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Research report

Support for the parental practice hypothesis: Subadult prairie voles exhibit similar behavioral and neural profiles when alloparenting kin and non-kin

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ABSTRACT

Parental care is critical for offspring survival in altricial species. Although parents are the most common caregivers, other individuals (e.g., older siblings) can also provide alloparental care. Some have argued that animals engage in alloparental behavior to practice providing care for their eventual offspring, whereas others have argued that alloparental behavior enhances indirect fitness. Proximate measures have the potential to test ultimate functions of behavior. A focus on neural expression of oxytocin and vasopressin (two neuropeptides modulating alloparental care) or neural activation following exposure to related and unrelated individuals could reveal whether practice or investment in indirect fitness explains alloparental behavior. This study examined alloparental behaviors and neural responses in prairie voles (*Microtus ochrogaster*), a species that engages in alloparental behavior. Subadult (independent, yet sexually immature) male prairie voles were exposed to one of four stimuli: same-age sibling, neonatal sibling, unrelated neonate, or inanimate neonate-sized object. We assessed alloparental behaviors and quantified cFos protein expression in oxytocin and vasopressin neuronal populations of the paraventricular nucleus of the hypothalamus and the supraoptic nucleus of the hypothalamus in response to stimulus exposure. We detected no differences in cFos and nonapeptide co-localization among stimulus groups. Subjects performed similar amounts of alloparental care toward related and unrelated neonates, but not other subadults or inanimate objects. Notably, caregiving did not differ based on kin-status. The lack of difference in alloparenting toward related and non-related neonates suggests that alloparental care in prairie voles primarily serves to provide subadults with parental practice.

1. Introduction

Offspring of altricial species depend on caregivers to survive. Although caregiving is most commonly provided by at least one parent, not all caregivers are the genetic parents of the offspring benefitting from parental effort [1]. In fact, parental care can also come from adoptive parents or other non-parent individuals in what is known as alloparental care [2–4]. Species differ in which individuals provide alloparental care, varying in traits such as the developmental stage and age when alloparenting occurs. Specifically, some species rely on fully mature adults, whereas others rely on sexually immature subadults for alloparental care in addition to or instead of adults. For example, adult nursing lionesses, (*Panthera leo*) [5], dwarf mongooses, (*Helogale parvula*) [6], and common marmosets (*Callithrix jacchus*) [7] commonly engage in alloparental care only when reaching adulthood. In contrast, subadults (i.e., sexually immature individuals that are independent of parents for food) regularly engage in alloparental care in vervet monkeys, (*Cercopithecus aethiops sabaeus*) [8], African striped mice, (*Rhabdomys pumilio*) [9], ring-tailed lemurs, (*Lemur catta*) [10], and humans (*Homo sapiens*) [11]. The extent to which alloparents are related to the young who receive care can also vary. Importantly, the examples of adult alloparents mentioned above are usually unrelated to the offspring to which they provide care. However, adult alloparents providing care to unrelated offspring is not universal. For example, adult banded mongooses (*Mungos mungo*) care for related and unrelated young indiscriminately [12], whereas Seychelles warblers (*Acrocephalus sechellensis*) and long-tailed tits (*Aegithalos caudatus*) are more likely to provide alloparental care to full siblings than half siblings and provide no care to unrelated young [13,14]. This variation in who provides alloparental care (related vs. unrelated individuals) and when they provide it (adult vs. subadult) has implications for the evolution of alloparental care itself.

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Arguably, the primary benefit of providing parental care is to increase the chance of offspring survival, further enhancing an individual's reproductive success and fitness [15,16]. Caregiving, however, requires extensive effort, and daily energy expenditures are greater for many species during caregiving compared to non-caregiving periods [17]. Because of these high costs, caregiving should have substantial benefits if they are to be selected for by evolution, and these behaviors should enhance individual lifetime fitness. Indeed, parental and alloparental care are widespread, suggesting that the benefits of caregiving often outweigh the associated costs. Although the benefits of providing parental care to one's offspring are direct, it is less clear what the benefits are for exhibiting alloparental care. Alloparents face much of the same costs that parents do when providing care to young, but may receive different benefits depending on the life history of a species [18]. The degree to which the potential benefits sustain alloparental care varies from species to species and often reflects the natural history of the species in question [18,19]. There are multiple hypotheses that attempt to explain what drives alloparental care, and, importantly, these hypotheses are not necessarily mutually exclusive.

Perhaps the most prominent explanation for alloparental care is that non-parent caregivers increase their own indirect fitness by rearing siblings [20]. Hamilton's rule provides a framework for this hypothesis, and states that - all things being equal - individuals can increase their fitness by investing in the most closely related individuals [21]. For this to occur, individuals should have the ability to determine relatedness through kin discrimination. Indeed, kin discrimination abilities increase as the benefits to the alloparent increase [19]. Species such as white-fronted bee-eaters (*Merops bullockoides*) [22], Seychelles warblers [13], and long-tailed tits [14] demonstrate kin discrimination and increase alloparental care as the relatedness of the young increases. Importantly, however, kin discrimination is not always necessary for gains in indirect fitness to occur.

Not all species in which alloparental care exists demonstrate kin recognition. This could result when kin recognition is unnecessary. For example, species in which natal dispersal is limited or delayed might still increase indirect fitness by engaging in alloparental care because their natal groups will always be composed of close relatives [23,24]. On the other hand, benefits other than indirect fitness can also explain alloparental care. For instance, alloparental care appears to have evolved in some species to provide opportunities to practice raising offspring, thereby benefitting future offspring of the now experienced, and presumably competent, parents [25,26]. Indeed, practice via alloparental care increases offspring survival in many species, including primates [26–29], birds [30–33], and rodents [34–36]. For example, male Mongolian gerbils (*Meriones unguiculates*) with alloparental experience produce their first litter sooner, and their pups gain weight faster than litters from alloparentally inexperienced males [35]. Similarly, prairie voles (*Microtus ochrogaster*) with alloparenting experience during adolescence produce pups that gain weight at a greater rate when they become parents than those without alloparental experience [36]. Thus, in instances such as these, alloparental care appears to enhance the probability of survival and success of an individual's own offspring.

Although the ultimate explanations for alloparental care can vary across species, the mechanisms governing it appear to be relatively similar. The neural mechanisms behind alloparental care are traditionally framed with reference to maternal care because the specific behaviors observed in parental (particularly maternal) care and alloparental care are demonstrably similar [37]. Indeed, post-adolescent alloparents in species ranging from mammals to fish show hormonal profiles extremely similar to their parents, suggesting conserved mechanisms behind parental and alloparental behaviors [37]. Although there are several hormones that modulate parental and alloparental care throughout the brain (for reviews, see [38] and [39]), oxytocin (OT) and arginine vasopressin (VP) are two closely related neuropeptides that are frequently implicated in parental care [40]. OT, in particular, is correlated with alloparental care in multiple mammalian species, from common marmosets to prairie voles [41–46]. For example, the density of OT receptors in various brain regions has been hypothesized to explain both species and individual differences in rodent alloparental care [46]. Despite its structural similarity to OT, relatively little research has examined the role that VP plays in alloparental care [39]. Parker and Lee [47] demonstrated that VP facilitates alloparental behaviors in male meadow voles (*Microtus pennsylvanicus)*. Furthermore, intracerebroventricular injections of VP in adult virgin female rats induces alloparental behavior, however, these effects are slower than those produced by OT injections [48]. In contrast, both sexually naïve female and male marmosets exhibit increased care towards infants in response to VP, but not OT [49,50].

Building upon this foundation, research with prairie voles has provided a powerful opportunity to explore the function of alloparental care and understand the neural mechanisms that facilitate it. Prairie voles are small arvicoline rodents in which both sexes reliably demonstrate parental care [51–53]. A majority of both virgin adult and subadult males and females also exhibit spontaneous alloparental care in the lab [52,54], with females tending to be less alloparental than males [54]. Notably, approximately 27% of breeding units sampled in the field are communal (i.e., consisting of male-female breeders, plus infant, subadult and/or adult offspring, and occasionally unrelated adults) [55,56]. Indeed, approximately 30% of the members of a communal groups are unrelated, reproductive adults [56]. Subadults typically disperse from their natal nest when they are between 45 and 55 days old [57]. Thus, subadults are likely present for the birth of another litter; prairie voles reliably mate within 24 h of giving birth and will have a second litter 21–23 days later [58]. Prairie voles therefore have the opportunity to care for related and/or unrelated young, making alloparental care an important part of their natural behavioral repertoire.

In the present study, we investigated alloparental caregiving in subadult male prairie voles, a stage of development when they readily engage in these behaviors. Furthermore, subadults have not yet reached the age of dispersal, and this could represent a particularly important time to gain caregiving experience during prairie vole development, an opportunity to contribute to their indirect fitness through alloparental care, or both. Because adolescent male prairie voles with alloparental experience produce healthier offspring in their first litter [36], there is reason to favor the hypothesis that alloparenting serves a role in parenting practice. We note, however, that alloparental care can both enhance parental practice and favor kin, and thus these are not mutually exclusive. Nevertheless, we predicted that if subadult prairie voles perform alloparental care to increase indirect fitness, they would be more likely to invest in related siblings than unrelated conspecifics. If, however, caregiving practice explains alloparental care, then subadult voles should preferentially provide care to neonates regardless of relatedness.

We also examined whether subadult prairie voles exhibit differences in neural responses to stimuli associated with kin or neonate pups to inform whether prairie vole alloparenting might relate to direct fitness, indirect fitness, or both. We used cFos, an immediate early gene (IEG), to examine the functional differences within neurons of the paraventricular nucleus of the hypothalamus (PVN) and the supraoptic nucleus of the hypothalamus (SON), as these regions express cFos in both parenting and alloparenting contexts [59–61]. Specifically, we sought to determine whether hypothalamic nonapeptides differentially respond to exposure to either a related neonate, an unrelated neonate, a related same-sex cagemate (i.e. a littermate serving as a social control in a non-alloparental context), or a neonate-sized novel object (a nonsocial control). We predicted that OT- and VP-positive neurons would be differentially active after engaging in alloparental care. Furthermore, we predicted that if subadult prairie voles perform alloparental care to increase indirect fitness, then OT- and VP-positive cells should show relatively more neural activation after exposure to related siblings than unrelated conspecifics. This result would suggest some form of kin recognition, as OT and VP play a role in this behavior [62]. On the other

hand, if caregiving practice explains alloparental care, OT- and VP-positive cells should show similiar activation following exposure to related and unrelated neonates, but be more active compared to same-aged siblings.

By presenting behavioral and hypothalamic nonapeptide activity profiles of subadult males interacting with conspecifics in alloparental and non-alloparental contexts, we explore the possible functional origins of alloparental care in the prairie vole.

2. Methods

2.1. Subjects

Forty unique breeding pairs were created using prairie voles from our colony breeders, which are primarily wild caught animals from Champagne County, Illinois, USA. To control for litter size effects, only breeding pairs that produced 3–5 pups were used in this study. All animals were housed in standard polycarbonate rodent cages (29 × 18 × 13 cm) lined with Sani-chip bedding and provided nesting material. Animals were kept on a 14L:10D cycle and were provided with rodent chow (Laboratory Rodent Diet 5001, LabDiet, St. Louis, MO, USA) and water ad libitum. Ambient temperature was maintained at 20 °C (±2 °C). All procedures were approved by and in compliance with the Institutional Animal Care and Use Committee of Cornell University (Protocol 2013–0102).

Prairie voles are induced ovulators and will frequently mate during and immediately after parturition, usually becoming pregnant with another litter within 24 h [58]. Subjects from the primiparous litter were kept with their families after the second litter of siblings had been born until testing (post-natal day [PND] 28). This allowed all subjects to have seven days of experience with newborn pups and the opportunity to perform alloparental care at the natal nest. The number of pups in this second litter was also kept between 3 and 5 pups. All subjects used in this experiment were males because studies in several rodent species (including prairie voles) have shown that subadult males more reliably exhibit parental behaviors, whereas females demonstrate comparably less caregiving, more infanticide, and more aggression [63–65].

2.2. Stimulus exposure

We modeled our stimulus exposure after work exploring the activation of OT-ir and VP-ir containing neurons in parents separated and reunited with their offspring [61]. Stimulus exposure took place when the subjects reached PND 28 and was conducted between 1130 h and 1530 h. A video camera (Sony Handycam [CX405] Sony, New York City, NY, USA) above the cage recorded the entire stimulus exposure for later behavioral analysis (see Section 2.3). To begin stimulus exposure, subjects were transferred to a clean, novel cage containing clean Sani-chip bedding to habituate for 30 min by themselves. We included this habituation time to reduce the likelihood that neuronal activation (assessed by cFos, see Section 2.4) would be attributable to the transfer of subjects to the apparatus and/or exploration of a novel environment. Testing was done in a neutral cage to avoid creating a resident-intruder context, which could affect behavior due to territorial dominance [66]. After the habituation period, one of four stimuli was placed in the cage with the subject, and the subject was then allowed to interact with the stimulus for 90 min. The four potential stimuli were: i) a same-aged sibling (PND 28), ii) a younger sibling (between PND 2 and PND 4), iii) a neonate from an unrelated litter (between PND 2 and PND 4), or iv) a pup-sized inanimate object. We used a sample size of $N = 12$ for each stimulus group. The inanimate object was a small plastic Mardi Gras King Cake figurine (BABY100R, Space Age Plastics Inc, Jefferson Twp, PA), which measured approximately 4 cm × 1.5 cm × 1 cm. Although alloparental studies in rodents have traditionally used a wooden dowel as an inanimate stimulus, we chose to use the figurine because it more closely resembles a neonate (i.e., size and presence of curves resembling limbs; see Fig. 1) and has features that are salient to rodents [67]. We closely monitored subjects during exposure to neonates because some adult male prairie voles can be infanticidal and might attack unrelated pups [68]. Neonates were gently washed with warm soap and water before being returned to their nest to ensure they would not be attacked by their parents; pups were never reused as stimuli. None of the twelve stranger neonates or twelve related neonates were attacked by the subadult male subjects during testing or the pups' parents upon return to their home cage.

2.3. Behavioral analysis

We exposed each subject to one of the four aforementioned stimulus contexts for 90 min to capture neuronal activation that resulted from exposure to each stimulus (see Section 2.4). We recorded and scored the behavioral interactions contained within the first 30 min of stimulus exposure because this is the most representative window for relating behavior to neural activity [69–71]. We analyzed the resulting videos using Observer XT v13 software (Noldus Information Technology, Leesburg, VA, USA). We scored the following behaviors for all subjects: *Grooming* (subject is manipulating stimulus with its mouth and front paws), *Huddling* (more than 50% of the subject's body is in contact with the stimulus, e.g., side-by-side with a same-age sibling or on top of a pup), *Miscellaneous Contact* (less than 50% of the subject's body is in contact with the stimulus, e.g., anogenital sniffing), and *No Contact* (no part of the subject is in contact with the stimulus). Grooming, huddling, and miscellaneous contact were combined to give a measure for the total amount of contact spent with the stimulus. We observed no aggressive behavior in any subject and therefore aggression was not scored.

Precisely 90 min after the introduction of the stimulus, subjects were sacrificed by isoflurane overdose and transcardially perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde dissolved in 0.1 M borate buffer (pH 9.5). Brains were extracted, post-fixed overnight in 4% paraformaldehyde dissolved in 0.1 M borate buffer (pH 9.5) before cryoprotection in 30% sucrose dissolved in PBS for 48 h to protect the brains before slicing. Note that one brain collected from a subject serving in the inanimate object condition was damaged during sectioning, and we accordingly excluded that individual from the following analyses (inanimate object condition $N = 11$).

2.4. Histology and immunocytochemistry

To assess the degree to which cells in the SON and PVN were functionally active during stimulus exposure, we quantified the number of OT and VP positive cells and determined the proportion of these cells that concomitantly contained the cFos protein. The immediate early gene cFos rapidly increases gene expression and results in cFos protein synthesis in response to cell surface signals [72]. cFos protein expression peaks between 60 and 90 min after neuronal stimulation and remains measurable until 120 min after exposure [69–71]. We chose to quantify neuronal activation and nonapeptide expression at 90 min after stimulus exposure to ensure we did not miss any neural responses to stimulus exposure [73,74]. Thus, neurons that co-label cFos and either OT or VP indicate that cells containing OT or VP were functionally activated as a result of the previous experience.

To visualize OT, VP, and cFos, tissue was sectioned into three 40 μm series. One series of tissue was immunofluorescently triple labeled for VP, OT, and cFos. Tissue sections were rinsed 5x for 10 min in 0.1 M PBS (pH 7.4), incubated at room temperature for 1 h in blocks (PBS + 10% normal donkey serum + 0.3% Triton-X-100), and then incubated at 4 °C for approximately 48 h in primary antibodies diluted in PBS containing 5% normal donkey serum + 0.3% Triton-X-100. Primary antibodies used were guinea pig anti-VP (1:1000; Peninsula Laboratories, San Carlos, CA), mouse anti-OT (3:1000; Millipore, Billerica, MA), and rabbit anti-Fos (5:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Note that the Santa Cruz rabbit anti-Fos antibody was from a lot prior to

Fig. 1. Comparison of inanimate object (a plastic Mardi Gras King Cake figurine) to a live prairie vole pup (postnatal day [PND] 3). Both stimuli are approximately the same size and shape.

2014, which has been previously validated in numerous labs. Antibodies were previously validated with preadsorption controls to verify specificity [61]. The primary incubation was followed by two 30 min rinses in PBS. Tissue was incubated for 1 h in a biotinylated donkey anti-guinea pig secondary (8:1000; Jackson Immunoresearch, West Grove, PA), rinsed twice for 15 min in PBS, and incubated for 2 h at room temperature in streptavidin conjugated to Alexa Fluor 488 (3:1000), donkey anti-mouse secondary conjugated to Alexa Fluor 680 (6:1000), and donkey anti-rabbit secondary conjugated to Alexa Fluor 594 (5:1000). All secondary antibodies were diluted in PBS containing 5% normal donkey serum + 0.3% Triton-X-100. Alexa Fluor conjugates were obtained from ThermoFisher Scientific (Waltham, MA). Following two 30 min rinses in PBS, sections were mounted on microscope slides and cover-slipped with Prolong Gold antifade containing a DAPI nuclear stain (ThermoFisher Scientific).

2.5. Neural quantification

Images of brain regions for cell counting were obtained using a Zeiss AxioImager II microscope outfitted with an AxioCam Mrm, z-drive, and an Apotome optical dissector (Carl Zeiss Inc., Gottingen, Germany). Observers were blind to the stimulus exposure for each subject. We used flattened z-stack images to conduct cell counts in the open-source image editor GIMP (GNU Image Manipulation Program; http://gimp.org) as previously described [61,75].

We examined the percentage of VP immunoreactive (-ir) and OT-ir cells that were double labeled for cFos (VP-cFos or OT-cFos colocalization) out of all nonapeptide containing cells. Using this percentage normalizes across individuals with different total numbers of cells. VP-cFos colocalization and OT-cFos colocalization were both quantified in the SON and PVN and were combined across two brain sections (one caudal and one rostral). See Fig. 2 for a diagram of each cell group location, which corresponds roughly with the Allen Mouse Brain Atlas [76]. There were no significant differences in VP-cFos colocalization or OT-cFos colocalization between rostral and caudal measurements, and thus cell counts were collapsed for analysis. Data are reported as the percentage of VP-ir or OT-ir cells that were colocalized with cFos (% VP-cFos or %OT-cFos colocalization).

2.6. Analysis and statistical methods

Data were analyzed using R [77]. We used one-way ANOVAs with Tukey's post hoc tests to determine if behavior or neuronal activity differed among the four stimulus exposure types. We used Pearson's correlation to examine the relationship between neuronal activity and alloparental care, specifically focusing on licking and grooming of the stimulus. Significance threshold was set at $p < 0.05$ for all statistical tests. Multiple helper packages were used to import and visualize the

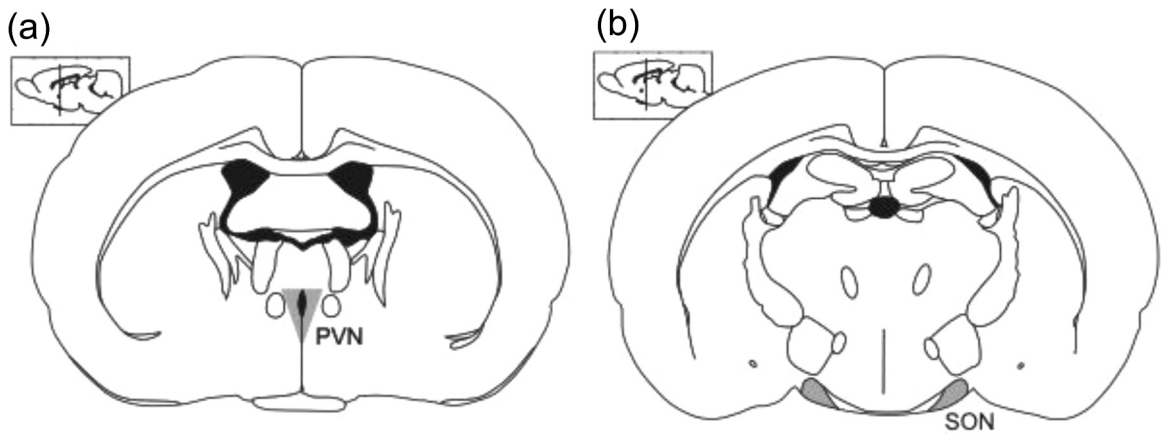

Fig. 2. Coronal diagram showing the relative location of the (a) PVN and (b) SON shaded in gray. The small inlay shows a sagittal view of the brain, with the vertical black line indicating location of coronal slice. Contour lines represent other structures for reference. Ventricles are marked in black.

data [78,79].

3. Results

3.1. Behavioral results

We first examined how total positive contact varied in response to our four stimulus types. The total time in contact with the stimulus varied significantly across the groups (main effect: $F_{(3,\ 44)} = 77.67$, $p < 0.001$; Fig. 3**a**). Subjects spent more time in contact with living stimuli than with the inanimate object (object vs related neonate: TukeyHSD: −1364.75 (s), CI: [−1643.69, −1085.81], $p < 0.001$; object vs unrelated neonate: TukeyHSD: −1198.33 (s), CI: [−1477.27, −919.39], $p < 0.001$; object vs same-age sibling: TukeyHSD: −388.33 (s), CI: [−677.27, −109.39], $p = 0.003$). Subjects also spent more time in contact with either of the neonate stimuli than the same-age sibling (same-age sibling vs related neonate: TukeyHSD: −976.42 (s), CI: [−1255.36, −697.48], $p < 0.001$; same-age sibling vs unrelated neonate: TukeyHSD: −810.00, CI: [−1088.94, −531.06], $p < 0.001$). However, we found no difference in the amount of time subjects spent between either related or unrelated neonates (unrelated neonate vs related neonate: TukeyHSD: −166.42 (s), CI: [−445.36, 112.52], $p = 0.39$).

We then examined how more specific types of contact (i.e.,

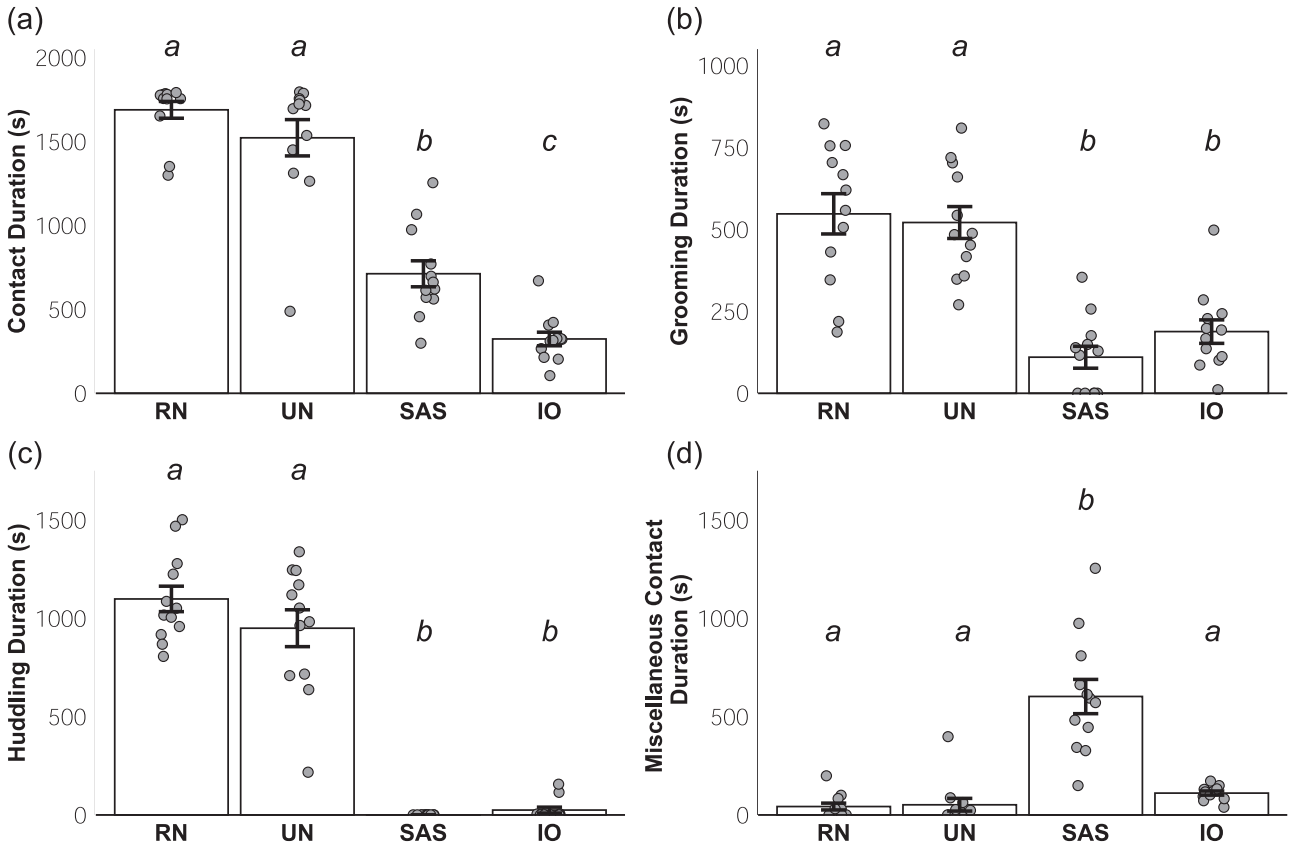

Fig. 3. (a) Mean (+SE) time (in seconds, s) subjects spent in contact with stimuli. (b) Mean (+SE) time subjects spent grooming stimuli. (*c*) Mean (+SE) time subjects spent huddling stimuli. (*d*) Mean (+SE) time subjects spent in miscellaneous contact. Related neonate (RN), unrelated neonate (UN), same-age sibling (SAS), and inanimate object (IO). Dots represent the individual data in each group. Different letters indicate statistical differences where $p < 0.05$.

grooming, huddling, miscellaneous contact) differed in response to the various living stimuli. Given that subjects spent little time in contact in general with the non-living stimulus, we focused this part of the analysis on exclusively the living stimuli. Our results show a main effect of stimulus type for all three types of contact (grooming: $F_{(3,\ 44)} = 23.64$, $p < 0.001$; huddling: $F_{(3,\ 44)} = 104.20$, $p < 0.001$; miscellaneous contact: $F_{(3,\ 44)} = 31.94$, $p < 0.001$; Fig. 3b–d). Subjects spent more time grooming both related and unrelated neonates than same-age siblings (same-age sibling vs related neonate: TukeyHSD: −437.58, CI: [−612.24, −262.93], $p < 0.001$; same-age sibling vs unrelated neonate: TukeyHSD: −410.92, CI: [−585.57, −236.26], $p < 0.001$). Huddling behavior showed a similar pattern, with subjects spending more time huddling with neonates than with same-age siblings (same-age sibling vs related neonate: TukeyHSD: −1098.17, CI: [−1315.40, −880.93], $p < 0.001$; same-age sibling vs unrelated neonate: TukeyHSD: −949.25, CI: [−1166.49, −732.01], $p < 0.001$). In contrast, miscellaneous contact (which included behaviors such as nose-to-nose contact and nose-to-flank contact) demonstrated the opposite effect, with subjects spending more time in contact with same-age siblings than with either of the neonates (same-age sibling vs related neonate: TukeyHSD: 559.33, CI: [379.97, 738.70], $p < 0.001$; same-age sibling vs unrelated neonate: TukeyHSD: 550.17, CI: [370.80, 729.54], $p < 0.001$). We also examined whether subjects demonstrated any behavioral differences towards related and unrelated neonates. We found no difference between related and unrelated siblings for all three behaviors (grooming: TukeyHSD: −26.67, CI: [−201.32, 147.99], $p = 0.98$; huddling: TukeyHSD: −148.92, CI: [−366.15, 68.32], $p = 0.27$; miscellaneous contact: TukeyHSD: 9.17, CI: [−170.20, 188.53], $p = 0.99$).

3.2. Immunocytochemistry results

We next asked whether hypothalamic OT and VP differentially responded to the four distinct stimulus types. Our results demonstrated that OT-cFos colocalization did not differ across any group for either the PVN or the SON (SON: $F_{(3,\ 43)} = 0.89$, $p = 0.45$; PVN: $F_{(3,\ 43)} = 1.93$, $p = 0.14$; Fig. 4a, b). Similarly, we found no differences in VP-cFos colocalization following exposure to the four experimental conditions (SON: $F_{(3,\ 43)} = 0.63$, $p = 0.60$; PVN: $F_{(3,\ 43)} = 0.49$, $p = 0.69$; Fig. 4c, d). These results indicate that OT- and VP-positive neurons were not differentially activated following exposure to neonates (related or unrelated), same-aged siblings, or an inanimate object. Consistent with these results, we found that the degree to which subjects engaged in alloparental care, as measured by time spent huddling and grooming, did not correlate with VP-cFos co-localization (SON: $r_{(45)} = 0.05$, $p = 0.73$; PVN: $r_{(45)} = -0.02$, $p = 0.90$) or OT-cFos co-localization (SON: $r_{(45)} = 0.06$, $p = 0.68$; PVN: $r_{(45)} = 0.008$, $p = 0.96$). We also found that the total amount of VP or OT, independent of cFos co-localization, did not differ as a function of stimulus exposure type (SON OT: $F_{(3,\ 43)} = 1.53$, $p = 0.22$; SON VP: $F_{(3,\ 43)} = 1.34$, $p = 0.28$; PVN OT: $F_{(3,\ 43)} = 1.26$, $p = 0.30$; PVN VP: $F_{(3,\ 43)} = 0.17$, $p = 0.92$; Fig. 5). The total amount of cFos across the SON and PVN also did not differ or correlate with alloparental care (SON cFos: $F_{(3,\ 43)} = 0.037$, $p = 0.99$; SON correlation: $r_{(45)} = 0.11$, $p = 0.44$; PVN cFos: $F_{(3,\ 43)} = 0.66$, $p = 0.58$; PVN correlation: $r_{(45)} = 0.13$, $p = 0.37$).

4. Discussion

We used subadult male prairie voles with previous opportunities for alloparenting to examine alloparental behavior and nonapeptide functional profiles in response four different stimulus types: a related neonate, an unrelated neonate, a same-sex littermate/cagemate, or a novel, inanimate object. The behavioral results demonstrate that, consistent with our expectation and with other alloparental studies, prairie voles spend more time investigating and interacting with a living

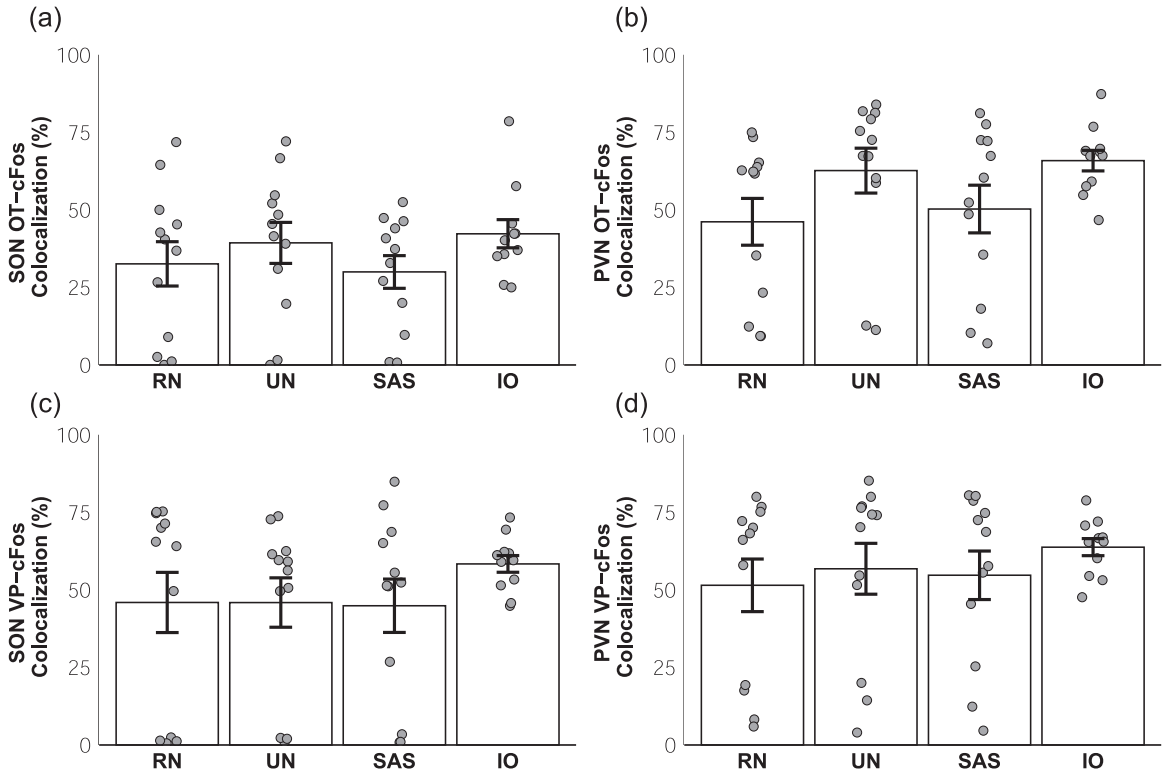

Fig. 4. Mean (+SEM) percentage of oxytocin (OT) immunoreactive (ir) neurons expressing cFos-ir in the (a) supraoptic nucleus of the hypothalamus (SON) and (b) paraventricular nucleus of the hypothalamus (PVN), or vasopressin (VP)-ir neurons expressing cFos-ir in the (c) SON or (d) PVN. RN: subjects exposed to related neonate. UN: subjects exposed to unrelated neonate. SAS: subjects exposed to same-age sibling (SAS). IO: subjects exposed to inanimate object during the 90-min IEG test. Dots represent the individual data in each group.

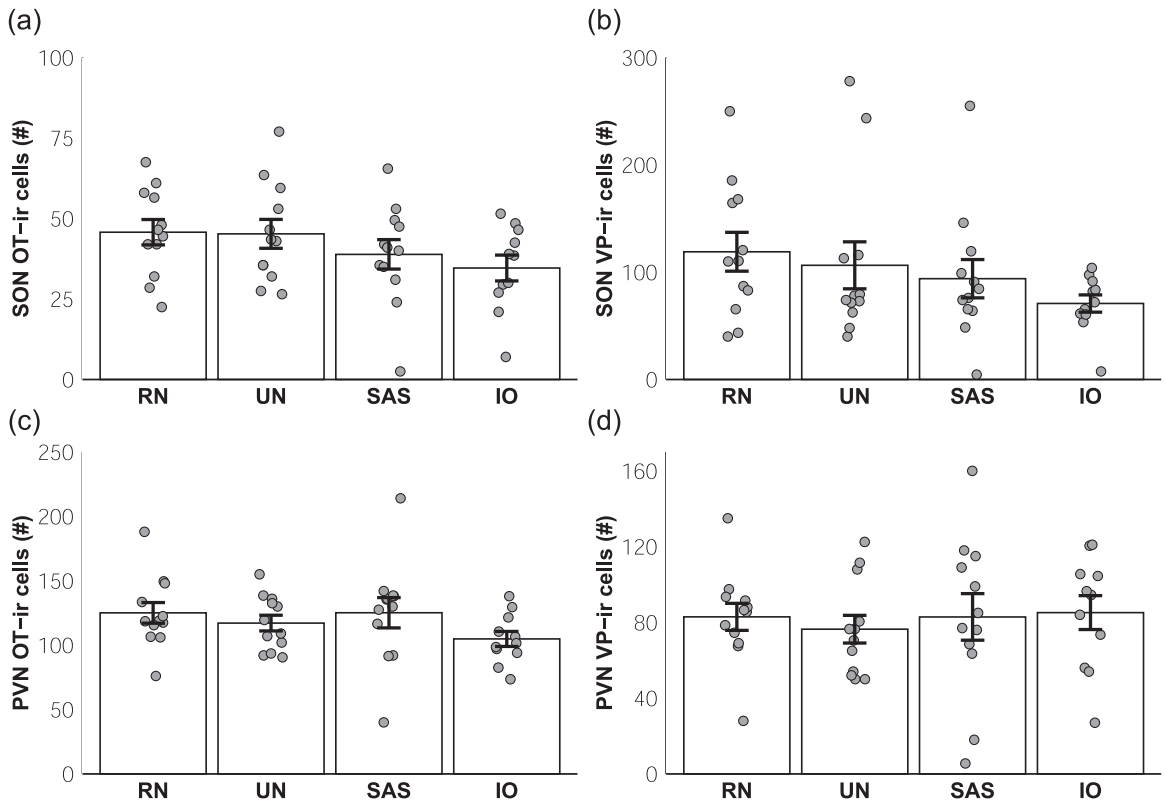

Fig. 5. Mean (+SEM) number of nonapeptide containing neurons in supraoptic nucleus of the hypothalamus (SON) and (b) paraventricular nucleus of the hypothalamus (PVN). (a) Number of oxytocin (OT) immunoreactive (ir) cells in the SON. (b) Number of vasopressin (VP)-ir cells in the SON. (c) Number of OT-ir cells in the PVN. (d) Number of VP-ir cells in the PVN. Dots represent the individual data in each group.

stimulus than with an inanimate object [59]. We sought to determine whether subadults exhibit more alloparental care with kin than unrelated neonates, and found that subadult males exhibited similar behavior toward neonates, irrespective of kin status. To our surprise, we observed no differences in PVN and SON VP or OT neural activity (as indicated by colocalization with cFos) between groups, suggesting that hypothalamic nonapeptides do not differentially respond to the social contexts we examined in subadult male prairie voles. These findings may reflect the dynamic nature of hypothalamic nonapeptides, which are involved not only in alloparental care, but in various social and non-social behaviors [80] and/or may be a result of under-developed functions of nonapeptide neurons in subadults (discussed below).

4.1. Do subadult prairie voles provide care differently to related and unrelated neonates?

Our subjects showed no difference in the amount of alloparental care provided to related and unrelated neonates. This lack of support for kin discrimination may suggest that subadult prairie voles engage in alloparenting to gain practice with parenting. If the practice hypothesis provides a plausible explanation for the evolution of alloparental care among these animals, then they should derive benefits for their own future offspring as a result of practicing during the subadult period. Indeed, alloparenting experience during the subadult experience improves parenting, with experienced prairie vole alloparents having pups that gain weight faster than inexperienced alloparents [36]. Moreover, if subadults are indeed engaging in alloparental care as a way to practice for having their own offspring, then they should reduce alloparental care of younger siblings around sexual maturity (~PND 45) in favor of providing care for their own offspring to maximize direct reproductive success. In support of this prediction, virgin male and female prairie voles over 40 days old are more likely to attack unrelated pups than voles under 40 days old [54]. Notably, this observation is more pronounced in female prairie voles; subadult female prairie voles (~PND 21–45) are alloparental, but become infanticidal soon after (by ~PND 90) [81]. Taking our results together in the context of other studies, our data are consistent with the interpretation that alloparental care among subadult male prairie voles offers valuable practice for when these individuals have pups of their own.

4.2. Does subadult alloparental care align with adult alloparental care?

Although lack of support for kin discrimination is a new finding in subadult prairie voles, this result is consistent with data from adults also showing that adult prairie voles do not exhibit differences in alloparental care based on kin-status [82]. Instead, adults appear to discriminate between kin on the basis of familiarity. For example, cross-fostering prairie vole pups at an early age eliminates sibling discrimination and can lead to biological siblings mating with each other upon reintroduction [83,84]. Similar to other rodents, adult prairie vole parents appear to be insensitive to kinship of their own offspring, and they will readily accept neonates as long as cross-fostering occurs when the neonates are young [85,86]. Adult prairie voles will, however, attack unrelated, novel weanlings but demonstrate parental behaviors toward their own weanlings [87]. Thus, social familiarity appears to be more salient to adult prairie voles than relatedness.

Our study is unique in its focus on how *subadults* behave with neonates; previous prairie vole alloparental studies have centered on *adult* alloparental behavior. Studies focused on adult alloparental care have shown that although some individuals will engage in alloparental care, adult prairie voles are generally more likely than subadults to attack novel neonates [54,81,87]. It is currently unclear why adults might express more infanticidal tendencies, whereas subadults appear to be more alloparental. For instance, it could be more adaptive for adult male

prairie voles to increase their own direct fitness by killing unfamiliar pups and subsequently mating with the female instead of gaining parenting experience through alloparenting [88–90]. In contrast, subadults cannot yet sire their own pups, possibly increasing the possible pay-off value of continued practice parenting on neonates. Still, the fact that some adults are alloparental suggests that parental practice alone cannot account for the existence of alloparental care. Nevertheless, the reduction in the ubiquity of alloparental care for neonates (familiar or unfamiliar) over advanced stages of life history suggests that unlike adults, subadult prairie voles might benefit from providing alloparental care indiscriminately and for reasons other than primarily enhancing immediate reproductive success.

At a proximal level, the behavioral differences between subadults and adults could be due to differences in hormonal profiles. For example, a study that examined developmental trajectories in behavioral interactions with same-sex, novel conspecifics in prairie voles found that aggressive behavior increases and prosocial behavior decreases as male prairie voles reach adulthood [73]. Such changes in social behavior are sure to be accompanied by developmental changes in hormone profiles associated with the transition to sexual maturity. It is tempting to speculate that such changes in hormonal milieu could be permissive for age-specific indiscriminate subadult alloparenting, possibly for the ultimate purpose of parental practice. As animals approach adulthood, these hormonal changes might decrease the probability of engaging alloparental care, in effect enabling individuals to invest preferentially in direct fitness enhancing behaviors. Although it is always advisable to take caution when mixing proximate and ultimate levels of analysis, using them to inform each other is a very powerful approach to achieve a deeper understanding of the fully behaving animal [91]. Additional work will benefit from this approach as it explores the developmental impacts of hormonal shifts on alloparental care and other social behaviors.

4.3. Can providing alloparental care benefit subadult prairie voles both directly and indirectly?

Preferential treatment of nestmates, irrespective of kinship, could still promote indirect fitness benefits if there is a high probability that animals at the nest are related. Furthermore, indirect benefits by investment in kin (even without evidence for kin recognition) and direct benefits associated with parental practice resulting from alloparental caregiving are not mutually exclusive. Indiscriminate care for neonates at the natal nest could be a kin-based approach for providing alloparental care, but only if the likelihood of caring for unrelated neonates is low [92,93]. Indeed, species are more likely to show kin discrimination when the average relatedness within groups is lower and more variable [92]. The relatively low level of natal dispersal among prairie voles [57] suggests a generally high level of relatedness among alloparents and neonates in the nest. Although there is a relatively high prevalence of extra-pair copulations and cuckoldry in prairie voles [94], the chances of sharing a genetic relationship are still high in members of the natal nest because prairie voles at a nest most likely share the same mother. Therefore, we cannot exclude the possibility that indiscriminate alloparental care offers some indirect fitness benefits to prairie voles. However, a non-insignificant portion of communal nests also contain unrelated breeding adults [56], and subadults would gain no indirect fitness benefits caring for these unrelated offspring. Additionally, more than half of dispersing subadults do so after reaching sexual maturity, suggesting a benefit for remaining at the natal nest at the cost of direct reproduction [57]. Thus, although we cannot definitively rule out the potential for indirect fitness benefits, we believe that alloparental care in subadult prairie voles may be best explained by the parental practice hypothesis. At a minimum, our results suggest that subadult alloparental care is unlikely to have solely evolved to promote indirect fitness, and could have primarily evolved for the purpose of providing practice for parental behaviors.

4.4. Alloparental care and interactions with the brain

Our neural data may provide further support for the hypothesis that parental practice primarily explains alloparental care in subadult prairie voles. Unlike studies in adult prairie voles that showed increased activity of OT-ir and VP-ir containing neurons [59], our subjects did not demonstrate context-specific activation of these neurons in any of the stimulus groups. This suggests that 1) OT and VP may not mediate alloparental care in subadults, 2) subadult brains may be inherently different from adult brains, and 3) experience alloparenting as subadults may alter developmental trajectories to bring the neural structures underlying kin discrimination in adulthood online.

Juvenile and adult PVN and SON neuronal activity clearly appear to differ, however, we acknowledge that we did not directly test adult neural responses to various stimuli in this study and methodological differences between labs could explain why our subadults demonstrate different neural activity in response to neonates than adults. Nevertheless, both the PVN and SON are remarkably inert during early development in prairie voles. Specifically, pups at PND 2, PND 9, and PND 21 do not show VP and OT neural activity (i.e., cFos co-labeling) in the PVN and SON, as would be expected in adults [74]. On the other hand, the PVN and SON of adult prairie voles show context-specific activation of OT and VP containing cells. For example, exposure to neonates increases double-labeling with cFos in OT-ir and VP-ir neurons in adult male prairie voles [59]. The PVN and SON are clearly undergoing changes between the juvenile period and the adult period in prairie voles that ultimately lead to context-specific activation of the PVN and SON. Given the lack of differences in OT-ir and VP-ir neuronal expression between any of the four stimulus groups to which we exposed subadult males, our results suggest that subadults have a PVN and a SON that are more similar to juveniles than adults. This, in turn, indicates that the brains of subadults are still under development and must undergo further maturation to eventually allow the neural responses observed in adult alloparents. Taken further, although they clearly play a role in adult alloparental care, OT and VP neurons within the SON and PVN do not appear to be involved in subadult alloparental care, and alloparental care in subadults might be facilitated by a different set of neural mechanisms than adults.

Additionally, although it is expected that neural structures, and the function of signaling molecules and modulatory mechanism therein, naturally change as subadults age to support adult behavior, practice parenting through indiscriminate alloparenting of neonates likely plays an important role in shaping these neural structures. Prior work in prairie voles has shown alloparenting experience can have a direct effect on brain signaling. For instance, experience in the natal nest as an alloparent increases anxiety-like behavior and decreases exploration in prairie voles, and decreases brain-derived neurotrophic factor in the CA1 region of the hippocampus [95]. Similar effects have been shown in other species as well. For example, exposing adult male mandarin voles (*Microtus mandarinus)* to a novel pup for 10 min increases OT neurons in the SON and PVN one week later [96]. Recall that alloparental experience as a subadult has important implications for the health of pups in adulthood [36]. Considering that brains are shaped by alloparenting practice, it is plausible that practice parenting may contribute to shaping and fine-tuning the neural structures important for quality parental care in adulthood, thus providing a potential mechanistic explanation for how parental practice might benefit subsequent adult behavior.

4.5. Other potential mechanisms driving alloparental care in subadults

Our results suggest that OT and VP are not modulating alloparental care in subadult prairie voles. We focused our study on the major sites of nonapeptide production (the PVN and SON), but the targets of these signaling molecules could also offer insight into the neural modulation of alloparental care. If OT and VP are modulating alloparental behavior, brain regions that are integral to alloparental care should contain OT

and VP receptors and show cFos activation in response to alloparental stimuli. There is little evidence, however, that other OT- and VP- containing brain regions are activated by alloparental contexts. For instance, adult prairie voles exposed to novel pups show no difference compared to control subjects in cFos, OT, or VP expression in the bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA) [59]. In subadult prairie voles, Perkeybile et al. found no relationship between alloparental care and OT or VP receptor densities in any of the structures commonly associated with alloparental care (e.g., the medial preoptic area, BNST, CeA, lateral septum, and nucleus accumbens) [86]. Furthermore, although one study has shown OT receptor density in the nucleus accumbens to be positively correlated with alloparental behavior [46], overexpressing OT receptors in this region does not increase alloparental behavior [97]. These results together suggest that OT and VP action in these brain regions is minimally involved in alloparental behavior in subadults.

A more plausible interpretation of the aforementioned studies and our data is that alloparental behavior in subadults is mediated by mechanisms other than OT and VP. One likely mechanism may be estrogen receptors [96,98,99]. For instance, the activation of the estrogen receptor ERα in subadult prairie voles increases aggression towards pups [98]. Dopamine and glucocorticoids also appear to modulate alloparental behavior [100–103]. For example, pharmacological blockade of the dopamine D1 receptor in the ventromedial hypothalamus impairs the onset of adult male alloparental behavior [101], and female subadult prairie voles treated with corticosterone show reduced alloparental behavior [102]. Future research should explore other possible mechanisms important for alloparental care in subadult prairie voles.

5. Conclusion

Our results suggest that the practice parenting hypothesis (possibly alone or in combination with benefits associated with indirect fitness benefits resulting from indiscriminate care) provides a good explanation for the presence of alloparental care in subadult male prairie voles. Neural differences between the subadults in our study and adult prairie voles in other studies further imply that, at the very least, alloparenting in subadults is facilitated by different neural structures and/or mechanisms than in adulthood. Notably, our results indicate that practice parenting as subadults could facilitate the fine-tuning of the neural mechanisms important for adult parenting. Overall, these results implicate the subadult period in prairie voles as an important developmental stage that prepares animals for optimal parental care in adulthood [104]. Future studies should examine just how important subadult alloparental practice is for subsequent parenting quality and the long-term reproductive success of individuals.

Declarations of interest

None.

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