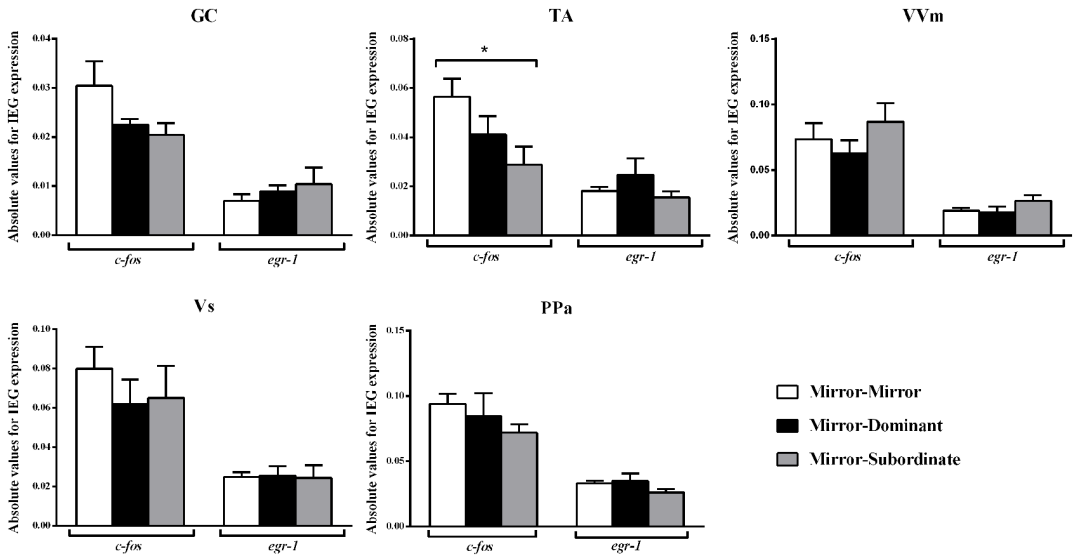

FIGURE 3 | Expression of the immediate early genes (IEG) *c-fos* and *egr-1* in several brain areas of the social decision-making network (SDMN). GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. *Significant difference for $p < 0.05$. Results are expressed as mean ± standard error of the mean (SEM).

TABLE 2 | Effect of treatment on immediate early genes expression in social decision-making network (SDMN) areas.

	Main effects		MM vs. MD			MM vs. MS			MD vs. MS		
Areas	***F***	***p***	***t***	***p***	***d***	***t***	***p***	***d***	***t***	***p***	***d***
c-fos											
VVm	0.816	0.462	0.550	0.583	0.031	0.820	0.583	0.050	1.268	0.583	0.086
Vs	0.821	0.458	1.004	0.473	0.072	1.170	0.473	0.061	0.160	0.873	0.008
TA	3.839	**0.042**	1.250	0.211	0.081	2.770	**0.017**	0.140	1.421	0.211	0.069
GC	0.426	0.663	0.910	0.363	0.091	0.319	0.750	0.017	0.591	0.555	0.036
PPa	0.970	0.400	1.027	0.457	0.047	1.277	0.457	0.119	0.286	0.775	0.016
egr-1											
VVm	1.528	0.247	0.675	0.500	0.038	1.119	0.395	0.070	1.729	0.252	0.087
Vs	0.156	0.857	0.166	0.868	0.010	0.552	0.868	0.030	0.362	0.868	0.018
TA	1.176	0.333	0.808	0.419	0.040	0.831	0.419	0.057	0.1.533	0.376	0.074
GC	0.918	0.419	1.094	0.411	0.066	1.174	0.411	0.059	0.130	0.897	0.008
PPa	1.705	0.213	0.078	0.938	0.004	1.600	0.164	0.109	1.618	0.164	0.081

Main effects and post hoc comparisons between treatments. d: effect size estimate (Cohen's d); Treatments: MM, Mirror-Mirror; MD, Mirror-Dominant; MS, Mirror-Subordinate; GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon; c-fos degrees of freedom for F-test: GC: (2, 12); PPa: (2, 16); TA: (2, 17); VVm: (2, 14); Vs: (2, 16); egr-1 degrees of freedom for F-test: GC: (2, 16); PPa: (2, 16); TA: (2, 17); VVm: (2, 16); Vs: (2, 17); statistically significant values are in bold.

TABLE 3 | Association between the correlation matrices for immediate early gene (IEG) expression in the brain areas of the SDMN.

		MM		MD	
		r	***p***	***r***	***p***
c-fos	MS	−0.202	0.291	−0.119	0.409
	MD	0.148	0.367		
egr-1	MS	−0.222	0.259	−0.134	0.501
	MD	−0.489	0.189		

Quadratic assignment procedure (QAP) for c-fos and egr-1 co-activation matrices. Treatments: MM, Mirror-Mirror; MD, Mirror-Dominant; MS, Mirror-Subordinate.

to acute changes in social interactions. Only 2 min of exposure to different fight outcomes (i.e., MD vs. MS) of an interaction that was already going on for 15 min was sufficient to trigger different patterns of *c-fos* and *egr-1* expression. Given the pivotal role of these immediate early genes in orchestrating integrated transcriptome changes (Clayton, 2000), these short-term responses of *c-fos* and *egr-1* to acute changes in the perceived dynamics of the interaction suggest that the neurogenomic state of the SDMN can change rapidly in response to perceived social interactions.

Our results also confirm the hypothesis, that the expression of social behavior is better explained by the overall pattern of activation of the SDMN rather than by the activity of a specific region in the brain (e.g., a specific node of the network; Teles et al., 2015). Indeed, there were no significant correlations between the expression of any of the immediate early genes

TABLE 4 | Characterization of the SDMN for each experimental treatment using *c-fos* and *egr-1* as reporters of neuronal activity.

		c-fos			*egr-1*		
		MM	**MD**	**MS**	**MM**	**MD**	**MS**
Density		0.559	0.360	0.535	0.243	0.391	0.553
eigenvector	GC	0.550	0.175	0.565	0.532	0.459	0.542
	PPa	0.408	0.579	0.382	0.127	0.576	0.518
	TA	0.455	0.264	0.398	0.644	0.374	0.380
	VVm	0.456	0.523	0.375	0.454	0.188	0.444
	Vs	0.342	0.538	0.486	0.282	0.532	0.310

Values reported correspond to network cohesion (density) and centrality (eigenvector) of each node of the network. Treatments: MM, Mirror-Mirror; MD, Mirror-Dominant; MS, Mirror-Subordinate; GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon.

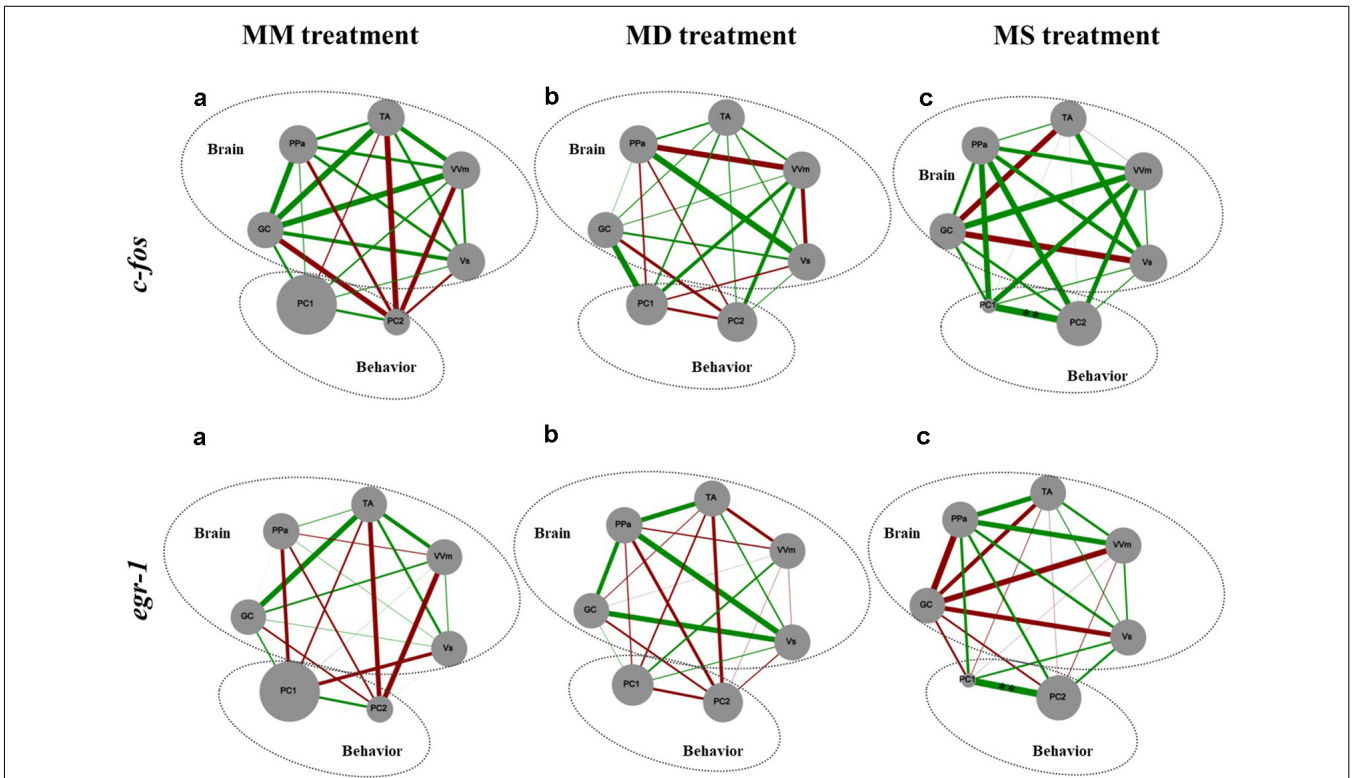

FIGURE 4 | Representation of the state of the SDMN and the behavior for all the experimental treatments. Node size of each brain area indicates the activity level at each network node using *c-fos* and *egr-1* as reporters of neural activity. PC1 and PC2, component loadings obtained with the PCA of aggressive behavior were used as behavioral network nodes, where the node size corresponds to the average of principal component scores within each treatment. Line thickness indicates the strength of the connection between nodes (measured with Pearson correlation coefficients, *r*-value); green lines represent positive correlations; red lines represent negative correlations. GC, central gray; PPa, anterior part of the periventricular preoptic nucleus; TA, nucleus anterior tuberis; VVm, medial part of the ventral subdivision of the ventral telencephalon; Vs, supracommissural nucleus of the ventral telencephalon. PC1, first component loading interpreted as "overt aggression"; PC2, second component loading interpreted as "aggressive motivation." **Significant correlations after *p*-value adjustment for $p < 0.01$.

tested and the expression of aggressive behavior. In contrast, the correlation matrices for the expression of each IEG across the nodes of the SDMN, which capture the co-activation or reciprocal inhibition between brain regions, were specific for each experimental treatment. Moreover, only the expression of *c-fos* in the TA was significantly different between experimental treatments (i.e., MM and MS treatments). The TA is the putative homolog of the ventromedial hypothalamus in mammals, and its ventrolateral subdivision has been strongly associated with aggression. For instance, pharmacogenetic inactivation of this area in mice stops inter-male aggressive behavior while optogenetic activation induces attacks towards females or inanimate objects (Lin et al., 2011). Other study analyzed the *c-fos* expression in the brain of subordinate hamsters after a fight and detected elevated activation in several areas including the lateral part of the ventromedial hypothalamus in comparison with dominant males (Kollack-Walker et al., 1997). In a recent review, Hashikawa et al. (2017) proposed the

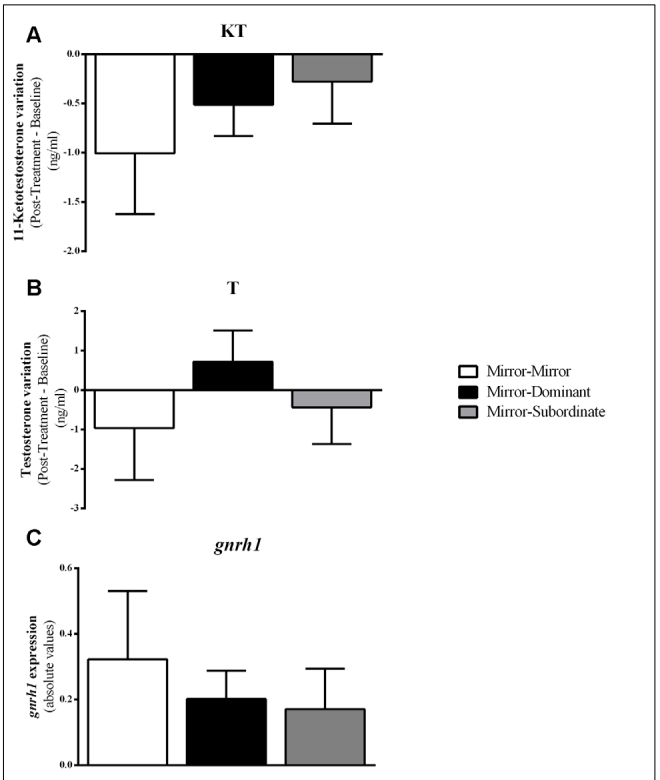

FIGURE 5 | Variation in androgen levels and expression of gnrh1 in the Ppa of the focal fish for each experimental condition. **(A)** 11-Ketotestosterone (KT) levels; **(B)** Testosterone (T) levels; **(C)** *gnrh1* expression. Results are expressed as mean ± standard error of the mean (SEM).

involvement of this particular sub-nucleus in the following aspects of aggression: aggressive motivation, specifically that the activation of this area heightens aggressive state (Falkner and Lin, 2014); detection of aggressive signals, such as for example olfactory cues (Falkner and Lin, 2014; Lin et al., 2011); and in the start and execution of aggressive behavioral patterns (Falkner and Lin, 2014). Our results only partially agree with this research in mammals since we report an accentuated expression of *c-fos* only in one of the two treatments (i.e., in MM but not in MD) in which fish express high levels of aggression and a decreased expression of this IEG when fish see a subordinate male after interacting with a mirror (MS) and consequently stop performing attacks and bites. In another cichlid fish (the Burton's mouthbrooder, *Astatotilapia burtoni*) it has been demonstrated that males that were given an opportunity to rise in social rank have higher expression of *c-fos* and *egr-1* in all the areas of the SDMN, including the TA, when compared to stable males, either of a dominant or a subordinate social status (Maruska et al., 2013b). On the other hand, a social descending male has an increase of *c-fos*, and not *egr-1*, expression in this area (Maruska et al., 2013a), corroborating its involvement also in social status transitions, as observed in the current study.

Moreover, a very interesting finding was that fish that saw a subordinate male after fighting with a mirror (MS) showed an increase in the density of the structure of the SDMN, namely on the density of the *egr-1* network, when compared to the other treatments. This evidence suggests that the perception of the fight outcome (which only unequivocally occurred in this treatment) originated a denser brain network, which is characterized by redundant connections and hence a higher robustness to changes in its nodes (i.e., it is less likely affected by the removal of nodes at random (Makagon et al., 2012). Looking into centrality measures obtained with the network analysis it is possible to ascertain that the TA is a more central area while the PPa is a less important node of the *egr-1* network in the MM condition while in the MD and MS conditions the reversed pattern is observed. These results strengthen the idea of the main role of TA in status changes and of the PPa as a link to the bodily changes (e.g., androgen response) that should accompany the changes in brain state.

Androgens are known to respond to social interactions and this response has been hypothesized to play an adaptive role in the adjustment of aggressive behavior to the competitive demands of the social environment (challenge hypothesis, Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990). Therefore, in this study, we have also investigated how androgens responded to the fighting assessment and how the changes in activation of the PPa, where GnRH1 neurons that control the HPG axis are located, were linked to a putative androgen response. Surprisingly, we found no significant changes in androgen levels in any of the treatments with social challenges (MD, MS). Concomitantly, we also did not find a change in the expression of gnrh1 in the PPa in response to the MD or MS treatments, and there were no correlations between gnrh1 expression and circulating androgen levels. Moreover, there were no correlations between the expression of any of the immediate early genes and that of *gnrh1*, indicating that the observed activation of the PPa in response to the experimental treatments does not correspond to an activation of the HPG axis. These negative results may result from the short time span of the staged fights with the real opponents, and/or from the failure to induce a perception of fight outcome in the case of the MD treatment.

In summary, our results support the view that it is the assessment that animals make of ongoing fights, and not the perception of the outcome, which triggers rapid changes in gene expression across the SDMN and that the TA is a key node in this network.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Direcção Geral de Alimentação e Veterinária, Lisbon, Portugal.

AUTHOR CONTRIBUTIONS

OA and RO designed the experiments. OA performed behavioral experiments. AF processed the samples. GO, JL and AF analyzed the data. AF, GO and RO wrote the article.

FUNDING

This study was funded by a grant (EXCL/BIA-ANM/0549/2012) from Fundação para a Ciência e a Tecnologia (FCT, Portugal) given to RO. OA and AF were supported by FCT PhD fellowships (SFRH/BD/37187/2007 and SFRH/BD/102892/2014, respectively), and GO was supported by a FCT post doc fellowship (SFRH/BPD/123616/2016).

ACKNOWLEDGMENTS

We would like to thank all members of the Oliveira Lab for valuable discussions of this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00229/full#supplementary-material

REFERENCES

Baerends, G. P., and Baerends-Van Roon, J. M. (1950). *An Introduction to the Study of the Ethology of Cichlid Fishes*. Leiden: BRILL.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x

Borgatti, S. P., Everett, M. G., and Freeman, L. C. (2002). *Ucinet 6 for Windows: Software for Social Network Analysis*. Harvard, MA: Analytic Technologies.

Cardoso, S. D., Teles, M. C., and Oliveira, R. F. (2015). Neurogenomic mechanisms of social plasticity. *J. Exp. Biol.* 218, 140–149. doi: 10.1242/jeb.106997

Clayton, D. F. (2000). The genomic action potential. *Neurobiol. Learn. Mem.* 74, 185–216. doi: 10.1006/nlme.2000.3967

Dijkstra, P. D., Schaafsma, S. M., Hofmann, H. A., and Groothuis, T. G. G. (2012). "Winner effect" without winning: unresolved social conflicts increase the probability of winning a subsequent contest in a cichlid fish. *Physiol. Behav.* 105, 489–492. doi: 10.1016/j.physbeh.2011.08.029

Falkner, A. L., and Lin, D. (2014). Recent advances in understanding the role of the hypothalamic circuit during aggression. *Front. Syst. Neurosci.* 8:168. doi: 10.3389/fnsys.2014.00168

Faykoo-Martinez, M., Mooney, S. J., and Holmes, M. M. (2018). Oxytocin manipulation alters neural activity in response to social stimuli in eusocial naked mole-rats. *Front. Behav. Neurosci.* 12:272. doi: 10.3389/fnbeh.2018.00272

Field, K. E., and Maruska, K. P. (2017). Context-dependent chemosensory signaling, aggression and neural activation patterns in gravid female African cichlid fish. *J. Exp. Biol.* 220, 4689–4702. doi: 10.1242/jeb.164574

Foo, J. T. W., and Lam, T. J. (1993). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone level in the tilapia, *Oreochromis mossambicus*. *Aquaculture* 115, 145–158. doi: 10.1016/0044-8486(93)90365-6

Fryer, G., and Iles, T. D. (1972). *The Cichlid Fishes of the Great Lakes of Africa: Their Biology and Evolution*. Edinburgh: Oliver & Boyd.

Goodson, J. L. (2005). The vertebrate social behavior network: evolutionary themes and variations. *Horm. Behav.* 48, 11–22. doi: 10.1016/j.yhbeh.2005.02.003

Hashikawa, Y., Hashikawa, K., Falkner, A. L., and Lin, D. (2017). Ventromedial hypothalamus and the generation of aggression. *Front. Syst. Neurosci.* 11:94. doi: 10.3389/fnsys.2017.00094

Hirschenhauser, K., and Oliveira, R. F. (2006). Social modulation of androgens in male vertebrates: meta-analyses of the challenge hypothesis. *Anim. Behav.* 71, 265–277. doi: 10.1016/j.anbehav.2005.04.014

Hsu, Y., Earley, R. L., and Wolf, L. L. (2006). Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. *Biol. Rev.* 81, 33–74. doi: 10.1017/s146479310500686x

Hsu, Y., Earley, R. L., and Wolf, L. L. (2011). "Aggressive behaviour in fish: integrating information about contest costs," in *Fish Cognition and Behavior*, eds C. Brown, K. Laland and J. Krause (Oxford: Wiley-Blackwell), 108–134.

Jain, R. B. (2010). A recursive version of Grubbs' test for detecting multiple outliers in environmental and chemical data. *Clin. Biochem.* 43, 1030–1033. doi: 10.1016/j.clinbiochem.2010.04.071

Kabelik, D., Weitekamp, C. A., Choudhury, S. C., Hartline, J. T., Smith, A. N., and Hofmann, H. A. (2018). Neural activity in the social decision-making network of the brown anole during reproductive and agonistic encounters. *Horm. Behav.* 106, 178–188. doi: 10.1016/j.yhbeh.2018.06.013

Kalendar, R., Khassenov, B., Ramankulov, Y., Samuilova, O., and Ivanov, K. I. (2017). FastPCR: an *in silico* tool for fast primer and probe design and advanced sequence analysis. *Genomics* 109, 312–319. doi: 10.1016/j.ygeno.2017.05.005

Kime, D. E., and Manning, N. J. (1982). Seasonal patterns of free and conjugated androgens in the brown trout Salmo trutta. *Gen. Comp. Endocrinol.* 48, 222–231. doi: 10.1016/0016-6480(82)90020-x

Kollack-Walker, S., Watson, S. J., and Akil, H. (1997). Social stress in hamsters: defeat activates specific neurocircuits within the brain. *J. Neurosci.* 17, 8842–8855. doi: 10.1523/jneurosci.17-22-08842.1997

Koressaar, T., and Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23, 1289–1291. doi: 10.1093/bioinformatics/btm091

Lin, D., Boyle, M. P., Dollar, P., Lee, H., Perona, P., Lein, E. S., et al. (2011). Functional identification of an aggression locus in the mouse hypothalamus. *Nature* 470, 221–226. doi: 10.1038/nature09736

Makagon, M. M., McCowan, B., and Mench, J. A. (2012). How can social network analysis contribute to social behavior research in applied ethology? *Appl. Anim. Behav. Sci.* 138, 152–161. doi: 10.1016/j.applanim.2012.02.003

Maruska, K. P., Becker, L., Neboori, A., and Fernald, R. D. (2013a). Social descent with territory loss causes rapid behavioral, endocrine and transcriptional changes in the brain. *J. Exp. Biol.* 216, 3656–3666. doi: 10.1242/jeb.088617

Maruska, K. P., Zhang, A., Neboori, A., and Fernald, R. D. (2013b). Social opportunity causes rapid transcriptional changes in the social behaviour network of the brain in an African cichlid fish. *J. Neuroendocrinol.* 25, 145–157. doi: 10.1111/j.1365-2826.2012.02382.x

Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network. *Ann. N Y Acad. Sci.* 877, 242–257. doi: 10.1111/j.1749-6632.1999.tb09271.x

O'Connell, L. A., Fontenot, M. R., and Hofmann, H. A. (2011). Characterization of the dopaminergic system in the brain of an African cichlid fish, Astatotilapia burtoni. *J. Comp. Neurol.* 519, 75–92. doi: 10.1002/cne.22506

O'Connell, L. A., and Hofmann, H. A. (2011a). Genes, hormones and circuits: an integrative approach to study the evolution of social behavior. *Front. Neuroendocrinol.* 32, 320–335. doi: 10.1016/j.yfrne.2010.12.004

O'Connell, L. A., and Hofmann, H. A. (2011b). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *J. Comp. Neurol.* 519, 3599–3639. doi: 10.1002/cne.22735

O'Connell, L. A., and Hofmann, H. A. (2012). Evolution of a vertebrate social decision-making network. *Science* 336, 1154–1157. doi: 10.1126/science.1218889

Oliveira, R. F. (2012). Social plasticity in fish: integrating mechanisms and function. *J. Fish Biol.* 81, 2127–2150. doi: 10.1111/j.1095-8649.2012.03477.x

Oliveira, R. F., and Almada, V. C. (1996). Dominance hierarchies and social structure in captive groups of the Mozambique tilapia *Oreochromis mossambicus* (Teleostei, Cichlidae). *Ethol. Ecol. Evol.* 8, 39–55. doi: 10.1080/08927014.1996.9522934

Oliveira, R. F., and Almada, V. C. (1998). Mating tactics and male-male courtship in the lek-breeding cichlid *Oreochromis mossambicus*. *J. Fish Biol.* 52, 1115–1129. doi: 10.1111/j.1095-8649.1998.tb00959.x

Oliveira, R. F., Carneiro, L. A., and Canário, A. V. M. (2005). Behavioural endocrinology: no hormonal response in tied fights. *Nature* 437, 207–208. doi: 10.1038/437207a

Oliveira, R. F., Silva, A., and Canário, A. V. M. (2009). Why do winners keep winning? Androgen mediation of winner but not loser effects in cichlid fish. *Proc. Biol. Sci.* 276, 2249–2256. doi: 10.1098/rspb.2009.0132

R Core Team. (2015). *R: A Language and Environment for Statistical Computing*. Available online at: http://www.r-project.org/. Accessed April 2019.

Roleira, A., Oliveira, G. A., Lopes, J. S., and Oliveira, R. F. (2017). Audience effects in territorial defense of male cichlid fish are associated with differential patterns of activation of the brain social decision-making network. *Front. Behav. Neurosci.* 11:105. doi: 10.3389/fnbeh.2017.00105

Taborsky, B., and Oliveira, R. F. (2012). Social competence: an evolutionary approach. *Trends Ecol. Evol.* 27, 679–688. doi: 10.1016/j.tree.2012.09.003

Teles, M. C., Almeida, O., Lopes, J. S., and Oliveira, R. F. (2015). Social interactions elicit rapid shifts in functional connectivity in the social decision-making network of zebrafish. *Proc. Biol. Sci.* 282:20151099. doi: 10.1098/rspb.2015.1099

Teles, M. C., Dahlbom, S. J., Winberg, S., and Oliveira, R. F. (2013). Social modulation of brain monoamine levels in zebrafish. *Behav. Brain Res.* 253, 17–24. doi: 10.1016/j.bbr.2013.07.012

Teles, M. C., Sîrbulescu, R. F., Wellbrock, U. M., Oliveira, R. F., and Zupanc, G. K. H. (2012). Adult neurogenesis in the brain of the Mozambique tilapia, *Oreochromis mossambicus*. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 198, 427–449. doi: 10.1007/s00359-012-0721-6

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Res.* 40:e115. doi: 10.1093/nar/gks596

Williamson, C. M., Klein, I. S., Lee, W., and Curley, J. P. (2019). Immediate early gene activation throughout the brain is associated with dynamic changes in social context. *Soc. Neurosci.* 14, 253–265. doi: 10.1080/17470919.2018.1479303

Wingfield, J. C., Hegner, R. E., Dufty, A. M. J., and Ball, G. F. (1990). The challenge hypothesis: theoretical implications for patterns of testosterone secretion, mating systems and breeding strategies. *Am. Nat.* 136, 829–846. doi: 10.1086/285134

Zhao, S., and Fernald, R. D. (2005). Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J. Comput. Biol.* 12, 1047–1064. doi: 10.1089/cmb.2005.12.1047

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