Published in final edited form as:

Horm Behav. 2013 August ; 64(3): . doi:10.1016/j.yhbeh.2013.07.004.

VPAC Receptor Signaling Modulates Grouping Behavior and Social Responses to Contextual Novelty in a Gregarious Finch: A Role for a Putative Prefrontal Cortex Homologue

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Abstract

In both mammals and birds, vasoactive intestinal polypeptide (VIP) neurons and fibers are present in virtually every brain area that is important for social behavior. VIP influences aggression in birds, social recognition in rodents, and prolactin secretion in both taxa, but other possible functions in social modulation remain little explored. VIP effects are mediated by VPAC receptors, which bind both VIP and pituitary adenylate cyclase activating peptide. Within the lateral septum and medial bed nucleus of the stria terminalis, VPAC receptors are found at higher densities in gregarious finch species relative to territorial species, suggesting that VPAC receptor activation promotes social contact and/or preference for larger groups. Here we here test this hypothesis in zebra finches (*Taeniopygia guttata*), and also examine the relevance of VPAC receptors to anxiety-like processes. Intraventricular infusions of the VPAC receptor antagonist, neurotensin6–11–mouseVIP7–28, strongly reduce social contact when animals are tested in a novel environment, and exert sex-specific effects on grouping behavior. Specifically, VPAC receptor antagonism reduces gregariousness in females but increases gregariousness in males. Interestingly, VPAC antagonism in the medial pallium (putative prefrontal cortex homologue) significantly reduces gregariousness in both sexes, suggesting site-specific effects of VIP signaling. However, VPAC antagonism does not modulate novel-familiar social preferences in a familiar environment or general anxiety-like behaviors. The current results suggest that endogenous activation of VPAC receptors promotes social contact under novel environmental conditions, a function that may be accentuated in gregarious species. Moreover, endogenous VIP modulates gregariousness in both males and females.

Introduction

Vasoactive intestinal polypeptide (VIP) is a neuropeptide that is produced and released by numerous hypothalamic and extrahypothalamic cell groups and is perhaps best known for its role in the stimulation of prolactin release (Christensen and Vleck, 2008; Christian et al., 2007; El Halawani et al., 1996; Lam, 1991; Macnamee et al., 1986; Maney et al., 1999; Vleck and Patrick, 1999). In addition to preoptic and hypothalamic areas (e.g. anterior hypothalamus, AH, and ventromedial hypothalamus, VMH), VIP receptors, cells and/or fibers are also present in many other areas that comprise a social behavior network

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(Goodson, 2005; Newman, 1999), including the medial extended amygdala (medial amygdala and medial bed nucleus of the stria terminalis, BSTm), midbrain central gray, ventral tegmental area and lateral septum (LS) (Antonopoulos et al., 1987; Bottjer and Alexander, 1995; Hof et al., 1991; Joo et al., 2004; Kuenzel and Blahser, 1994; Kuenzel et al., 1997; Roberts et al., 1982; Sims et al., 1980). Collectively, these brain areas regulate social recognition, affiliation, grouping, aggression, appetitive and consummatory sexual behavior, parental behavior and responses to social stress (Goodson, 2005; Newman, 1999), and thus VIP may play important roles in numerous aspects of social behavior.

Not surprisingly, given these anatomical observations, central VIP elements are extensively regulated by steroid hormones and in turn influence steroid production. Central VIP elements are also seasonally variable. For instance, seasonally breeding sparrows exhibit winter decreases in VIP immunoreactivity across many socially relevant brain areas, including the preoptic area, multiple hypothalamic nuclei, multiple subnuclei of the septum, BSTm, midbrain central gray, ventral tegmental area and nucleus accumbens (Goodson et al., 2012c). Experimental data in sparrows further indicate that some of this seasonal variation is estrogen-dependent (Wacker et al., 2008), and similarly, VIP mRNA is rapidly upregulated in the hypothalamus and other brain areas by estrogen treatment in rats (Gozes et al., 1989; Kasper et al., 1992). VIP in turn modulates the secretion of gonadotropin-releasing hormone in rats (Christian and Moenter, 2008). Finally, central VIP release stimulates secretion of adrenocorticotrophic hormone and corticosterone, and at least part of this effect is mediated via VIP release in the paraventricular nucleus of the hypothalamus (Alexander and Sander, 1994, 1995).

Despite these VIP-hormone interactions and the prevalence of VIP peptide and receptors throughout socially relevant brain areas, very little is known about the specific roles of VIP signaling in social behavior. VIP is thought to be important for parental behavior, given that it is the primary prolactin releasing factor in birds (El Halawani et al., 1997) and prolactin secretion is particularly important for egg incubation in galliforms (El Halawani et al., 1984). Correlational studies show that levels of plasma prolactin mirror levels of VIP cell density within the infundibular hypothalamus, both of which are highest during egg incubation and rearing, and lowest following nest deprivation and in non-rearing hens (Chaiyachet et al., 2012; Cloues et al., 1990). Similarly, parallel changes in VIP cell size and prolactin levels are observed during the incubation and brooding stages in ring doves (Cheng and Burke, 1983; Cloues et al., 1990; Goldsmith et al., 1981). Interestingly, VIP-induced prolactin secretion is reduced by testosterone, apparently at the level of the pituitary (Knapp et al., 1988), suggesting a potential mechanism for the tradeoffs commonly observed between testosterone-mediated behaviors (e.g., aggression) and parental behavior (McGlothlin et al., 2007). VIP innervation of the LS also varies between color morphs of the white-throated sparrow (*Zonotrichia leucophrys*) that differ in their levels of parental care and aggression (Maney et al., 2005).

Only a handful of studies have directly manipulated VIP signaling *in vivo* and examined the consequences for social behaviors. Reduction of VIP signaling during mouse embryogenesis via VPAC antagonism *in utero*, or through the use of VIP deficient mothers (VIP +/- or VIP -/-), leads to deficits in normal play behavior, affiliative behavior, social recognition and responses to social novelty in the adolescent and adult offspring (Hill et al., 2007a; Hill et al., 2007b; Lim et al., 2008; Stack et al., 2008).

Recent findings in birds also establish an important role for VIP in aggressive behavior, most notably through the identification of an aggression-specific VIP cell group in the AH of finches (Goodson et al., 2012b). Antisense knockdown of VIP production in the AH essentially abolishes resident-intruder aggression in territorial violet-eared waxbills

(Estrildidae: *Uraeginthus granatina*) and greatly reduces aggression in the context of nest defense in colonial zebra finches (Estrildidae: *Taeniopygia guttata*). However, VIP knockdown in the AH has no impact on other aspects of behavior such as social contact, gregariousness, courtship, pair bonding and anxiety-like behaviors. Interestingly, aggression in the context of mate competition is not influenced by VIP knockdown, and thus the AH VIP neurons are both behaviorally and contextually specific in their functions. Similarly, infusions of VIP into the septum (an AH projection target; (Atoji and Wild, 2004) facilitate aggression in the context of territory defense, weakly reduce aggression in the context of mate competition (Goodson, 1998b; Goodson and Adkins-Regan, 1999), and inhibit territorial dawn song in male field sparrows (Goodson, 1998a). Also consistent with these findings is the observation that VIP innervation densities in the AH and caudocentral septum (an area partially homologous to the posterior septal nuclei in mammals) correlate positively with aggression in free-living sparrow species, and differentiate the more aggressive song sparrow (Emberizidae: *Melospiza melodia*) from the less aggressive field sparrow (Emberizidae: *Spizella pusilla*) (Goodson et al., 2012c).

In contrast to these findings for the AH and caudocentral septum, VIP cell numbers in the tuberal hypothalamus are negatively related to aggression in violet-eared waxbills (Goodson et al., 2012b), and VIP innervation density in an adjacent area, the VMH, is negatively correlated with aggression in sparrows. Again, the innervation densities differentiate field sparrows from song sparrows (Goodson et al., 2012c). These findings suggest that different VIP circuits are functionally distinct, and indeed, whereas the VIP innervation of some brain areas is associated with aggression, innervation of the BSTm and paraventricular hypothalamus is greater in sparrow species that flock during winter as opposed to those that do not (Goodson et al., 2012c). Similarly, flocking finch species exhibit higher densities of VIP binding sites in the BSTm and subpallial LS (Goodson et al., 2006). Peptide circuits of the BSTm and LS are particularly important for grouping behavior and social affiliation in birds and mammals, although this has been primarily studied with regard to the nonapeptides (Curley et al., 2009; Curley et al., 2012; Goodson et al., 2012a; Goodson et al., 2009a; Goodson et al., 2009b; Goodson and Wang, 2006; Goodson et al., 2012c; Kelly et al., 2011; Liu et al., 2001; Ophir et al., 2009).

Given these links to flocking, we hypothesized that VIP signaling modulates social contact and/or gregariousness (i.e., preference for larger groups). In addition, based on the findings in mice (Hill et al., 2007a; Stack et al., 2008), we hypothesized that VPAC receptor activation would promote approach to novel social stimuli. To test these hypotheses, we performed a variety of social behavior assays following central antagonism of VPAC receptors in male and female zebra finches, a highly colonial finch species. VIP effects in birds and other vertebrates are mediated by VPAC receptors (i.e. VPAC1 and VPAC2), which bind both VIP and pituitary adenylate cyclase activating peptide (PACAP) (Vaudry et al., 2000). PACAP also acts through a single PACAP receptor, PAC1, which binds PACAP with high affinity and VIP with very poor affinity (Vaudry et al., 2000; Zawilska et al., 2003). We here used a selective VPAC receptor antagonist (neurotensin6–11-mouseVIP7–28), which is known to block VIP stimulation of cyclic AMP production in chick brain slices without blocking PAC1 receptors (Nowak et al., 2003). We assessed antagonist effects primarily following infusions into the lateral ventricle, but also report findings for infusions into the medial telencephalon.

Methods

Subjects

A total of 61 adult zebra finches (22 females and 39 males) were used for the present study. Prior to surgery, male and female subjects were housed with same-sex conspecifics in 61 cm

W × 36 cm D × 41 H cm wire cages. Following surgery, same-sex subjects were housed together in groups of 6 or 7. Three days before the start of behavioral tests, a 91 cm W × 36 cm D × 41 H cm wire cage containing 12 untreated same-sex conspecifics was placed directly adjacent to the subject cage in order to acclimate subjects to their “familiar” stimulus birds. An additional group of 12 untreated same-sex conspecifics served as “novel” stimulus birds and were housed together in a 91 cm W × 36 cm D × 41 H cm cage that was not visible to subject animals. Subjects had not been exposed to the novel birds for at least 4 months. Cannulated subjects within a cohort were exposed to the same novel and familiar stimulus animals. All animals were maintained on a 14L:10D photoperiod and provided seed mix and water *ad libitum*. Experiments were performed in accordance with federal and institutional guidelines for the ethical treatment of animals and were approved by the Animal Care and Use Committee of Indiana University.

Surgeries

Subjects were anesthetized with isoflurane vapor, administered at 1.8–3.5% of a compressed air flow. Each subject was stereotaxically fitted with a unilateral 26-gauge chronic guide cannula (Plastics One, Arkon, OH) directed at the right lateral ventricle. Based on interesting results from “misses” that fell just short of the ventricle and thus targeted the medial telencephalon, we complemented the intracerebroventricular (ICV) dataset with a smaller dataset for the medial telencephalon (TEL; see Results for placements). ICV cannulae were positioned 3.0 mm rostral of the cerebellum, 0.75 mm lateral of the midline and 2.5 mm deep at an angle of 15° to avoid midline vasculature. Cannulae were secured to the skull using dental acrylic (Stoelting Co. Wood Dale, IL) and veterinary-grade tissue adhesive (3M Vetbond, Fisher Scientific Pittsburgh, PA) and kept closed by a dummy wire affixed to a threaded cap (Plastics One). Animals were allowed 6 days for recovery before infusions and testing.

Infusions

Thirty min before the start of behavioral testing subjects were restrained by hand and unilaterally infused with either 0.5 µl of isotonic saline or 0.5 µl isotonic saline containing 250 ng of neurotensin6–11-mouseVIP7–28 (H-9935, Bachem, Torrance, CA) over a 2 min period. This dose produces significant behavioral effects *in vivo* that mirror those obtained through antisense knockdown of VIP production (Goodson et al., 2012b). Previous experiments have shown that the neurotensin6–11-mouseVIP7–28 antagonist completely blocks VIP stimulated cAMP production after a 30 min incubation period (Nowak et al., 2003). Infusions were performed using a microsyringe (Hamilton Co., Reno, NV) connected to polyethylene tubing and an injector (Plastics One) that extended 1 mm below the guide cannula tip.

Behavioral testing

Gregariousness and social contact—Subjects were first tested for social contact and gregariousness using the choice apparatus shown in Fig. 1. Antagonist and vehicle infusions were delivered in a counterbalanced order, with 2 days allowed between tests (repeated measures design). The choice apparatus was set up in the housing room on the day of testing and was screened from view with the use of blinds. Thus, the first test was conducted in a novel environment. The apparatus consisted of a testing cage (1 m W × 43 cm H × 36 cm D) subdivided into zones by seven perches with a stimulus cage (46 cm × 43 cm H × 36 cm D) placed at each end. The stimulus cages contained either two or ten familiar same-sex conspecifics, with the side of presentation counterbalanced across subjects. The percent of time spent on the two end perches combined (within 6 cm of either stimulus cage) provided a measure of “social contact,” and the percent of that contact time that the subject spent next

to the large group provided a measure of “group size preference” or “gregariousness.” Subject location was recorded every 15 sec for 5 min by an experimenter blind to treatment condition and hidden from view through the use of a blind.

Novel-familiar preference test—Novel-familiar choice tests were performed as described above for social contact and gregariousness, with the exception that the stimulus cages contained either 6 familiar (selected from the same group of familiar subjects used for gregariousness testing) or 6 novel same-sex birds. We again measured social contact, and operationally defined “preference for novel individuals” as the percent of social contact time that was spent with the group of novel birds.

Anxiety-like behavior—On the final testing day, subjects were administered two tests of anxiety-like behavior using a between subjects design. The first test measured the subject’s latency to feed in the presence of a novel object. Novelty suppression of feeding tests are increasingly used as measures of anxiety (Bilkei-Gorzo et al., 2002; Catts et al., 2008; Greene et al., 2009; Leuner et al., 2010) and have been readily adapted to birds (Kelly et al., 2011). For this test, food was removed from the subject’s cage just prior to lights-on in the morning. Subjects were then immediately infused with either the VPAC receptor antagonist or saline and placed back into their home cage. Forty-five min after infusion, each subject was placed into a small cage (31 cm W × 20 cm H × 36 cm D) that contained a small food dish and a purple nitrile glove (“novel object”) hanging beside the food dish. A 45 min delay (as opposed to 30 min for choice tests) was based on logistical considerations, given that infusions for all subjects in a cohort had to be completed before the early AM tests of novelty suppressed feeding. Testing cages were placed in the housing room but subjects were visually isolated from all other birds through the use of blinds. Subjects were videotaped for 45 min and the latencies to approach the food dish and to feed were recorded by an experimenter blind to treatment condition.

We additionally measured exploration of a novel environment, an anxiety assay widely used in birds (Biondi et al., 2010; Groothuis and Carere, 2005; Kelly et al., 2011; Martins et al., 2007). For this second test, each subject was placed into a testing cage (122 cm W × 42 cm H × 36 cm D) containing 3 clusters of novel evergreen branches. The testing cage was located in a small, unfamiliar room. The number of hops, number of visits to evergreen clusters (i.e. zone changes), and total number of branches touched were recorded during a 3 min test by an experimenter blind to the subject’s condition and hidden from view through the use of a blind.

Because the anxiety assays were conducted in a between-subjects design (not within-subjects, as with other tests), the *n*’s for each treatment condition were effectively halved relative to the social preference assays. Thus, in order to bolster subject numbers for the anxiety assays, we used additional cannulated subjects that had previously been tested for social preferences in a similar design as that here, but with other peptide treatments not relevant to the current study. Therefore, only anxiety data are included here for those subjects.

Histology

Immediately following the exploration assay, subjects were sacrificed by isoflurane overdose and transcardially perfused with 0.1M phosphate buffered saline (PBS) followed by 10% formalin. Brains were removed, post-fixed for 1 hour and cryoprotected in 30% sucrose in PBS overnight at room temperature. Brains were sectioned on a cryostat at 100 μm and tissue was mounted onto chrom-alum subbed slides to verify cannula placement and infusion site.

Statistics

Data from social preference tests were conducted using repeated measures ANOVA with Sex and Treatment as between-subjects factors. Initial analyses also included Treatment Order as a factor, although for simplicity, we eliminated this factor from the final analyses if it made no significant contribution to the model. Data from anxiety assays were analyzed using ANOVA, with Sex and Treatment as between-subjects factors. Data for ICV and TEL infusions were analyzed separately.

Results

Cannula placements

Data are reported here for a total of 61 subjects, 30 (13 females and 17 males) with cannula placements in the right ventricle, and 31 with placements in the medial telencephalon (TEL, 9 females and 22 males). The majority of telencephalic placements were centered in the medial nidopallium, with other placements falling either slightly more dorsally (in the mesopallium or apical part of the hyperpallium) or slightly more ventrally (in the medial striatum). Based on relatively low n 's for these placements, they were combined for analysis into a single TEL group. However, based on diffusion and the clustering of placements in and near the nidopallium, it is likely the nidopallium was consistently affected by telencephalic infusions. Notably, the medial nidopallium exhibits a very high density of VIP binding sites (Goodson et al., 2006) and occupies a similar position to the prefrontal cortex in a limbic loop that includes the thalamus and nucleus accumbens (Husband and Shimizu, 2011).

VPAC receptor antagonism decreases social contact in a novel environment

Following drug infusions on the first two days of testing (days 1 and 3), subjects were placed into the choice apparatus and given a chance to affiliate with 2 or 10 same-sex conspecifics (Fig. 1). For the total amount of time spent in social contact (i.e., next to both stimulus cages combined), initial ICV analyses including Treatment Order revealed a significant contribution of Treatment Order ($F_{19,1,1,1} = 8.45$; $P < 0.01$) and a strong trend towards an interaction between Treatment Order and Treatment ($P = 0.06$; partial eta squared = 0.167), such that the antagonist reduced social contact primarily on the first day of testing, when the testing environment was novel. Because we observed no effect of Sex, we ran the analysis again omitting it, which yielded a significant interaction of Treatment Order and Treatment ($F_{21,1,1} = 5.28$; $P = 0.03$; partial eta squared = 0.201). Results of this simplified ANOVA are much easier to interpret in graphical form, and are shown in Fig. 2.

In contrast to the ICV findings, no significant effects of Treatment Order were observed for TEL infusions (main effect: $F_{11,1,1,1} = 0.08$; $P > 0.50$; Treatment Order*Treatment: $F_{11,1,1,1} = 0.14$; $P = 0.72$) and TEL infusions of the VPAC antagonist produced no effects on social contact ($F_{13,1,1} = 0.07$; $P = 0.79$).

VPAC receptor antagonism has site- and sex-specific effects on gregariousness

Analyses of gregariousness (group size preference) for ICV subjects did not reveal a significant contribution of Treatment Order ($F_{15,1,1,1} = 0.51$; $P > 0.40$) or an interaction of Treatment Order and Treatment ($F_{15,1,1,1} = 0.63$; $P > 0.40$). Thus, Treatment Order significantly influenced *social contact* in this assay, as reported above, but did not influence *gregariousness*. However, we did observe a significant interaction of Sex and Treatment in this first analysis that included Treatment Order in the model ($F_{15,1,1,1} = 5.32$; $P < 0.04$) and also in a subsequent analysis that omitted it ($F_{17,1,1} = 6.54$; $P < 0.02$; partial eta squared = 0.278). Specifically, ICV infusions of the VPAC antagonist tended to decrease

gregariousness in females while increasing it in males (Fig. 3A). Interestingly, TEL analyses revealed a significant main effect of Treatment ($F_{12,1,1} = 5.84$; $P < 0.03$; partial eta squared = 0.327) such that VPAC antagonism at this site significantly decreased preferences for the larger group of conspecifics in both males and females (Fig. 3B). An interaction effect was not observed for Sex and Treatment ($F_{10,1,1,1} = 0.05$; $P > 0.80$) nor for Treatment Order when Treatment Order was included in the analyses ($F_{10,1,1,1} = 0.42$; $P > 0.50$).

VPAC receptor antagonism does not influence novel-familiar social preferences

Following drug infusions on the third and fourth days of testing (days 5 and 7), subjects were placed into the choice apparatus and given a chance to affiliate with 6 novel or 6 familiar same-sex conspecifics. Unlike the first testing day, ICV VPAC antagonism on these later test days did not affect social contact time ($F_{19,1,1,1} = 1.98$; $P = 0.18$) nor was there a significant contribution of Treatment Order ($F_{19,1,1,1} = 0.33$; $P > 0.50$) or an interaction of Treatment Order and Treatment ($F_{19,1,1,1} = 0.28$; $P > 0.60$). Sex also did not contribute significantly to the model ($F_{19,1,1,1} = 0.12$; $P > 0.70$).

For subjects with ICV placements, an analysis of time spent with novel individuals that included Treatment Order and Sex did not reveal a significant contribution of Treatment Order ($F_{17,1,1,1} = 0.48$; $P > 0.40$) nor an interaction effect for either Treatment Order ($F_{17,1,1,1} = 0.26$; $P > 0.60$) or Sex ($F_{17,1,1,1} = 0.39$; $P > 0.50$) with Treatment.

Similar to findings for ICV subjects, VPAC antagonism within the TEL on tests days 3 and 4 did not affect social contact time ($F_{19,1,1,1} = 1.98$; $P > 0.17$) nor was there a significant contribution of Treatment Order ($F_{19,1,1,1} = 0.33$; $P > 0.50$) or an interaction effect for Treatment Order with Treatment ($F_{19,1,1,1} = 0.28$; $P > 0.60$). Sex also did not contribute significantly to the model ($F_{19,1,1,1} = 0.14$; $P > 0.70$). Likewise, VPAC TEL antagonism did not affect the percent of time spent next to novel conspecifics ($F_{17,1,1,1} = 0.97$; $P > 0.33$), nor were there any significant effects when Sex and Treatment Order were included in the model ($P > 0.25$).

VPAC receptor antagonism has no effect on non-social anxiety-like behavior

The results obtained from the first two testing days demonstrate that VPAC receptor antagonism decreases social contact in a novel environment, suggesting that endogenous VIP may reduce anxiety in novel social situations. To further examine VIP's role in anxiety, we performed two behavioral tests, novelty suppressed feeding and exploration of a novel environment on the fifth day of testing (day 9 overall), that have previously been used to measure general anxiety-like behavior in zebra finches (Kelly et al., 2011; Martins et al., 2007). Regardless of the infusion site, we observed no differences in either the latency to approach the food dish (ICV, $F_{26,1,1} = 1.10$; $P > 0.30$; TEL, $F_{28,1,1} = 0.05$; $P > 0.80$) or the latency to feed (ICV, $F_{26,1,1} = 1.26$; $P > 0.20$; TEL, $F_{28,1,1} = 0.04$; $P > 0.80$) in the presence of a novel object for animals that received antagonist infusions relative to those that received saline. Similarly, during the exploration of a novel environment, subjects that received antagonist infusions did not differ from control subjects in the number of zone changes (ICV, $F_{24,1,1} = 0.01$; $P > 0.90$; TEL, $F_{28,1,1} = 0.94$; $P > 0.30$), the number of hops exhibited (ICV, $F_{24,1,1} = 1.07$; $P > 0.30$; TEL, $F_{28,1,1} = 0.16$; $P > 0.60$) or the number of branches touched (ICV, $F_{24,1,1} = 1.83$; $P > 0.18$; TEL, $F_{28,1,1} = 1.09$; $P > 0.30$). There was also no significant contribution of Sex to any of the dependent measures in the novelty suppressed feeding paradigm ($P > 0.30$) or in the exploration of a novel environment test ($P > 0.18$).

Discussion

Although numerous observations suggest that VIP and VPAC receptors may be important for a diversity of social behaviors, many potential functions remain unexplored. We now show that VPAC receptor antagonism exerts site- and sex-specific effects on gregariousness, and strongly reduces the amount of time subject animals spend in social contact when they are first tested in a novel environment. Interestingly, VPAC antagonism has no effect on subsequent testing days with the same testing apparatus, even when novel stimulus animals are presented on test days 3 and 4. These results suggest that endogenous activation of VPAC receptors promotes social contact under novel environmental conditions. Furthermore, we observed no effect of VPAC antagonism on two tests of general anxiety-like behavior, despite the fact that these testing apparatus were also novel to the subjects. Together, these findings suggest that endogenous VIP signaling specifically promotes social contact in response to contextual novelty, but does not modulate anxiety processes more generally.

Site- and sex-specific effects of VPAC antagonism on gregariousness

Effects of VPAC antagonism are site- and sex-specific. Delivered ICV, the VPAC antagonist tends to reduce gregariousness in females but increase gregariousness in males. However, VPAC antagonism in the medial telencephalon (centered in and around the medial nidopallium) reduces gregariousness in both sexes. Taken together, these results suggest that gregariousness is modulated via VPAC activation in the medial nidopallium and one or more other brain areas. We hypothesize that at least a portion of the effects observed following ICV infusions are due to the antagonism of VPAC receptors in the BSTm and LS. These two brain areas are directly adjacent to the ventricular infusion site and numerous studies have shown that these two sites are extremely important for the regulation of grouping behavior and affiliation, particularly with respect to nonapeptide signaling (Curley et al., 2009; Curley et al., 2012; Goodson et al., 2012a; Goodson et al., 2009a; Goodson et al., 2009b; Goodson and Wang, 2006; Goodson et al., 2012c; Kelly et al., 2011; Liu et al., 2001; Ophir et al., 2009).

As suggested by a variety of correlational data, VIP likely acts within the BSTm and LS to modulate grouping behavior. For instance, in finch species that exhibit stable grouping phenotypes year-round, flocking species exhibit higher densities of VIP binding sites in the BSTm and subpallial (ventral) LS as compared to non-flocking species (Goodson et al., 2006). Similarly, emberizid sparrows that switch from summer territoriality to winter flocking display greater VIP innervation of the BSTm in winter compared to sparrows that do not flock. This latter pattern in sparrows is also observed in the paraventricular nucleus of the hypothalamus (Goodson et al., 2012c), and thus the paraventricular nucleus may also be a site of action in the present experiments.

Because no significant sex differences in VIP receptor binding densities have been observed in the BSTm and subpallial LS of zebra finches (Goodson et al., 2006), we hypothesize that the sex-specific effects observed following ICV infusions arise from sex differences in VIP innervation and/or sex-specific activation of VIP neurons. Indeed, sex-specific modulation of gregariousness by the nonapeptides is now known to be associated with sex-specific neuronal responses to social stimuli (A.M. Kelly and J.L. Goodson, unpubl. obs.).

In contrast to ICV infusions, infusions of the VPAC antagonist into the medial telencephalon decrease gregariousness in *both* sexes, suggesting that endogenous VIP at this site promotes flocking. Telencephalic infusions were primarily centered on the medial nidopallium, with some placements just dorsal or ventral to it, and thus the medial nidopallium is likely the only area that was consistently affected by our telencephalic

infusions. Notably, the medial nidopallium exhibits a higher density of VIP binding sites than do other nearby regions such as the mesopallium, medial striatum and the apical part of the hyperpallium, although binding sites are observed throughout the majority of the telencephalon (Goodson et al., 2006). Based on these results, we revisited the sparrow dataset of Goodson et al. (2012c) to quantify VIP innervation density in the medial nidopallium, and find that winter flocking sparrows have significantly higher VIP innervation densities in winter than do year-round territorial sparrows (unpubl. obs.), providing convergent evidence that VIP acts in the medial pallium to promote gregariousness.

Interestingly, the results of a recent anatomical study suggest that the medial pallium in pigeons (which includes the medial nidopallium, mesopallium and apical part of the hyperpallium; areas which make up our TEL category), is likely homologous to the mammalian prefrontal cortex, based on 1) the medial pallium's extensive connections with the nucleus accumbens, 2) the finding of a direct connection from the anterior dorsomedial nucleus of the thalamus (homologous to the mammalian mediodorsal nucleus; (Metzger et al., 1996; Montagnese et al., 2003; Veenman et al., 1997) to the pigeon medial pallium and 3) the location of the medial pallium within an avian "limbic loop" that is remarkably similar to a mammalian "limbic loop" that includes the nucleus accumbens, prefrontal cortex, ventral pallidum and mediodorsal thalamus (Husband and Shimizu, 2011). Projections from the mediodorsal thalamus are a major defining feature of the mammalian prefrontal cortex (Divac et al., 1978; Kuroda et al., 1998), and thus it is notable that the dorsomedial thalamus does not project to the caudolateral nidopallium, an area sometimes compared to the prefrontal cortex (for an extensive consideration, see Husband and Shimizu, 2011).

The mammalian prefrontal cortex and nucleus accumbens are part of the mesolimbic dopaminergic reward pathway and are involved in the motivation and reinforcing effects of abusive drugs, as well as natural stimuli (McBride et al., 1999). Not surprisingly, these two key areas of the mesolimbic reward pathway are particularly important for affiliation and social attachment behavior, as demonstrated in prairie voles (Young et al., 2001). Furthermore, the medial pallium also shows increased immediate early gene induction in response to both appetitive and consummatory sexual behavior in Japanese quail (Ball and Balthazart, 2001). Together, these studies suggest that the medial pallium in birds is an important brain area for social affiliation and approach to rewarding stimuli.

It is important to consider that the effects of VPAC receptor antagonism, including the site-specific effects on gregariousness, may also be due to the contributions of PACAP and/or VIP, both of which can bind to VPAC receptors with equal affinity (Harmar et al., 2012; Vaudry et al., 2009). Of note is that PACAP also acts through a single PACAP receptor, PAC1, which binds PACAP with high affinity and VIP with very poor affinity (Vaudry et al., 2000; Zawilska et al., 2003). The selective VPAC receptor antagonist used in the present study, neurotensin6-11-mouseVIP7-28, is known to block VIP stimulation of cyclic AMP production in chick brain slices without blocking PAC1 receptors (Nowak et al., 2003) and also produces behavioral effects *in vivo* that mirror those of antisense-mediated VIP knockdown (Goodson et al., 2012b). Unfortunately, exogenous peptide infusions cannot clarify whether the effects observed here are due to endogenous release of PACAP or VIP, although future studies may resolve this issue by identifying relevant peptidergic afferents (e.g., to the medial pallium) followed by site-specific peptide knockdown.

Thus while we cannot rule out that PACAP acting through VPAC1 and VPAC2 may mediate some of our observed effects, based on the previous data linking VIP to gregariousness (Goodson and Wang, 2006; Goodson et al., 2012c), we hypothesize that the

effects observed here are predominately mediated by VIP. Consistent with this hypothesis, reduction of VIP signaling *in utero* in mice via VPAC antagonism and through the use of VIP deficient mothers (VIP +/- or VIP -/-) leads to significant deficits in affiliative behavior, social recognition, social approach and responses to social novelty (Hill et al., 2007a; Hill et al., 2007b; Lim et al., 2008; Stack et al., 2008). Furthermore, many of PACAP effects on social behavior, including anxiety, stress and affiliative behavior, appear to be mediated specifically via the PAC1 receptor (Hammack et al., 2009; Nicot et al., 2004; Otto et al., 2001).

VPAC receptor signaling modulates social approach during contextual novelty

We here observed a reduction in social contact when animals were tested in a novel environment, but this effect is obtained only following ICV infusions of the VPAC antagonist, and is thus likely mediated outside of the medial telencephalon. One possibility is that circuitry of the ventral hippocampus and LS mediate this effect, given that interconnections between these areas influence anxiety-like behavior and regulate context-specific appetitive behavior in rodents (Belzung, 1992; Luo et al., 2011; Poucet, 1989; Trent and Menard, 2010). Both the hippocampus and LS contain VIP receptors (Goodson et al., 2006), and the hippocampus contains a fairly large population of VIP neurons (Bottjer and Alexander, 1995; Erichsen et al., 1991; Luo et al., 2011). Notably, several studies implicate circuitry of the hippocampus and LS in reactions to novelty and exploration of novel environments, but without a social component (Belzung, 1992; Poucet, 1989; Trent and Menard, 2010), whereas our effects to contextual novelty were *only* observed in a social context. However, the exposure of low novelty-seeking versus high novelty-seeking mice to an intruder in their homecage differentially activates these same brain areas, suggesting that novelty seeking behavior may impact responses to social stimuli (Clinton et al., 2011).

Conclusions

We here show that signaling through VPAC receptors is important for mediating social approach to conspecifics in instances of contextual novelty. Furthermore, activation of VPAC receptors modulates gregariousness in site-specific and sex-specific ways, with VPAC activation promoting gregariousness via action in a putative homologue of the mammalian prefrontal cortex. Given that 1) anatomical studies link VIP signaling to gregariousness in multiple species of birds (Goodson et al., 2006; Goodson et al., 2012c), 2) VIP manipulations significantly impact social behavior in mice and birds (Goodson et al., 2012b; Hill et al., 2007a; Hill et al., 2007b; Lim et al., 2008; Stack et al., 2008) and 3) the antagonist employed here selectively binds to VPAC, but not PAC1, receptors (Nowak et al., 2003) we hypothesize that the effects observed are primarily mediated by VIP signaling. The present study, along with other recent studies (Goodson et al., 2012a; Goodson et al., 2012b), indicates that VIP has significant, yet site-specific, roles in many aspects of social behavior, including aggression, grouping, and social responses to novelty. Clearly more work on the social functions of VIP and VPAC receptors is required, and many potential functions remain to be explored.

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Highlights

We examine the role of VPAC receptor signaling for social affiliation and grouping.
VPAC receptor signaling modulates social contact behavior in unfamiliar contexts.
ICV VPAC antagonism reduces gregariousness in females and increases it in males.
Telencephalic VPAC receptor antagonism reduces gregariousness in both sexes.
Results propose a new role for medial pallium (putative prefrontal cortex homologue).

Fig. 1.

Choice apparatus design. A 1 M wide testing cage was subdivided into zones by seven perches with stimulus cages at either end. The percent of test time spent on the two end perches combined (within 6 cm of a stimulus cage) provides a measure of “social contact time”. Sociality test: stimulus cages contained either two or ten familiar same-sex conspecifics. The percent of contact time spent with the large group provides a measure of “gregariousness” (group size preference). Novel-familiar choice test: stimulus cages contained either six familiar or six unfamiliar same-sex conspecifics (not shown). A “preference for novel individuals” was operationally defined as the percent of contact time spent with the group of novel birds. Sides were counterbalanced across subjects. Subject location was recorded every 15 sec for 5 min. Figure from (Kelly et al., 2011).

Fig. 2. VPAC receptor antagonism decreases social contact in a novel environment. During group size preference tests, ICV VPAC antagonist infusions decrease *social contact* on the first test day when testing paradigm is novel, but not on the second test day.

Fig. 3.

VPAC antagonism has site- and sex-specific effects on gregariousness. (A) A significant interaction of Sex and Treatment ($F_{17,1,1} = 6.54$; $P < 0.02$) is observed following ICV infusions. (B) A significant main effect of Treatment is observed following TEL infusions of the VPAC antagonist ($F_{12,1,1} = 5.84$; $P < 0.03$).