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Oxytocin and the Warm Outer Glow: Thermoregulatory Deficits Cause Huddling Abnormalities in Oxytocin-Deficient Mouse Pups

Christopher Harshaw^{a,b}, Joseph K. Leffel^b, and Jeffrey R. Alberts^b

^aDepartment of Psychology, University of New Orleans, New Orleans, LA

^bDepartment of Psychological & Brain Sciences, Indiana University, Bloomington, IN

Abstract

Oxytocin is a social and reproductive hormone that also plays critical roles in a range of homeostatic processes, including thermoregulation. Here, we examine the role of oxytocin (OT) as a mediator of brown adipose tissue (BAT) thermogenesis, cold-induced huddling, and positive thermotaxis in eight-day-old (PD8) OT ‘knock out’ (OTKO) mouse pups. We tested OTKO and wildtype (WT) pups in single- and mixed-genotype groups of six, exposing these to a period of ambient warmth (~35°C) followed by a period of cold (~21.5°C). Whether huddling exclusively with other OTKO or alongside WT pups, OTKO pups showed reduced BAT thermogenesis and were significantly cooler when cold-challenged. Huddles of OTKO pups were also significantly less cohesive than WT huddles during cooling, suggesting that thermoregulatory deficits contribute to contact abnormalities in OTKO pups. To further explore this issue, we examined thermotaxis in individuals and groups of four OTKO or WT pups placed on the cool end of a thermocline and permitted to freely locomote for 2 hrs. When tested individually, male OTKO pups displayed abnormal thermotaxis, taking significantly longer to move up the thermocline and settling upon significantly lower temperatures than WT pups during the 2 hr test. OTKO mouse pups thus appear to have deficits in both thermogenesis and thermotaxis—the latter deficit being specific to males. Our results add to a growing body of work indicating that OT plays critical roles in thermoregulation and also highlight the entanglement of social and thermoregulatory processes in small mammals such as mice.

Keywords

Oxytocin; Huddling; Brown adipose tissue; Behavioral Phenotyping; Mice

*Correspondence should be addressed to Christopher Harshaw, Department of Psychology, Geology/Psychology Building, 2000 Lakeshore Drive, New Orleans, LA 70148. charshaw@uno.edu.

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Introduction

Oxytocin (OT) is a nonapeptide hormone that plays prominent roles in social and reproductive behavior across a range of taxa (e.g., Carter et al., 2008; Donaldson and Young, 2008; Goodson and Bass, 2001; Keverne and Curley, 2004). In addition to these well known functions, OT is now known to be involved in numerous non-social, homeostatic processes (cf. Lee et al., 2009), including ingestion (see Blevins et al., 2004; Leng et al., 2008), smooth muscle tone and peristalsis (e.g., Altura and Altura, 1984; Babygirija et al., 2010), fluid and electrolyte balance (e.g., Bernal et al., 2010), and metabolic and body temperature homeostasis (see Argiolas and Gessa, 1991; Blevins and Ho, 2013; Chaves et al., 2013).

Although interest in the social versus homeostatic functions of OT has fueled largely separate areas of research, it is unlikely that social and homeostatic processes are the output of entirely separate systems, particularly for highly social species (see Harshaw et al., 2017). This principle is particularly evident in species that modulate their gregariousness or sociability with fluctuations in ambient temperature and/or humidity. For example, in many species of rodents thermal energy (i.e., warmth) can be characterized as both an internal, physiological resource and a kind of ‘social commodity’ (see Haig, 2008; Harshaw et al., 2014). When challenged with cold, that is, mice and other rodents rely upon a combination of thermogenesis, heat conservation, and behavioral thermoregulation, involving positive thermotaxis (i.e., moving toward warmth) and huddling with conspecifics (Gordon, 2012; 1990; Leon, 1986; Satinoff, 1996). As an efficient means of reducing heat loss, huddling plays an important role in the social lives of many rodents and other small mammals (e.g., Alberts, 2006; Bautista et al., 2008; Harshaw and Alberts, 2012).

Heat produced by brown adipose tissue (BAT) thermogenesis appears to make a critical contribution to huddling. When BAT is pharmacologically inactivated, for example, huddles of infant rats are markedly less cohesive—a result that suggests that the thermal attractiveness of pups to each other drives the formation and maintenance of huddles (Sokoloff and Blumberg, 2001). Differences in BAT activation have also been shown to drive spontaneous *assortment* within huddles, whereby warmer individuals preferentially contact each other, while cooler individuals are driven to the periphery (Harshaw et al., 2014; Sokoloff and Blumberg, 2001). Situated in the natural ecological niche—i.e., a group of huddling individuals—BAT can thus be seen as both a thermal and “social” effector (Harshaw et al., 2014).

Interestingly, OT manipulations have been shown to result in significant shifts in huddling, in species as diverse as rats (*Rattus norvegicus*; Alberts, 2006; Kojima and Alberts, 2011), mice (Arakawa et al., 2015), meadow voles (*Microtus pennsylvanicus*; Beery and Zucker, 2010), naked mole rats (*Heterocephalus glaber*; Mooney et al., 2014), and marmoset monkeys (*Callithrix penicillata*; Smith et al., 2010). There has also long been evidence that central OT acts as an endogenous *pyrogen* or producer of metabolic heat (e.g., Lipton and Glyn, 1980; Mason et al., 1986), though the mechanism(s) underlying this effect were unclear.¹ Recent studies have shed a great deal of light on this question. For example, when exposed to a 5°C cold challenge, adult male OT ‘knockout’ (OTKO) and OT-receptor (OTR) KO mice have difficulty maintaining core temperature and lose significantly more body heat

than wildtype (WT) mice (Kasahara et al., 2013; 2007; Takayanagi et al., 2008). A number of abnormalities suggestive of reduced BAT activation (e.g., shifts in beta-adrenergic receptor expression; accumulation of lipids) have also been identified in the BAT of adult OTR KO mice (Kasahara et al., 2013; 2015). When OTR is restored to the DMN/VMN of the hypothalamus via injection of an OTR viral vector, OTR KO mice nevertheless show full recovery of cold-induced BAT thermogenesis (Kasahara et al., 2013). A recent study moreover demonstrated that ablating OT neurons² resulted in both reduced BAT activation and impaired peripheral vasoconstriction (i.e., heat retention) in response to cold (Xi et al., 2017). Thus, a picture has emerged in which central OT must be seen as a regulator of multiple components of the homeostatic response to cold in rodents, including BAT thermogenesis and thermolysis via peripheral vasoconstriction (e.g., Deis et al., 1963; Kasahara et al., 2015; 2013; 2007; Lin et al., 1983; Xi et al., 2017).

Given the importance of BAT thermogenesis as an organizer of cold-induced huddling, mice lacking a functional OT system may experience significant disruption to a core feature of their social lives beginning shortly after birth. More specifically, if OTKO and OTR KO pups have impaired BAT thermogenesis, they are likely to be less *attractive* to each other and thus experience disruption to the typical, spontaneous emergence of huddling during periods of maternal absence from the nest (e.g., Harshaw et al., 2014; Sokoloff and Blumberg, 2001). Variation in early behavior within the huddle has also been shown to predict variation in later social and emotional phenotypes in rabbits and rats (e.g., Reyes-Meza et al., 2011; Rödel and S. S. Meyer, 2011), suggesting that this issue may also be important for interpreting the many studies in which adult OTKO and OTR KO mice display differences in social-emotional behavior compared to WT conspecifics (e.g., Ferguson et al., 2000; Mantella et al., 2003; Winslow et al., 2000). Here, we present an initial exploration of these questions in OTKO mice, examining the effects of oxytocin deficiency on huddling behavior, BAT thermogenesis, and thermotaxic behavior during early development. Based on findings in adult OTKO mice, we hypothesized that OTKO mouse pups would show reduced BAT thermogenesis and impaired huddling ability relative to WT pups when challenged with cold. Additionally, we hypothesized that the normally positive correlation between the thermal status of individual pups and the number of contacts they receive while huddling (see Harshaw et al., 2014) would be absent or reduced in OTKO pups. Lastly, we hypothesized that thermoregulatory differences would be sex-specific, given known sex differences in nonapeptide hormones and receptors (e.g., Dumais and Veenema, 2016; Tamborski et al., 2016).

Methods and materials

Animals and housing

All animals were derived from B6;129S-Oxt^{tm1Wsy/J} heterozygous stock purchased from Jackson Labs (Bar Harbor, Maine) and bred in the Animal Behavior Laboratory at Indiana

¹Although a number of studies have shown that peripheral administration of large doses of OT generally triggers a *hypothermic* rather than pyrogenic response (e.g., Murzenok et al., 1989; Ring et al., 2006), Hicks et al. (2014) demonstrated that such OT-induced hypothermia is due to the action of OT on AVP (V_{1A}) rather than OT receptors.

²Via injection of diphtheria toxin in mice expressing both an OT promoter for *cre* expression and a *cre*-inducible diphtheria toxin receptor (Xi et al., 2017).

University. The F1 offspring were genotyped (Transnetyx, Cordova, TN) shortly after weaning and housed thereafter in same-sex groups, separated by genotype ($Oxt^{+/+}$ or $Oxt^{-/-}$). Males and females of each genotype were then bred to produce homozygous litters (F2). Pups born to $Oxt^{-/-}$ dams were immediately fostered to actively lactating $Oxt^{+/+}$ or $Oxt^{+/-}$ dams, to ensure their survival (Winslow et al., 2000). All mice were born and reared in standard mouse cages ($30 \times 13 \times 19$ cm) with food and water available *ad libitum*. The colony was maintained on 14:10 h light/dark cycle (lights on at 0700 h) at $22 \pm 2^\circ\text{C}$.

All animal care and procedures were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC) at Indiana University (IU #12-024) and were conducted in accordance with both international standards and the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Care was taken at every step to minimize pain and discomfort to animals.

Tests and apparatus

Huddling and activity—Huddling tests involved simultaneous testing of six Postnatal Day 8 (PD8; day of birth = PD0) pups: either three male-female sibling pairs, drawn from the same litter and thus of the same genotype (Exp. 1) or three same-sex OTKO-WT pairs, drawn from two different litters (Exp. 3). PD8 pups were employed because pups of this age show robust huddling and locomotor competence, whereas fur development impedes thermography after PD8 (Harshaw et al., 2014; Harshaw and Alberts, 2012). PD8 pups drawn from different litters do not huddle preferentially with littermates over same-age non-littermates and thus do not spontaneously assort by litter during huddling tests (Harshaw et al., *unpublished data*).

Pups were weight-matched within-pairs, to minimize advantage due to weight (see (Bautista et al., 2010; Rödel et al., 2008)). In Exp. 1, the average weight difference within male-female pairs was $.00 \pm .03$ g for both genotypes, with pups from OTKO litters being heavier, on average, than pups from WT litters ($5.35 \pm .06$ vs. $5.04 \pm .05$ g; $t_{90.2} = 4.08$, $d = -.83$, $p < .0001$). In Exp. 3 the average difference within mixed-genotype OTKO-WT pairs was $.04 \pm .02$ g, with OTKO pups slightly outweighing WT pups (4.76 vs. 4.72 g; $t_{41} = -2.28$, $d = -.35$, $p < .03$). Although congruent with reports of lower energy expenditure and obesity proneness in OTKO and OTR KO mice (Camerino, 2009; Kasahara et al., 2013; Nishimori et al., 2008; Takayanagi et al., 2008), this difference was likely also influenced by the smaller litter size of OTKO compared to WT litters (8.4 vs. 9 pups, across both experiments; $t_{37.6} = 1.33$, $d = -.40$, $p = .193$).

Apparatus: All huddling tests were performed within a double-walled glass chamber (height = 30 cm; dia = 15.2 cm) on a circular platform (dia = 11.25 cm), 21.5 cm from the chamber's upper edge. The platform was constructed of 1.27 cm Styrofoam insulation (Dow Chemical Company), circled by a polyethylene mesh wall (height = 15 cm), to prevent pups from making contact with the glass wall of the chamber, covered with a circular piece of clear plastic sheeting, permitting cleaning between sessions. Ambient temperature (T_a) was controlled by circulating either chilled or heated water through its walls. An ICI 7320 P-Series infrared thermal imaging camera (Infrared Cameras Inc., Beaumont, TX) and Sony

DXC-151A video camera were mounted above the testing chamber, such that both cameras could simultaneously capture images of pups at angles nearly perpendicular to the testing platform.

Procedure: Pups were carefully removed from their dams, weighed, and checked for milk bands; only pups displaying such bands were tested. Each pup's tail was then color-coded with a non-toxic marker to prevent errors in returning pups to their dams. Pups' mid-backs and heads were also marked with a water-based paint for individual identification. All pups were then placed in the testing chamber, wherein T_a was stabilized at $\sim 35^\circ\text{C}$. A Plexiglas divider created a separate compartment for each pup, preventing contact prior to the onset of data collection. Pup location in the chamber was randomized. However, the sex (Exp. 1) or genotype (Exp. 3) of pups was alternated such that at the start of each warm phase (described below) each pup had only opposite sex or genotype huddlemates to its left and right, biasing initial contacts toward opposite-sex or opposite-genotype contact.

Once all pups were in the chamber, a 20 min acclimation period was provided to allow for recovery of body heat lost during transfer (Blumberg et al., 1992a). Following acclimation, the divider separating pups was removed and data collection initiated. Testing involved a standardized sequence consisting of a warm and a cool phase. During the warm phase, the initial warm T_a (mean: $34.8 \pm .01^\circ\text{C}$) was maintained for a further 51 min. Water baths were then switched and T_a within the chamber cooled rapidly to $\sim 21.5^\circ\text{C}$ (mean: $21.6 \pm .06^\circ\text{C}$). The cool phase consisted of a further 51 min, initiated once T_a reached $\sim 23^\circ\text{C}$. Trials thus lasted ~ 130 min (20 min acclimation + 51 min warm + 6-7 min cool down + 51 min cool).

Ambient air temperature (T_a) was monitored continuously and logged at 1 min intervals using a Type K thermocouple (located 1.5 cm above the platform) connected to an Omega HH802U thermometer and Omega Software for Windows, Ver. 1.6 (Omega Engineering, Inc., Stamford, CT). Thermal images and video frames were captured simultaneously, once per minute. Thermographs were acquired via IR Flash ver. 2.0 (Infrared Cameras Inc., Beaumont, TX) running on a Dell Latitude E6400 laptop. Video frames were captured using Scion Image 1.62a, running on a Power Mac 64 (Mac OS 9.1).

Thermotaxis—Tests of thermotaxis involved simultaneous testing of four PD8 littermates (2 male and 2 female) in an enclosed alley on a thermocline, alongside a singleton (male or female) selected from the same litter, placed in a non-adjacent alley. These tests allow quantification of *positive thermotaxis* or oriented movements toward warmer regions of a temperature gradient. The behavior is regulatory, in the sense that pups do not move continuously or maximally up the gradient but, instead, tend to settle in a zone of the gradient where the surface temperature is favorable to normothermia (see Farrell and Alberts, 2007).

Apparatus: The thermocline was composed of a brass surface (89.7 cm long) painted black, housed within a Plexiglas incubator. The incubator was encircled by a black curtain, with homogenous lighting from above. Air circulation was perpendicular to the length of the thermocline, which minimized air temperature gradients above the thermocline surface (see Alberts et al., 2004). Surface temperature was set to range from 23.5 to 39°C along the

thermocline, with the average obtained gradient being $0.19^{\circ}\text{C}/\text{cm}$. Comparing OTKO and WT runs, temperatures at neither the warm ($38.7 \pm .07$ vs. $38.7 \pm .05$ $^{\circ}\text{C}$, respectively) nor cool ($23.8 \pm .13$ vs. $23.7 \pm .12$ $^{\circ}\text{C}$, respectively) ends of the thermocline differed significantly ($t_{58,3} = -.57$, $d = -.14$, $p = .573$ and $t_{61,1} = .92$, $d = -.23$, $p = .36$, respectively). Likewise, the steepness of the gradient did not significantly differ between OTKO and WT runs ($t_{58,2} = -1.3$, $d = -.32$, $p = .198$).

Procedure: Pups were tested in 2.5 cm wide alleys, separated by plastic partitions. For coding purposes, the length of the thermocline was divided into 9 equal zones (~ 9.96 cm each), with the warmest temperature always on the left side of the apparatus (i.e., zone 1). The temperature of each zone was recorded prior to each testing session. At the onset of testing, pups (mixed sex groups and individuals) were placed in plastic corrals on the cool end of the apparatus (i.e., zone 7; $\sim 26^{\circ}\text{C}$). After one min, corrals were removed, at which time pups could move freely within their assigned alley for the duration of the test (120 min). Pup locations were captured every 3 min via frame grabs from a video camera mounted above the apparatus. These frames were subsequently scored for the location of pups within the zones of the thermocline, as well as how many pups were in contact (i.e., aggregates). A pup was considered in a zone if $>50\%$ of its body was in that zone. For group runs, locations were obtained for all pups in the group and zone temperatures averaged between these.

Data collection and analyses

Experiments 1 and 3—In Experiments 1 and 3, groups of pups were tested once on PD8, from which activity levels, cohesiveness, and contact behavior were measured, and infrared thermographs were also obtained. In Experiment 1, a total of eight replicates per genotype ($N = 96$ pups, total) were drawn from 16 uncultured litters of 6-11 pups (mean litter size = $9.0 \pm .5$ for WT; $8.5 \pm .5$ for OTKO). Two additional OTKO litters were not included: one because a pup left the group and huddled with the thermocouple for nearly the entire run (i.e., the experimenter failed to move the thermocouple, per our protocol) and the other because there was a large, systematic weight difference between males and females. In Exp. 3, seven replicates per sex ($N = 84$ pups, total; 42 per genotype) were obtained. These mixed-genotype groups were composed of pups drawn from 26 uncultured litters of 5-12 pups³. An additional male replicate (not included in the above total) was disqualified because T_a was outside our target range during the run.

Experiment 2—In Experiment 2, a total of 70 OTKO pups (56 tested in groups, 14 as singletons) and 95 WT pups (76 tested in groups, 19 as singletons), drawn from 16 OTKO litters and 19 WT litters, were tested. Two additional OTKO groups and one WT group were run but not included in the above totals and analyses: one OTKO litter because the thermocline was abnormally warm (Grubb's test: $G = 3.75$, $p < .0005$) and another because the sex ratio of the group deviated from an even male/female ratio. The singleton from the latter litter was nevertheless retained. An additional WT litter was not included because pups

³This number is 26 rather than 28 litters because on two occasions both male and female replicates were obtained from the same WT and OTKO litters, to minimize litter usage.

from the litter were significantly heavier than pups from the other WT litters (Grubb's test: $G = 3.38$, $p < .005$).

Data analysis

Scoring and analysis of contact behavior: We employed a modified version of the system used by Sokoloff and Blumberg (2001) to score contacts, as described previously (Harshaw et al., 2014). As illustrated in Figure 1, each pup in the huddle is scored at each time point for the number of male and female (Exp. 1) or OTKO and WT (Exp. 3) pups with which it is in contact, excluding contacts made only via tails or outstretched paws. Categorization of specific contact patterns or *contactogons*, permitted analysis of relations between pup interscapular temperature (see below) and overall number of contacts, contacts by sex/genotype, as well as whether the overall distribution differed from that expected by chance.

All analyses were performed in *R*, version 3.3.1 (The R Foundation for Statistical Computing, Vienna, Austria), using $\alpha = .05$ (two-tailed). To determine whether the distribution of contact types or contactogons deviated from that expected by chance between -sex (Exp. 1) or -genotype (Exp. 3), G-tests of goodness of fit (see Fisher, 1950) were performed on data for each phase. The G-test is similar to the chi-square goodness of fit test, but superior in several respects, including how well it approximates the chi-square distribution (Harremoës and Tusnády, 2012). Expected values were generated by multiplying the observed number of occurrences of each contact type by the ratio of probabilities for that contactogon for each type of pup. For example, in Exp. 1, 1M 1F and 2M 2F were equally likely for males and females, giving a 1:1 ratio if contacts are made at random, whereas the probability of 2M 0F would be .1 for males and .3 for females, yielding a 1:3 ratio. All contact types displayable by only a single sex or genotype (e.g., 3M 2F) were collapsed into a single category. In the case of a significant G-test (indicating that the *overall* distribution of contactogons differed from chance expectations), post-hoc Fisher's exact tests were performed, using a Bonferroni correction for multiple tests ($\alpha = .05/10 = .005$).

Scoring and analysis of activity: To assess differences in locomotor activity between genotypes we measured pup location and movement from captured video frames sampled once per min, using a custom NIH ImageJ script (Harshaw and Alberts, 2012). The tip of each pup's nose and the base of its tail were identified (or estimated, when obscured) and marked within each video frame. An x,y coordinate was automatically abstracted from these points and the midpoint of a line between these calculated, such that pup activity (i.e., movement of pup midpoints (cm/min) could be calculated for consecutive frames.

Analysis of thermal images: All thermographic measurements were obtained using ICI IR Flash ver. 2.0, by an experimenter blind to the sex (Exps. 1 and 3) and genotype (Exp. 3) of pups. Body surface temperatures from the interscapular (T_{IS}) and/or rump (T_{rump}) regions were measured for each pup. The interscapular (IS) region lies above the largest BAT deposit in the body and the temperature of the IS skin increases when BAT is active. A pup's rump contains no BAT and thus provides a reference temperature. The difference between these ($T = T_{IS} - T_{rump}$) provides a proxy for BAT activation (Blumberg and Alberts, 1990; Harshaw and Alberts, 2012; Sokoloff and Blumberg, 2001). Figure 2A depicts how circular

zones superimposed on the regions of interest, centered on the body's midline, provided an average temperature for all enclosed pixels. Zone diameters corresponded to an actual size of .55 cm or approximately half of the average body width of a PD8 pup. If only the IS or rump were visible, then measurement was obtained only for that region and no T was calculated for that pup at that time step. If a pup was lying on its side, was under the huddle or otherwise obscured, then no thermal measurements were obtained for that pup for that frame. Our primary measures for correlational and causal analyses were a pup's T_{IS} and body weight relative to those of its huddlemates (T_{IS}^{rel} and $weight^{rel}$, respectively). A pup's T_{IS}^{rel} was calculated for each frame by subtracting its T_{IS} from the average T_{IS} for the group ($T_{IS}^{rel} = T_{IS}^{pup} - T_{IS}^{group}$) at that time point. Each pup's weight relative to its huddlemates was calculated similarly ($weight^{rel} = weight^{pup} - weight^{group}$). Only frames in which three or more pups had visible interscapular regions were used for calculating T_{IS}^{rel} . Average T_{IS}^{rel} thus provides a better indicator of the relative attractiveness of a pup in its immediate context and over the course of a trial than either T_{IS} or T , while also making possible comparisons that are independent of T_a (see Harshaw et al., 2014).

Sex and genotype differences in T_{IS} , T_{rump} , T and contacts were evaluated using two complementary statistics. First, the *consistency* and *directionality* of sex difference was determined using Sign tests performed on time series obtained from averages for each time step, with missing data points for either sex/genotype excluded for the other sex/genotype within each group. Next, *magnitude* of difference was analyzed using Wilcoxon matched-pairs signed rank tests on average values for each pup, with each pup paired against the closest weight-matched opposite sex/genotype huddlemate, and missing values for either pup removed for the paired pup. Only pairs of measurements obtained under identical T_a were thus included in between-sex and between-genotype analyses and in overall averages for the two genotypes (e.g., Tables 1 and 2). For contact behavior, these analyses were performed by sex (average number of contacts), type (same sex, opposite sex) and sub-type (male-male, female-female, male-female).

Correlational and causal analyses: Bivariate relationships between contacts, pup thermal status, and other variables, were examined using Pearson correlations. For multivariate analyses, we used the 'lmer' function from the 'lme4' package in *R* (Bates et al., 2015) to construct linear mixed models (LMM) using the restricted maximum likelihood (REML) approach. In all such LMMs, the number of pup marking (i.e., pup number) and run (i.e., replicate) of the huddling test were controlled as random effects. F-statistics, p-values, and degrees of freedom were obtained via Satterthwaite's approximation, using the 'lmerTest' package (Kuznetsova et al., 2016). Given the limitations of correlational analyses, we also performed causal modeling on T_{IS}^{rel} and pup contacts using Granger analysis (Granger, 1988; 1969), to more closely examine interactional dynamics within mixed-genotype OTKO/WT huddles (see Harshaw et al., 2014). Specifically, we first calculated average time-series for all OTKO or WT pups within each litter/run. Using Eviews ver. 9.5 (IHS Global, Irvine, CA) for Windows, we tested all time series for stationarity (a pre-requisite of Granger analysis), using the Augmented Dickey-Fuller (ADF) test for unit roots. Next, we used the Dumitrescu-Hurlin method for testing for Granger causality in heterogeneous panel data (Dumitrescu and Hurlin, 2012), examining the relationships (bi-directionally) between

time series for T_{IS}^{rel} and total contacts, contacts with OTKO pups, and contacts with WT pups in Exp. 3, with Bonferroni corrections for multiple tests ($\alpha = .05/6 = .0083$). At a minimum, a significant Granger test demonstrates (a) temporal precedence in change—a prerequisite for causality—and (b) that one time-series contains information useful in *forecasting* another (Granger, 1988). Given that a fully ‘causal’ interpretation of such tests is sometimes controversial, ‘Granger-cause’ is often used (and used here) as a substitute.

Experiment 2—Video frames were captured from the camera mounted above the thermocline at the start of the trial and at 3 min intervals thereafter. Because pups tended to move rapidly to warmer locations upon release, we used only the first frame to compare start locations between strains and groupings (i.e., groups of 4 vs. singletons). To compare end-of-trial locations, we calculated an average of the final two frames (obtained at 117 and 120 min). Because thermocline and ambient temperatures were highly correlated and varied slightly from day-to-day, we also calculated the *steepness* of the obtained temperature gradient for each run by subtracting the temperature of the cool end from that of the warm end of the thermocline and dividing the difference by the length of the apparatus, yielding a unit of °C/cm. We employed t-tests to compare group means and robust regression via MM estimation (‘rlm’ in ‘MASS’ package in R) to model multivariate relationships. For bivariate relationships, Pearson-product moment correlations were calculated. Because of the sensitivity of such correlations to the presence of outliers and our relatively low sample size, we used an iterated jackknife (i.e., an $N - 1$ bootstrap), running Grubb’s tests on the jackknife coefficients, dropping data point(s) that significantly modified the obtained correlation and statistical decision. This procedure ensures that reported correlations are robust to outlying data points (Ang, 1998).

Results

Experiment 1 – Temperature Regimes

During the warm phase, T_a did not differ for the two genotypes ($t_{9.7} = 1.15$, $d = .58$, $p = .277$). In contrast, there was a significant difference in T_a during the cool phase ($t_{13.3} = 2.30$, $d = 1.15$, $p < .04$), with OTKO experiencing slightly warmer T_a than WT litters ($21.4 \pm .03^\circ\text{C}$ vs. $21.3 \pm .02^\circ\text{C}$, respectively). Given that room temperature and equipment settings were identical, it appears that OTKO litters contributed slightly more heat (via heat loss) to the testing chamber than WT litters⁴. As a result, average T_a during each run was factored into all analyses.

Experiment 1 – Warm Phase

Activity levels—Pups maintained at warm ambient temperatures tend to be inactive, spending much of their time sleeping and moving only incidentally, as a result of myoclonic twitching (Blumberg et al., 1992b; Harshaw and Alberts, 2012). Under these conditions, OTKO pups showed consistently higher levels of activity than WT pups (sign test, $p < .00001$). This difference appeared to be driven by the (a) strong positive correlation between

⁴This interpretation is supported by a recent study showing that inactivation of OT neurons resulted in greater heat loss due to impaired peripheral vasoconstriction (Xi et al., 2017).

pup activity and weight ($r = .598$, $t_{94} = 7.30$, $p < .0001$), and (b) the fact that (b) OTKO pups were heavier than WT pups. An LMM, with weight, sex, genotype, and T_a as fixed effects, confirmed that weight and genotype were the only predictors of activity ($F_{1,59.6} = 14.2$, $p < .0004$ and $F_{1,13.7} = 20.2$, $p < .0006$, respectively). Moreover, once weight x T_a interaction was included in the model, the only remaining predictor of pup activity was genotype ($F_{1,14.1} = 19.7$, $p < .0006$).

Huddle cohesiveness and contacts—As shown in Figure 4, there was no difference in the cohesiveness of WT and OTKO litters during the warm phase (sign test, $p > .05$). However, OTKO pups made more contacts with other pups than did WT pups ($1.29 \pm .02$ vs. $1.16 \pm .03$, respectively; $t_{80.7} = 3.25$, $d = .66$, $p < .002$). In an LMM, with pup sex, weight^{rel}, T_a , and genotype x activity interaction as fixed effects, and run and pup number as random effects, the only significant predictors of contacts were genotype ($F_{1,69.4} = 4.64$, $p < .04$) and genotype x activity interaction ($F_{1,73.5} = 4.59$, $p < .04$).

Thermogenesis, contact, and activity—Despite similar ambient conditions, OTKO pups had warmer T_{IS} and T_{rump} values than did WT pups ($p < .00001$; see Table 1), suggesting that OTKO pups may have absorbed heat more quickly from the environment than did WT pups. As is typical of thermally comfortable pups, there was no relation between the relative warmth (T_{IS}^{rel}) of individual WT pups and contacts made with other pups during the warm phase ($r = -.12$, $t_{46} = -.80$, $p = .426$). In contrast, OTKO pups showed a positive correlation between the T_{IS}^{rel} and contacts ($r = .30$, $t_{46} = 2.1$, $p < .05$).⁵

Experiment 1 – Cool Phase

Activity levels, cohesiveness, and contacts—Upon cooling—and in contrast to the warm phase—groups of OTKO pups showed *reduced* levels of activity compared to WT groups (sign test, $p < .01$; see Fig. 3). There was also no relation between pup weight and activity ($r = -.16$, $t_{94} = -1.55$, $p = .125$), suggesting that the differing weights of the two genotypes did not account for their activity difference. This was confirmed by an LMM, in which the only significant predictor of activity was genotype ($F_{1,25.2} = 4.61$, $p < .05$). OTKO litters were also significantly less cohesive than WT litters (sign test, $p < .00001$) and thus had fewer contacts than did WT pups ($t_{90.5} = 4.01$, $d = .82$, $p < .0002$). An LMM confirmed that pup contacts were significantly predicted only by pup genotype ($F_{1,30.6} = 6.82$, $p < .02$) and weight^{rel} ($F_{1,83.1} = 6.54$, $p < .02$).

Thermogenesis and thermotaxis—OTKO pups were significantly cooler than WT pups, as indicated by lower T_{IS} and T_{rump} temperatures ($ps < .0005$; see Table 1). OTKO pups also had significantly lower T ($T_{IS} - T_{rump}$) scores ($p < .00001$), indicating reduced BAT thermogenesis. To explore whether oxytocin deficiency also modified positive *thermotaxis* (i.e., attraction to warmth) during cold-induced huddling, we examined the relation between the contacts that WT and OTKO pups received, each pup's relative thermal attractiveness (T_{IS}^{rel} ; see Fig. 2), and their weight relative to their huddlemates (weight^{rel}).

⁵Likely because of the significantly higher activity levels shown by OTKO pups. That is, more active pups move more and are thus more likely to make contacts than sleeping/less active pups.

As shown in Figure 5, there was a trend toward a positive correlation between T_{IS}^{rel} of WT pups and their contacts with other pups ($r = .26$, $t_{46} = 1.84$, $p = .072$). OTKO pups, in contrast, showed high correlation between these variables ($r = .64$, $t_{46} = 5.68$, $p < .00001$). The strength of the latter correlation relative to WT pups was likely driven by a number of factors, including lower cohesiveness and thus less sharing of heat in OTKO litters, leading to greater thermal stratification of pups.

Given that female pups overproduce heat relative to male siblings, an additional factor relevant to thermotaxis during huddling is the type of contact made (i.e., whether with male vs. female pups; see Harshaw et al., 2014). As is shown in Figure S1, which depicts the relationship between T_{IS}^{rel} and contacts during the cool phase by sex of contact and genotype, WT pups showed no correlation between T_{IS}^{rel} and contacts with males ($r = .00$, $t_{46} = .01$, $p = .996$) and a trend toward a positive correlation between T_{IS}^{rel} and contacts with females ($r = .27$, $t_{46} = 1.89$, $p = .065$).⁶ OTKO pups, in contrast, showed significant correlations between T_{IS}^{rel} and both contacts with males ($r = .33$, $t_{46} = 2.38$, $p < .03$) and females ($r = .30$, $t_{46} = 2.15$, $p < .04$). To verify this result and rule out modulation by other variables, we constructed LMMs for WT and OTKO runs, with T_{IS}^{rel} (i.e., relative warmth) as the dependent variable and pup sex, $weight^{rel}$, T_a , contacts with females, contacts with males, and pup activity x T interaction as fixed effects, and run and pup number as random effects. For WT pups, the only variable that predicted of T_{IS}^{rel} was contacts with females ($F_{1,38.8} = 6.91$, $p < .02$). For OTKO pups, in contrast, T_{IS}^{rel} was significantly predicted by contacts with females ($F_{1,33.4} = 38.8$, $p < .000001$), contacts with males ($F_{1,34.2} = 37.7$, $p < .000001$) and $weight^{rel}$ ($F_{1,36.6} = 15.3$, $p < .0005$). There are at least two possible explanations for the behavioral differences observed between OTKO and WT pups while huddling in response to cold. First, inactivation of BAT is known to impair huddling, by disrupting the attractiveness of pups to each other (Sokoloff and Blumberg, 2002; 2001). The behavioral differences observed here may thus be entirely the result of defective BAT thermogenesis in OTKO pups. Nevertheless, it is also possible that OT deficiency results in *sensory* or *motivational* deficits, affecting aspects of thermotactic behavior (c.f. Beets et al., 2012). To explore this possibility under controlled conditions, we examined the behavior of OTKO and WT pups on a thermocline.

Experiment 2

When tested as individuals on the thermocline, a dramatic difference was observed in the behavior of OTKO and WT pups (see Fig. 6; Fig S2). At the onset of testing there was no difference in location or surface temperature chosen between WT and OTKO pups (both $25.8 \pm .1^\circ\text{C}$; $t_{28.6} = .46$, $d = .16$, $p = .648$). Robust regression (RR), with pup genotype x sex x weight and weight x T_a x activity as predictors, confirmed that start temperature did not differ for the genotypes ($t_{18} = -1.09$, $p = .29$). WT and OTKO pups nonetheless diverged rapidly after this, with WT pups systematically choosing warmer regions of the thermocline than OTKO pups ($31.3 \pm .6^\circ\text{C}$ vs. $29.9 \pm .6^\circ\text{C}$, respectively; sign test, $p < .000001$). Although main effects of genotype ($t_{18} = 1.87$, $p = .076$) and sex ($t_{18} = 1.44$, $p = .166$) were

⁶This replicates a similar pattern of sex difference in contact observed in PD8 C57BL/6 mouse pups (Harshaw et al., 2014), though the difference observed here was *not* accompanied by significant sex differences in thermal measures (T_{IS} : $U = 148$, $p = .966$; T_{rump} : $U = 110$, $p = .264$; T_a : $U = 186$, $p = .317$).

not significant in a RR model, average temperature choice was predicted by genotype x sex ($t_{18} = -5.94, p < .0001$) and genotype x sex x weight ($t_{18} = 5.61, p < .0001$) interactions. Temperature choice at the end of the 2 hr test (WT: $32.8 \pm .6^{\circ}\text{C}$ vs. OTKO: $31.5 \pm .8^{\circ}\text{C}$) was similarly predicted by genotype x sex ($t_{18} = -5.40, p < .0001$) and genotype x sex x weight ($t_{18} = 4.95, p < .0002$) interaction; however, the main effects of genotype ($t_{18} = 2.16, p < .05$), weight ($t_{18} = -2.30, p < .04$), and T_a ($t_{18} = -2.25, p < .04$) were also significant predictors of position at the end of testing.

The crossover interaction implied by the above pattern of significance is displayed in Figure 6. Male OTKO pups were located on systematically cooler portions of the thermocline than OTKO females (sign test: $p < .000001$)—an effect that was significant considering pups' averages across the 2 hr trial ($t_{10,9} = -2.45, d = -1.32, p < .04$) and trended toward statistical significance at the end of the trial ($t_{11,1} = -2.02, d = -1.09, p = .068$). Male WT pups, in contrast, tended to be found on slightly warmer portions of the thermocline than WT females (sign test: $p < .0002$), particularly during the first half of the trial. This sex difference, however, was not significant when average location temperatures ($t_{11,1} = .41, d = .2, p = .69$) and pup positions at the end of trial ($t_{13,8} = .16, d = .07, p = .875$) were compared.

Taken together, these results indicate that OTKO pups are sluggish in responding to the cool surface of the thermocline and OTKO males, in particular, appear to show impaired positive thermotaxis compared to both WT pups and female OTKO pups. To explore the question of whether or not OTKO pups are insensitive to slight variations in the gradient, we examined the relationship between the steepness of the thermal gradient, which varied slightly from run to run, and behavior, finding a significant relationship between activity and steepness of the gradient for OTKO ($r = .534, t_{12} = 2.19, p < .05$) but not WT ($r = -.06, t_{16} = -.24, p = .812$) pups tested as individuals (see Fig. S4). Although it is possible that individual WT pups are more “distressed” by separation from their dams and litters than OTKO pups and thus may spend more time engaged in “search” for conspecifics during testing, this possibility is unlikely, given that the frequency with which individual WT and OTKO pups made zone transitions toward or away from warmth revealed no significant differences between genotypes ($t = .19, d = .07, p = .848$ and $t = -.30, d = -.10, p = .764$, respectively). OTKO pups also showed a strong positive correlation between the steepness of the gradient and their final positions on the thermocline ($r = .782, t_{11} = 4.16, p < .002$), whereas WT pups did not ($r = .19, t_{17} = .78, p = .445$; see Fig S5), indicating that they are indeed sensitive to slight temperature differences.

In contrast to tests of individuals, there was little difference in the thermotactic behavior of WT and OTKO pups when tested in groups of four (sign test, $p = .117$; see Fig. S2). This was true for beginning ($25.8 \pm .1^{\circ}\text{C}$ vs. $25.9 \pm .1^{\circ}\text{C}$; $t_{29,1} = .61, d = .21, p = .544$), average ($30.7 \pm .2^{\circ}\text{C}$ vs. $30.6 \pm .4^{\circ}\text{C}$; $t_{17,2} = -.16, d = -.06, p = .872$) and ending ($32.7 \pm .2^{\circ}\text{C}$ vs. $32.3 \pm .4^{\circ}\text{C}$; $t_{18,5} = .83, d = .32, p = .417$) locations/temperatures. Despite this, groups of OTKO pups were *more* cohesive than groups of WT pups over the course of the two-hour test (sign test: $p < .0002$; see Fig. S3). A RR model with average cohesiveness as the dependent variable nevertheless showed no significant effects of genotype ($t_{21} = -.29, p = .777$), with the strongest predictor of cohesiveness being the steepness of the thermocline ($t_{21} = 1.56, p = .134$). Like pups tested as individuals, OTKO groups showed positive correlation between

their activity and steepness of the thermal gradient ($r = .478$)—although this effect was short of significance ($t_{11} = 2.41$, $p = .098$)—whereas WT groups showed no relation between these variables ($r = -.01$, $t_{16} = -.06$, $p = .954$). In contrast, significant correlation between the final position of pups on the thermocline and the steepness of the gradient was found for both OTKO ($r = .588$, $t_{11} = 2.41$, $p < .04$) and WT ($r = .481$, $t_{16} = 2.19$, $p < .05$) groups (Fig. S5).

Experiment 3

The results of Experiment 2 suggested the presence of thermotactic differences in pups deficient in oxytocin, particularly male OTKO pups tested as individuals. To further explore this issue, we examined the behavior of mixed-genotype groups of male or female pups ($N = 3$ WT, $N = 3$ OTKO) in tests of huddling otherwise identical to those in Exp. 1. These mixed-genotype groups ensured that WT and OTKO pups experienced exactly the same ambient conditions for the duration of the test and also permitted us to apply causal modeling to elucidate interactions occurring between WT and OTKO pups in the huddle. Averages for all measures obtained from these tests for both warm and cool phases are presented in Tables 2 and 3, respectively.

Experiment 3 – Warm Phase

Activity levels and contacts—OTKO pups were more active than WT pups during the warm phase in both male and female runs (sign tests: $p < .007$ and $p < .001$, respectively). A LMM confirmed that genotype was the only significant predictor of activity ($F_{1,65.4} = 10.06$, $p < .003$). As a result, there were systematically elevated levels of contact with pups of the “opposite” or other compared to the same genotype in both male and female runs (sign tests: $p < .025$ and $p < .002$, respectively; see Fig. S9). There were also more OTKO-OTKO contacts than WT-WT during the warm phase of male runs (sign test: $p < .05$).

Thermal measures and total contacts—No between-genotype differences in temperature measures (i.e., T_{IS} , T_{rump} , or T) were observed during the warm phase (see Table 2). Despite the differences in specific contact patterns above, there was also no significant difference in the *total* number of contacts received by WT versus OTKO pups, in either male or female runs (sign tests: $p = .07$ and $p = .126$, respectively; see Fig. 7). An LMM confirmed that contacts received did not vary by genotype ($F_{1,63.4} = .36$, $p = .55$), with the only significant predictor of pup contacts being activity level ($F_{1,74} = 4.38$, $p < .03$).

Experiment 3 – Cool Phase

Activity levels—An LMM revealed that the only significant predictor of activity during the cool phase was sex x genotype interaction ($F_{1,63} = 7.26$, $p < .01$), with post-hoc tests showing significant genotype difference in activity only for females ($t_{63} = -2.31$, $p < .03$). In particular, OTKO pups tended to show lower activity than WT pups; a difference that was significant for female but not male runs (sign tests: $p < .005$ and $p = .085$, respectively; see Fig. S6).

Thermal measures and total contacts—OTKO pups were significantly cooler than their WT huddlemates during the cool phase (see Table 3; Figs. S12, S13). A LMM,

similarly, showed that the only significant predictor of pup thermal attractiveness (T_{IS}^{rel}) was genotype ($F_{1,71.4} = 31.4$, $p < .00001$), with WT pups being warmer than their OTKO huddlemates ($t_{63.7} = -4.41$, $p < .00001$). As a consequence, WT pups received more contacts than their OTKO huddlemates, in male and female runs (sign tests: $p < .05$ and $p < .0004$, respectively; Fig. 7).⁷

T_{IS}^{rel} and contacts by genotype—As shown in Figure 8, there was significant positive correlation between T_{IS}^{rel} and bias for WT contact (i.e., contacts with WTs adjusted for the differing chance ratios for the two genotypes; $r = .296$, $t_{82} = 2.80$, $p < .007$) and correspondingly negative correlation between T_{IS}^{rel} and OTKO bias ($r = -.29$, $t_{82} = -2.75$, $p < .008$). Previous studies have shown that warmer pups have competitive advantage for contacting each other while huddling (e.g., Harshaw et al., 2014). We thus examined whether the difference in T_{IS}^{rel} between the WT and OTKO pups resulted in assortment by genotype, finding evidence of such assortment during female (sign test: $p < .0005$) but not male (sign test: $p < .479$) runs (see Fig. S9). There were also more WT-WT than OTKO-OTKO contacts in female runs (sign test: $p < .0005$), with a similar trend in male runs (sign test: $p = .073$; see Fig. S10), suggesting that WT pups did garner some competitive advantage for contacting other WT pups.

A LMM examining T_{IS}^{rel} during the cool phase that included contacts with WT and OTKO pups as separate fixed effects revealed that the best predictors of T_{IS}^{rel} were (1) contacts with WTs ($F_{1,67.1} = 8.01$, $p < .007$), followed by (2) genotype ($F_{1,65.7} = 7.24$, $p < .01$), (3) three-way sex x genotype x contacts with WTs interaction ($F_{1,67.1} = 6.09$, $p < .02$), and (4) contacts with OTKOs ($F_{1,69} = 4.86$, $p < .05$). In light of the significant interaction with sex (see Fig. S11), we constructed separate LMMs for the two types of huddles. In male huddles, T_{IS}^{rel} was significantly predicted by genotype ($F_{1,31.1} = 10.28$, $p < .004$) and genotype x contacts with WTs ($F_{1,32.2} = 6.30$, $p < .02$). In female huddles, in contrast, T_{IS}^{rel} was significantly predicted only by contacts with WTs ($F_{1,34} = 13.33$, $p < .0009$).

Causal modeling of relationship between T_{IS}^{rel} and contacts—The preceding analyses indicate intriguing differences in how WT and OTKO pups behave when challenged with cold and allowed to interact in mixed-genotype huddles. To further explore these differences, we performed panel Granger analyses on the average time-series for WT and OTKO pups during the cool phase for male and female runs, using a 1 min lag. The results are displayed in Figure 9, along with lagged-correlations, indicating the directionality of these relationships. During the cool phase of female runs, T_{IS}^{rel} was found to Granger-cause contacts with WT pups for both WT ($W = 2.92$, $p < .002$) and OTKO ($W = 3.62$, $p < .00002$) females. Nevertheless, the direction of this relationship was opposite for the two genotypes, as lagged-correlation between these variables was negative (indicating a net loss of heat) for WT and positive (indicating a net gain in heat) for OTKO females. For WT females, T_{IS}^{rel} also significantly Granger-caused both total contacts ($W = 3.70$, $p < .00001$) and contacts with OTKO females ($W = 2.85$, $p < .003$). Combined with the results of the

⁷Confirming the importance of T_{IS}^{rel} for attracting contacts in mixed-genotype huddles, an LMM found that T_{IS}^{rel} was the best predictor of total contacts ($F_{1,60.2} = 26.4$, $p < .00001$), with T_{IS}^{rel} x genotype ($F_{1,67.2} = 7.2$, $p < .009$) T_{IS}^{rel} x sex ($F_{1,63.8} = 4.9$, $p < .04$) interactions also significant.

LMMs, this suggests that cooler WT females tended both to seek out and subsequently receive more contacts whereas warmer WT females either avoided contacts or simply showed normal “pup flow” or passive circulation to the periphery of the huddle (Alberts, 1978). In contrast, warmer OTKO females tended to subsequently have more contacts with WT females whereas cooler OTKO females tended to have fewer contacts with WT females, implying a process more akin to ‘cumulative advantage’ and ‘cumulative disadvantage’ (see Price, 1976) in OTKO females.

In male huddles the only significant Granger causality was found in OTKO males, in that their T_{IS}^{rel} negatively predicted subsequent contacts with OTKO pups ($W = 2.61$, $p < .008$), with a similar trend present in WT males ($W = 2.51$, $p = .0125$). That is, for male OTKO pups, being warmer relative to huddlemates at one time point tended to predict *fewer* contacts with other OTKO males at later time points. Given that WT males trended toward a similar pattern, this finding implies that either (a) warmer males—whether WT or OTKO—actively avoid contacts with OTKO males and/or (b) OTKO males are outcompeted for access to warmer, WT males.

Discussion and Conclusions

Several recent studies have reported defects in BAT thermogenesis in adult OTKO and OTR KO mice (Kasahara et al., 2015; 2013; 2007; Takayanagi et al., 2008). Here we explored whether such deficits are also present in OTKO pups in the developmentally important context of cold-induced huddling, prior to the development of potential compensatory adaptation (see (Cannon and Nedergaard, 2011). Huddling is a ‘group-regulatory behavior’ that is critical for conserving heat and energy during early development (Alberts, 1978; Harshaw and Alberts, 2012) and continues to be integral to social life in adulthood for mice and many other rodents (Arakawa et al., 2007; King and Cannon, 1955). Given that BAT thermogenesis plays a central role in the emergence huddling in groups of pups—providing the ‘glue’ of between-individual thermal attractiveness (Harshaw et al., 2014; Sokoloff and Blumberg, 2001)—we hypothesized that BAT abnormalities would disrupt huddling in OTKO pups.

To address this question we compared behavior under ambient conditions in which pups show minimal metabolic effort ($\sim 35^{\circ}\text{C}$) with conditions that provoked BAT thermogenesis and huddling ($\sim 21.5^{\circ}\text{C}$). Under conditions of warmth, BAT thermogenesis (i.e., elevated T) was absent in both genotypes. OTKO pups were nevertheless significantly warmer ($\sim .5^{\circ}\text{C}$) and more active (Fig. 3, left side) than WT pups, despite controlled and similar testing conditions. Pups made contacts largely at random under these conditions, though T_{IS}^{rel} correlated positively with contacts for OTKO but not WT pups. Despite these differences, the cohesiveness of OTKO and WT groups did not differ (Fig. 4). Under cool conditions both genotypes showed elevated BAT activity (Table 1). Nevertheless, OTKO pups showed less BAT thermogenesis and cooler T_{IS} ($\sim .9^{\circ}\text{C}$) and T_{rump} ($\sim .8^{\circ}\text{C}$) values than WT pups (Table 1). Huddles of OTKO pups were also significantly *less cohesive* (Fig. 4), making fewer contacts while huddling in response to cold than WT huddles. In addition, WT pups showed a sex-dependent modulation of the relation between T_{IS}^{rel} and number of contacts made while huddling in response to cold, with T_{IS}^{rel} being positively correlated with

contacts with females but not males.⁸ In contrast, OTKO pups showed similar positive correlations between T_{IS}^{rel} and contacts with both males and females (Fig. S1). Pups in OTKO litters were also *less active* than pups in WT litters, particularly during the first 25 min of cooling (Fig. 3). Overall, OTKO pups gave the appearance of being motivated to contact other pups during cold challenge, while also having difficulty maintaining such contact and being sluggish to respond when contact with littermates was broken.

Experiment 2 was designed to address the possibility that oxytocin deficient (i.e., OTKO) pups also have thermotactic deficits, by examining the behavior of individuals and groups of four pups on a thermocline. When tested as individuals, OTKO pups were sluggish compared to WT pups in moving up the gradient toward warmth. OTKO pups also selected ~1°C cooler regions than did WT pups after the 2 hr test (Fig. S2). As can be seen in Figure 6, this was primarily driven by an apparent deficit in positive thermotaxis in male OTKO pups. No such difference was observed in OTKO females or when OTKO pups were tested in groups of four (Fig. S2).

Experiment 3 was designed to further explore thermotactic differences in OTKO pups, in the context of a huddling test identical to that employed in Exp. 1, except that pups were tested in mixed-genotype (50% WT, 50% OTKO) groups of six, ensuring that WT and OTKO pups experienced identical T_a . Several findings from Exp. 1 were replicated. These included (a) OTKO pups being significantly cooler than their WT huddlemates when cold challenged (Table 3), (b) OTKO pups receiving fewer contacts while huddling in response to cold than WT huddlemates (Fig. 7), and (c) OTKO pups being more active during the warm phase and less active during cold challenge than WT pups (Fig. S6). Some degree of spontaneous assortment by genotype was also observed during cooling, particularly in female runs (Fig. S9). A greater number of WT-WT than OTKO-OTKO contacts also occurred (Fig. S10), suggesting that WTs outcompeted OTKO pups for access to WT pups.

These results suggest intriguing interactional differences between OTKO and WT pups in mixed-genotype huddles. An analysis of pup bias for contacting WT versus OTKO pups during cooling, for example, showed a positive relationship between the relative warmth of individual pups (T_{IS}^{rel}) their degree bias for WT contacts, as well as a negative relationship with bias for OTKO contacts (Fig. 8). OTKO pups also appeared to derive greater net benefit from contacting other pups than did WT huddlemates in correlational analyses (see Fig. S11). Granger analyses examining the temporal relationship between T_{IS}^{rel} , contacts with WT versus OTKO pups, and total contacts revealed key differences in the interactional dynamics between WT and OTKO pups in mixed-genotype huddles (Fig. 9). Significant Granger causality was detected in female huddles, with the direction of causality running primarily from T_{IS}^{rel} to contacts and the net result being negative for WTs—indicating, for example, that lower relative warmth predicted more downstream contacts—and positive for OTKO females—indicating lower warmth predicted fewer downstream contacts. In male huddles, in contrast, the only significant Granger causality detected was that the T_{IS}^{rel} of OTKO males predicted future contacts with other OTKO males, albeit in a negative fashion.

⁸In a previous study, we found a similar pattern in huddles of C57BL/6 pups that was ultimately driven by significant sex difference both in BAT thermogenesis and behavioral dynamics occurring in the huddle ((Harshaw et al., 2014).

This pattern indicates either active avoidance of OTKO pups by WT males or that cooler OTKO males had difficulty competing for or otherwise accessing the warmest spots in the huddle (i.e., WT pups).

Early studies of OTKO mice concluded that temperature homeostasis was unaltered in pups lacking a functional oxytocin gene (see Winslow et al., 2000; Winslow and Insel, 2002). The procedure that led to this conclusion consisted of a single rectal temperature measurement taken immediately after brief (two min) separation of a pup from their dam (Winslow et al., 2000). While variants of this method are common, they provide neither an adequate index of core body temperature nor measure of thermoregulatory competence in young mice. That is, rectal temperature is a localized, peripheral measure with a tenuous connection to the natural ecology of the neonatal rodent (see Blumberg, 2001). A study of C57BL/6 mice in our laboratory, for example, found that dams leave the nest in 10-25 min bouts, lasting up to 45-130 min (P. Meyer, 2010). Pups are seldom alone during such periods, as they have a nest of littermates with which they can huddle and thereby group-regulate (Harshaw et al., 2014; Harshaw and Alberts, 2012). Thus, temperature measurement of isolated individuals after brief separation has limited ecological validity as an assay of overall thermoregulatory functioning (cf. Blumberg, 2001). Such periods of brief isolation are also unlikely to be adequate for uncovering thermoregulatory deficits linked to BAT thermogenesis (see Harshaw and Alberts, 2012).

The present study provides compelling evidence of thermoregulatory dysfunction in OTKO mouse pups tested under conditions of greater ecological validity than those used by Winslow et al. (2000) (i.e., as a group of huddling pups faced with ~50 min cold challenge rather than brief maternal separation). Specifically, OTKO mouse pups exhibited significant differences in the modulation of their activity levels by both ambient warmth and ambient cooling. OTKO pups also clearly show deficient BAT thermogenesis, as has been documented in OTKO adults (Kasahara et al., 2007). Additionally, male OTKO pups appear to have a deficit in positive thermotaxis relative both to WT pups and OTKO females. There are a number of potential explanations for the latter deficit, including (a) learning over the first postnatal week, due to the lack of interscapular “hot spots” on littermates, (b) alterations in temperature hedonics or *alliesthesia*, and (c) nonobvious skin and/or thermosensory abnormalities. Further studies on this question are clearly warranted.

Interestingly, many of the deficits documented here in OTKO mouse pups (i.e., BAT thermogenesis, group cohesiveness while huddling, positive thermotaxis) are in the same direction, though not nearly as severe, as deficits shown by eight-day-old UCP1^{-/-} mouse pups provided identical tests of huddling and thermotaxis (Harshaw et al., *unpublished data*).⁹ This implies the possibility that OTKO pups may be buffered from the effects of OT gene deletion by OT ingested in the milk of their OT^{+/+} foster dams (see Higashida et al., 2017; 2010; Takeda et al., 1986). Though the quick digestion of OT in the gut and short plasma half-life of OT may make this possibility unlikely, OT and OTR are widely expressed in the gut (Ohlsson et al., 2006), indicating the likely presence of cells capable of detection,

⁹These data were not included in the present report largely due to the fact that the UCP1^{-/-} construct was produced on a C57BL/6 background, making direct comparisons of UCP1^{-/-} and OT^{-/-} pups difficult.

uptake, and storage of OT (e.g., Nelson et al., 1998; Qin et al., 2009). It is also possible that an intact arginine vasopressin (AVP) system aids in buffering in OTKO mouse pups from a total lapse of BAT thermogenesis, given the ability of AVP to bind to OT receptors (Audigier and Barberis, 1985). These questions thus deserve further attention (cf. Mishra et al., 2014).

Our findings add to a growing literature demonstrating that oxytocin and AVP play key roles in energy homeostasis (see Blevins and Ho, 2013; Chaves et al., 2013), likely including seasonal shifts in energetics and associated behaviors (cf. Ondrasek, 2016). A number of classic studies found that OT and OT analogues function as pyrogens when administered centrally (e.g., Lipton and Glyn, 1980; Mason et al., 1986; Robinson et al., 1988), whereas AVP and AVP analogues have often been linked to heat loss (e.g., Cooper et al., 1979; Kovács and de Wied, 1983; Naylor et al., 1986; Ramos et al., 2014; Wilkinson and Kasting, 1987). Until recently, the mechanism(s) underlying these effects were largely unknown. It is now clear that the hypothalamic nuclei responsible for adaptive cold-response (i.e., PVN, DMH) require OT and OTR for their normal functioning (Kasahara et al., 2013; 2007; Oldfield et al., 2002). In addition, two recent studies reported extensive OXTR expression in embryonic and neonatal BAT in C57BL/6 mice (Greenwood and Hammock, 2017; Hammock and Levitt, 2013), raising questions regarding the function of such receptors, the timing of their disappearance (given their absence in adults), and potential perinatal effects of OT on BAT during parturition and early development (e.g., Beck et al., 1979). Although the effects observed in OTKO mouse pups in the present study are almost certainly tied to deficiencies in BAT thermogenesis (Kasahara et al., 2007), it is also likely that OTKO mice possess abnormal *thermolysis* or heat conservation responses, due to changes in the ability to regulate vasoconstriction and/or cutaneous blood flow in response to cold (e.g., Deis et al., 1963; Lin et al., 1983; Xi et al., 2017).

The construct of *sociality* encompasses a wide range of affiliative behaviors and cognitive variables, including social motivation and empathy, pro-social affiliative behaviors such as pair bonding and huddling, as well as agonistic behaviors involved in territoriality and defense of social groups from outsiders (see Kelly and Ophir, 2015). As Kelly and Ophir (2015) have emphasized, these varied kinds of social behavior may have different regulators and these are likely to vary by species, sex, ecological context, season, etc. The results presented here highlight that specific social behaviors (e.g., huddling, aggregation) can be tightly linked to homeostatic processes, including thermoregulation and energy balance (cf. Porges, 2011). Social and energetic niches often covary significantly, whether considering circadian, seasonal, developmental, or evolutionary timescales (e.g., Harshaw et al., 2017; Ondrasek, 2016). Social and metabolic mechanisms are thus likely to be co-selected during phylogeny and co-regulated by environmental cues (e.g., light, temperature, the presence of conspecifics) during ontogeny (e.g., Kapheim et al., 2015; Woodard et al., 2014). As demonstrated here, the huddling behavior displayed by rodents and other small mammals and its regulation both by oxytocin (Alberts, 2006; Arakawa et al., 2015; Beery and Zucker, 2010; Bowen and McGregor, 2014; Kojima and Alberts, 2011; Mooney et al., 2014; Smith et al., 2010) and brown adipose tissue thermogenesis (Harshaw et al., 2014; Sokoloff and Blumberg, 2001) provides an excellent example of such likely co-evolution.

Within the framework adopted here, it should not be surprising that a molecule with such diverse effects on social and reproductive behavior as oxytocin also has critical consequences for metabolism and thermoregulation. We have shown that mouse pups deficient in oxytocin (e.g., OTKO) display impaired BAT thermogenesis, less cohesive huddling, and abnormal modulation of their activity levels by ambient temperature compared to WT pups. OTKO males moreover show evidence of impaired positive thermotaxis (i.e., reduced attraction to warmth) relative to WT pups and OTKO females. These results prompt a number of important questions regarding the regulation of thermoregulatory mechanisms by oxytocin that warrant further exploration. It is nevertheless clear that oxytocin must play an important role in orchestrating the energetic shifts and tradeoffs underlying simple forms of social behavior. In the case of huddling, this includes the production of a literal “outer glow.”

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- OTKO pups have impaired BAT thermogenesis and are thus significantly cooler than WT pups while huddling in response to cold.
- This deficit causes huddles of oxytocin knockout (OTKO) mouse pups to be less cohesive than huddles of WT pups when cold challenged.
- Analysis of mixed-genotype huddles revealed significant differences in the huddling behavior of OTKO and WT pups, related to both abnormal BAT thermogenesis and, in males, abnormal thermotaxis.
- Compared to WT pups, male OTKO pups display abnormal positive thermotaxis when placed on the cool end of a standard thermocline.
- These results highlight the entanglement of the social and thermoregulatory functions of oxytocin for mice.

Figure 1. Scoring of contact behavior

An illustration of the system of scoring contact behavior used (see Harshaw et al., 2014). Once per minute, the huddle is scored for overall cohesiveness and each pup scored for the other pups with which it is in contact, excluding contacts made via tails or outstretched paws. Each pattern of contact is represented by a unique "contactogon." In the example above, the black pup represents a target pup being scored for its contacts with same -sex or -genotype (dark grey) or different -sex or -genotype (white) pups. For example, in the case of a mixed huddle with three males and three females (e.g., in Exp. 1), 0M 2F designates contact with zero males and two females. Contactogons possible for only a single sex or genotype (e.g., 3M 0F) are not shown and are collapsed into a single category for the purposes of statistical analysis. Individual scores for total number of contacts made are also calculated based on these contactogons.

Figure 2. Infrared thermography

Sample thermographs from Experiments 1 and 3. The left panel (A) shows a group of OTKO pups (half male, half female) huddling in response to an ~21.5°C cold challenge (Exp. 1), with zones used for calculating interscapular and rump temperatures (T_{IS} and T_{rump} , respectively) illustrated on an OTKO pup that has separated from its huddlemates. The right panel (B) contains thermographs randomly chosen from three different mixed-genotype runs (2 female, 1 male), with the genotype of the pups (OTKO or WT) indicated for each thermograph (Exp. 3).

Figure 3. Pup activity in Experiment 1

Average activity level (cm/min) and 5 min moving averages for groups of six pups in OTKO and WT huddles during a 51 min period of ambient warmth (mean = $34.8 \pm .01^{\circ}\text{C}$) followed by a 51 min period of ambient cooling (mean = $21.6 \pm .06^{\circ}\text{C}$).

Figure 4. Group cohesiveness in Experiment 1

Average huddle cohesiveness for litters of WT (upper panel) and OTKO (middle panel) pups during a 51 min period of ambient warmth ($34.8 \pm .01^{\circ}\text{C}$) followed by a 51 min period of ambient cooling ($21.6 \pm .06^{\circ}\text{C}$). A huddle cohesiveness score of 1 indicates that all pups in the litter are in contact, forming a single aggregate. A score of 4, on the other hand, would indicate 4 separate individuals and/or aggregates (e.g., 2 groups of 2 pups and 2 isolated individuals). The lighter lines on the upper and middle panels are the data from individual litters. The lower panel presents a direct comparison of the average cohesiveness for WT and OTKO litters. Asterisks indicate a *systematic* difference (i.e., a non-random distribution of differences) between the two series, ascertained via a Sign test ($\alpha = .05$).

Figure 5. Correlation between T_{IS} relative to huddlemates and contacts (Exp. 1)

Linear regressions, coefficients of determination (R^2), and p -values for Pearson product moment correlations on T_{IS} relative to huddlemates (T_{IS}^{rel}) and average number of contacts per minute during the warm and cool phases of Experiment 1 (left and right portion of the figure, respectively). Asterisks indicate significant correlation, employing a criterion of $\alpha = .05$. As can be seen, the correlation between T_{IS}^{rel} and contacts was higher for pups in OTKO compared to pups in WT litters, likely because the greater cohesiveness and thus greater sharing of heat in WT litters resulted in less thermal stratification of pups.

Figure 6. Thermocline performance of male and female WT and OTKO pups, tested as individuals (Exp. 2)

Average location temperatures for WT (upper panel) and OTKO (lower panel) male and female pups over the course of a two-hour test. Pups are placed on the cool end of the thermocline at the onset of testing and free to locomote up and down the thermocline upon release. Asterisks indicate a *systematic* difference (i.e., a non-random distribution of differences) between the two series, ascertained via a Sign test ($\alpha = .05$).

Figure 7. Average number of contacts by WT and OTKO pups during huddling (Exp 3)
 Average number of contacts over time $\pm$ SEM during the huddling test for male (lower panel) and female (upper panel) runs. Asterisks indicate a *systematic* difference (i.e., a non-random distribution of differences) between the two series, ascertained via a Sign test ($\alpha = .05$).

Figure 8. Correlation between thermal attractiveness (T_{IS}^{rel}) and contacts (Exp. 3)
 Scatterplots of T_{IS} relative to huddlemates (T_{IS}^{rel} or average relative warmth) against pup bias for contacting WT pups (left panel) and bias toward contacting OTKO pups (right panel). Linear regressions, Pearson product moment correlations, and p -values are also shown. The contact bias measure depicted corrects for the fact that for any given pup there is a 2/5 chance of contacting another pup of the same genotype and a 3/5 chance of contacting a pup from the other genotype if contacts are random. A value of 1.0 would indicate chance responding (illustrated by the dashed vertical line), whereas a value of 1.25 would indicate 25% more contacts than expected by chance. Asterisks indicate significant correlation, using a criterion of $\alpha = .05$.

Figure 9. Causal modeling of the relationship between T_{IS}^{rel} and contacts during cooling (Exp. 3)

The results of panel Granger analyses on relative T_{IS} (T_{IS}^{rel}) and contact with OTKO and WT pups during the cool phase of mixed-genotype (male or female) huddling tests, using a 1 min lag. Asterisks indicate significant Granger causality, evaluated at $\alpha = .05/6 = .0083$. Non-significant results ($p < .05$) are also shown to illustrate trends. Arrows indicate that a change in one variable at time t_n predicts a change in another variable at t_{n+lag} . The coloration of arrows indicates lagged Pearson product moment correlations between the two variables, with positive and negative correlations indicated by green and red, respectively.

Table 1

Thermographic Measures by Genotype in Experiment 1

	measure	WT	OTKO	Independent Sample T-tests		
		average, in °C (SEM)	t-score	p-value	effect size (d)	
Warm phase	T _{IS}	35.58 (.05)	36.10 (.04)	8.54	0.00000*	1.74
	T _{rump}	35.46 (.05)	36.00 (.04)	8.47	0.00000*	1.73
	T	0.10 (.01)	0.10 (.01)	0.17	0.86390	0.04
Cool phase	T _{IS}	29.31 (.17)	28.40 (.18)	-3.67	0.00040*	-0.75
	T _{rump}	28.42 (.12)	27.65 (.15)	-4.02	0.00012*	-0.82
	T	.99 (.04)	0.70 (.04)	-4.77	0.00000*	-0.97

Note. Average interscapular temperatures (T_{IS}), rump temperatures (T_{rump}), and T_{IS} - T_{rump} (T) temperatures for WT and OTKO pups, obtained during group huddling tests with littermates on PD8. Two tailed, independent sample t-tests on between-genotype comparisons of these are also provided. Asterisks indicate a significant difference ($\alpha = .05 / 3 = .0167$). SEM = Standard error from the mean.

Table 2

Warm Phase Activity, Contact, and Thermal Measures for Experiment 3

	WT	OTKO	Matched-Pairs Signed Rank tests			
	measure	average (SEM)	Z	p-value	effect size (r)	
Males						
activity	weight (g)	4.76 (.08)	4.82 (.09)	-1.80	0.07220	-0.28
	cm/min	1.40 (.10)	1.82 (.16)	-2.22	0.02633*	-0.34
temperature	T _{IS} (°C)	35.32 (.10)	35.41 (.13)	-2.30	0.02157	-0.35
	T _{rump} (°C)	35.19 (.10)	35.29 (.12)	-2.26	0.02385	-0.35
contacts	T (°C)	0.13 (.01)	0.11 (.01)	-0.99	0.32050	-0.15
	total	55.8 (3.3)	59.0 (3.4)	-0.80	0.42390	-0.12
	same genot.	20.0 (2.0)	23.7 (2.4)	-1.80	0.06987	-0.28
	other genot.	35.8 (2.2)	35.3 (1.9)	-0.09	0.93070	-0.01
Females						
activity	weight (g)	4.68 (.06)	4.70 (.06)	-0.71	0.47490	-0.11
	cm/min	1.48 (.09)	1.75 (.14)	-2.03	0.04208*	-0.31
temperature	T _{IS} (°C)	35.27 (.08)	35.29 (.09)	-0.61	0.53920	-0.09
	T _{rump} (°C)	35.14 (.08)	35.18 (.09)	-1.20	0.22900	-0.19
contacts	T (°C)	0.12 (.01)	0.11 (.01)	-0.71	0.47610	-0.11
	total	54.1 (2.4)	52.0 (2.4)	-0.82	0.41090	-0.13
	same genot.	20.1 (1.7)	18.0 (1.5)	-0.83	0.40760	-0.13
	other genot.	34.0 (2.1)	34.0 (1.5)	-0.02	0.98610	-0.00

Note. Average for measures of weight, activity, thermal and contact for WT and OTKO pups, obtained during the Warm Phase of mixed-genotype huddling tests on PD8. Two tailed, Wilcoxon Matched-Pairs Signed Rank tests of these are displayed. Note that these analyses do not take into account the interactions presented in the mixed-effects models, in text. Asterisks indicate a statistically significant difference. Bonferroni correction was used within each family of measures ($\alpha = .05 / 3 = .0167$ for thermal and contact measures and $\alpha = .05$ for pup weight and activity). SEM = Standard error from the mean. T_{IS} = interscapular temperature. T_{rump} = rump temperature. T = T_{IS} - T_{rump}.

Table 3

Cool Phase Activity, Contact, and Thermal Measures During for Experiment 3

	measure	WT	OTKO	Matched-Pairs Signed Rank tests		
		average (SEM)	Z	p-value	effect size (r)	
Males						
activity	cm/min	0.99 (.04)	1.07 (.05)	-1.81	0.07014	-0.28
	T _{IS} (°C)	29.62 (.21)	29.12 (.24)	-3.43	0.00061*	-0.53
temperature	T _{rump} (°C)	28.61 (.19)	28.28 (.20)	-2.94	0.00328*	-0.45
	T (°C)	1.05 (.06)	0.87 (.06)	-2.45	0.01417*	-0.38
contacts	total	108.4 (3.5)	103.1 (3.4)	-1.21	0.22700	-0.19
	same genot.	47.3 (2.0)	41.8 (2.2)	-1.46	0.14390	-0.23
	other genot.	61.1 (3.0)	61.3 (3.6)	-0.06	0.95530	-0.01
Females						
activity	cm/min	1.15 (.06)	1.02 (.06)	-1.92	0.05469	-0.30
	T _{IS} (°C)	29.63 (.18)	28.94 (.19)	-3.72	0.00020*	-0.57
temperature	T _{rump} (°C)	28.57 (.15)	28.13 (.15)	-3.43	0.00061*	-0.53
	T (°C)	1.09 (.06)	0.86 (.05)	-3.38	0.00072*	-0.52
contacts	total	109.3 (3.1)	102.1 (5.2)	-1.48	0.14010	-0.23
	same genot.	49.0 (2.8)	41.8 (2.9)	-1.50	0.13480	-0.23
	other genot.	60.3 (3.2)	60.4 (4.2)	-0.15	0.88120	-0.02

Note. Average for measures of activity, thermal and contact for WT and OTKO pups, obtained during the Cool Phase of mixed-genotype huddling tests on PD8. Two tailed, Wilcoxon Matched-Pairs Signed Rank tests of these are displayed. Note that these analyses do not take into account the interactions accounted for in the mixed-effects models presented in text. Asterisks indicate a statistically significant difference. Bonferroni correction was used within each family of measures ($\alpha = .05 / 3 = .0167$ for thermal and contact measures and $\alpha = .05$ for activity). SEM = Standard error from the mean. T_{IS} = interscapular temperature. T_{IS} = rump temperature. T = T_{IS} - T_{rump}.