Social stress blocks energy conservation in rats exposed to an oxytocin-injected cage mate

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We previously found stress-reduction in rats exposed to an oxytocin-injected cage mate. Olfactory impairment and oxytocin antagonist treatment blocked the effect. Here, we investigated effects of social stress on the exposure-induced response and exposure on amygdaloid oxytocin concentrations. CT concentrations in exposed olfactorily impaired, CT antagonist-treated and saline-injected unexposed rats were reduced, compared to the significantly higher level in untreated and exposed saline-injected rats. Saline injections and group mixing enhanced heat dissipation. Exposure abolished the injection-induced, but not mixing-induced stress response, most likely via a social stress induced effect on the oxytocin-injected rat. The difference in exposure responsivity may relate to recognition, stress type and intensity affecting different stress—response systems. The mechanism could reinforce social attachment.

Key words: Amygdala; Group mixing; Olfactory stimulation; Oxytocin concentrations; Recognition; Social stress; Tail skin temperatures

INTRODUCTION
Recently, we reported that the increased heat dissipation and suppressed growth following mild challenges was reversed in saline-injected male rats when exposed to an oxytocin-injected cage mate [1,2]. ACTH and corticosterone plasma concentrations were also reduced [2]. No reversal occurred following olfactory impairment [1] or oxytocin-antagonist treatment [2], implying an olfactorily induced oxytocinergic, anxiolytic mechanism. If an olfactory mechanism is involved, one possibility is that a pheromone from the OT-injected rat stimulated the effects via the vomeronasal organ and accessory olfactory system. However, the method used for impairment, ZnSO4 nasal infusions, primarily affects the olfactory mucosa and main olfactory bulb [3] and individual recognition [4]. This suggests that higher order sensory and cognitive processing may be involved.

The main objective of this study was to further investigate mechanisms behind the socially induced process. Limbic pathways and higher-order sensory processing modulates conditioned, anticipatory fear or anxiety reactions. Here the central nucleus of the amygdala (CeA) has an important role in the integration of sensory input to cortical areas, hypothalamus and brain stem [5,6], allowing an influence of individual recognition.

Therefore, the importance of sensory input related to familiarity and dominance relationships was investigated. Secondly, amygdaloid tissue oxytocin-li concentrations were analyzed in saline-injected rats exposed to an oxytocin-injected cage mate. Repeated tail skin temperature (TST) assessments were used to estimate heat dissipation [1,2]. This method to monitor stress activation, using an infrared distant sensor, requires little training of the animals and it is non-invasive [1,2]. Mixing of unfamiliar rats was used to induce social stress. Stress-induced hyperthermia has been used in social stress models, in animal models including rats and mice [7,8].

MATERIAL AND METHODS
Animals and housing conditions: Adult male Sprague–Dawley rats (300–350 g; 400–450 g, B and K Universal AB, Sollentuna) were used in all experiments. They were housed in permanent groups as an ambient temperature of 21 ± 2°C. The rats were provided with standard laboratory food and water ad lib, and they were kept in a 12:12 h light: dark cycle, lights on at 07.00h. The rats were habituated to the laboratory facilities.

Drug treatments and olfactory impairment: The ‘signaling’ rats were always given oxytocin (1 mg/kg, s.c.), dissolved in saline (1 ml/kg) [1,2]. The experimental rats, exposed to an OT-injected rat, and the unexposed control rats were injected with saline (1 ml/kg) in the familiarity and dominance experiments.

Two unexposed groups were used for CeA oxytocin concentration analysis; one group received a saline injection and the other no treatment. Groups of exposed rats received injections of saline, or an oxytocin antagonist (Atosiban, Ferring AB, Malmö, Sweden), or they were saline injected
and olfactorily impaired. ZnSO₄ nasal infusions were used for the impairment [1,3].

**Tail skin temperatures assessments:** An infrared sensitive thermometer (Digitron, D805H, Farnell Components AB, Soina, Sweden) mounted on a 45 cm high tripod was used for the TST assessments. When measuring temperatures the rat is placed on a table and held gently across the scapula as previously described [1,2]. The rats were accustomed to handling with temperature assessments prior to the experiments. To avoid prior uncontrolled stressful events, and to reduce olfactory stimulation, between cages, the rats were placed in filter top cages (20 × 38 × 16, Macrolon IV) and moved at least 2 h before the experiments.

**The effect of group mixing on tail skin temperatures:** The effect of repeated handling was first assessed over 60 min in rats kept with their own permanent cage mates. At 80 min the experimental (n = 16), but not control (n = 8) rats were socially mixed. Social group mixing was accomplished by placing together one rat from each of four cages in new filter-top cages. Control rats were treated similarly but they were not mixed. These procedures took 5 min after which assessments continued for an additional 100 min.

**Effects of size on tail skin temperatures:** In four cages one randomly chosen male received an OT injection and their cage mates saline injections (n = 4 × 4). The first assessment was done directly after the injections, and then repeated over 60 min. The difference in bodyweight (range 300–350 g) between the OT and each saline-injected rat was calculated and size rank order was established for each caged group.

**Effects of social mixing on exposure-induced effects on tail skin temperatures:** The same rats were tested twice, 2 weeks apart. On the first occasion all rats were kept in their own permanent groups throughout the experiment. The effect of exposure to an OT-injected rat was assessed in its saline-injected (n = 12) cage mates. On the second occasion the rats were also subjected to group mixing when they received the OT or saline injections. Temperatures were assessed once before the injections and subsequently every 15–20 min for 65–70 min. In experimental groups three rats in each cage received a s.c. saline injection and the fourth largest individual received an oxytocin injection. In control groups (n = 16) all received a saline injection.

**Collection of tissue samples:** The rats were sacrificed by decapitation 50–60 min after the saline or drug injections. Their brains were placed in a mould and cut in 2.5 mm coronal sections using a stainless steel wire (diameter 0.07 mm). Samples (n = 10 for each treatment) from the central nucleus of the amygdala (CeA) were punched from the fourth and fifth slice, weighed and immediately frozen on dry ice. The samples were stored at −70°C until extraction and analysis.

**Extractions and radioimmunoassay (RIA):** The frozen samples were transferred to 2 ml boiling 0.05 M phosphate buffer (pH 7.4). They were boiled for 10 min, cooled, homogenised using a steel rod vortex mixer, and finally centrifuged (2800 × g, 4°C) for 10 min. The supernatant was incubated (100°C for 10 min), and the pellets were dissolved and mixed in 2 ml 1.0 M acetic acid, again followed by centrifugation and cooling. These procedures were repeated now dissolving the pellets in neutral medium (milli-Q water). The supernatant from the acetic acid and neutral extractions were mixed, and the samples were lyophilized overnight. The lyophilized samples were dissolved in 1 ml phosphate buffer and stored at −20°C until analysed.

A RIA using rabbit antiserum solution RAS-8152 and tracer solution [125I] oxytocin, Y-8152 (Peninsula Laboratories, Inc., CA, USA), was used for determination of oxytocin-li concentrations. Cross reactivity with [Arg8]-vasopressin was 0%, [Lys8]: Vasopressin < 0.01%, LH-RH 0% and with CRF (human, rat) 0%. The antiserum solution was incubated with 100 μl standard solution or the extracted samples at +4°C for 2 h. The tracer solution was added (100 μl), and the samples were incubated for another 24 h at 4°C. Separation of bound and unbound fractions was accomplished by the incubation with 500 μl of a second rabbit antibody, the Decanting suspension-3 (Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden) for 30 min at room temperature. Incubation was interrupted by the adding 1 ml water (milli-Q). The samples were centrifuged for 17 min (+4°C, 2800 × g) and the supernatant was decanted. A standard curve was prepared and the radioactivity in the sample precipitate was measured in a gamma counter for 6 min. The limit of the assay was 3.6 pmol/l. All samples were assayed in duplicate.

**RESULTS**

**Effects of group mixing on tail skin temperatures:** Before mixing (Fig. 1a; Time 0–60 min), the experimental and control rats showed a similar ANOVA (F(1,23) = 2.3, p < 0.15) variation in tail skin temperatures with repeated handling over time (F(3,66) = 25.6; p < 0.0001). The mixing at 80 min (F(1,22) = 8.9, p < 0.01) and repeated manipulations over time (F(6,132) = 20.8, p < 0.0001) induced a further variation. An increase of about 2.5°C had occurred 25 min, but not 5 min, after mixing. The tail skin temperatures of the briefly handled, but not mixed control rats showed little additional change (Fig. 1a). Tail skin temperatures of the mixed rats remained above those of the control rats (85 min ns, 105–165 min: p < 0.0001) until the final assessments.

**Effects of exposure to an OT-injected rat on mixing-induced stress:** Tail skin temperature, varied with exposure (F(1,52) = 20.4, p < 0.0001), group mixing (F(2,52) = 41.9, p < 0.0000) and repeated assessments over time (F(4,208) = 105.4, p < 0.0000) in the saline-injected rats (Fig. 2a, b). The interaction between mixing and time was significant (F(4,208) = 7.4, p < 0.0000). Basal temperatures did not vary, and tail skin temperatures increased significantly 15 min after the saline injections in all groups. Tail skin temperatures remained increased in unmixed control, but returned to basal levels in exposed rats (Fig. 2a). Following mixing TSTs did not return in either control or exposed rats (Fig. 2b). The mixed rats showed higher tail skin temperatures than unmixed ones throughout the experiment (Fig. 2a, b; 15–70 min; p < 0.0001).
Effects of OT injections plus mixing on tail skin temperatures: Basal tail skin temperature, were assessed, and rats in a cage were then either injected with OT or saline, directly followed new assessments. An overall effect of drug treatments ($F(2,3) = 7.8, p < 0.02$) was found, and the repeated assessments over time ($F(5,40) = 7.4, p < 0.0000$). Tail skin temperatures of the rats injected saline dropped acutely, increased within 30 min and then remained elevated during entire 2 h experiment (Fig. 3a: 30–120 min). Tail skin temperatures did not vary over time in the OT-injected rats (Fig. 3a).

Tail skin temperatures varied over time in the OT-injected rats kept with saline-injected rats ($F(4,24) = 15.1, p < 0.0000$). The interactive factor between mixing and time ($F(4,24) = 2.9, p < 0.04$) indicated that a significant increase only occurred when the rats were mixed (Fig. 3b). The temperatures were higher in mixed compared to unmixed groups at 30 min (Fig. 3b: $p < 0.04$).

Effects of size on exposure-induced TST-suppression: The OT-injected ‘signaling’ individual was not the largest rat in any test cage, unlike in the above experiments. The tail skin temperatures varied over time ($F(6,48) = 7.79, p < 0.0001$), with a peak 15 min after the injections in their cage mates (Fig. 1b). This peak temperature was used as set point to analyze the predicted tail skin temperature drop. Temperatures remained elevated for 30 min in all rats, and dropped subsequently in some. The size of the OT-injected cage mate had on overall effect on TST variation ($F(2,8) = 4.96, p < 0.05$). The interactive time–size factor ($F(12,48) = 3.60, p < 0.001$) indicated that a TST reduction only occurred when the ‘signaling’ rat was the second largest rat in the cage (Fig. 1b).

Consistently, the size rank (large to small: 2,3–4) of the OT-injected rat and the TST increase in the saline-injected rats at 35–65 min both loaded positively on the first factor in a principal components analysis (Table 1, eigen values 4.3,
2.2). The TST change during the increase phase 15 min after the injection, and the bodyweight of the saline-injected rats compared to their OT-injected cage mate loaded on the second factor. These two factors explained 48% and 24% of the total variance. Thus, the TST suppression in the saline-injected was related to the rank of the ‘signaling’ rat, and the initial energy mobilisation to their own size.

Effects of olfactory impairment, saline or OT antagonist injections on CeA OT concentrations in exposed cage mates: OT-li concentrations varied between treatment groups (Fig. 4; (F(4,45) = 18.42, p < 0.0001). The concentrations in exposed saline-injected rats were not different from those in untreated rats. The concentrations in unexposed saline-injected control rats, exposed olfactorily impaired saline-injected and exposed OT antagonist-injected rats were similar, all significantly suppressed compared to those of the untreated control rats (Tukey’s HSD test; p < 0.0001 and p < 0.0002).

DISCUSSION
Previously, effects consistent with energy conservation and suppression of anxiety were found in saline-injection stressed rats exposed to a systemically OT-injected cage mate [1,2], including reduced plasma concentrations of ACTH and corticosterone [2]. The response was blocked by OT antagonist treatment and olfactory impairment [1,2]. The radioimmunoassay (RIA) results of this study indicate that an oxytocinergic mechanism within the central nucleus of amygdala may be involved.

Tail skin temperature variation was previously used to assess injection and hypertherma induced by repeated handling [1,2]. Here we report that group mixing increased tail skin temperatures in non-injected rats. This demonstrates the feasibility to use tail skin temperature variation as an indicator of social stress. Encountering an unfamiliar conspecific is stressful, and commonly aggression promoting particularly in males of many species. Since little overt aggression was shown during testing an anticipatory stress response was likely induced. The social stress induced by group mixing blocked the exposure effect in saline-injected rats, as indicated by further increased rather than decreased tail skin temperature.
The reduced hyperthermia found in saline-injected exposed rats among cage mates confirms previous results [1,2]. Possibly, individual recognition contributed to the exposure-induced response. Several reports concern the impact of OT on learning, memory, recognition, olfactory preferences and stress coping [9]. Dose-dependent negative and positive effects of OT on recognition have been reported [10–15]. The effects of OT could partly concern the stress response rather than recognition per se.

Here, OT may have a dual function: an influence on the potential anti-stress signal induced by the OT injections, and, an anxiolytic impact on the exposed rats. This is demonstrated here in terms of maintained CeA OT concentrations and reduced heat dissipation in injection-stressed rats, and previously by maintained bodyweight and reduced plasma concentrations of ACTH and corticosterone [2]. Consistently, an inhibitory effect of endogenous OT on basal and physical stress-induced ACTH release has been indicated by i.c.v. infusions of an OT antagonist into the paraventricular nucleus [16].

The reduced CeA OT concentrations in olfactorily impaired rats could reflect the absence of individual specific olfactory input [3,4]. However, the rank-related impact on the effect of exposure to OT-injected rat on cage mates support the idea that individual recognition per se is not a sufficient factor. The level of psychosocial stress may be important.

Increased tail skin temperatures in response to mixing were observed in all rats, including the OT-injected ‘signaling’ rat. An acute drop in tail skin temperature precedes the increase in saline-injected rats [2]. However, neither a drop nor an increase was observed in OT-injected when all rats in a cage were injected the same. This lack of cutaneous vascular response could indicate a modified SNS response, possibly affecting a potential anti-stress signaling. The mixing and rank-related stress-effects may have modified this signal. It has been shown that the odour of stressed conspecifics will induce stress [17,18]. Thus, a stress signal may override an anti-stress signal from the OT-injected rat. The initial injection-induced drop in tail skin temperature and peak was associated with the exposed saline-injected rats’ own status. The presence or absence of an exposure effect may also depend on stress type and intensity differences. The saline injection probably induced a mild physical stress, rather than the more intensive mixing-induced social stress response, as judged from the temperature responses. Thus, different stress response systems were probably involved [19–21].

Finally, if the experimentally induced increase in OT plasma concentrations mimics a natural situation, this could be occurring in interaction between bonded individuals. Oxytocinergic mechanisms are involved in social bonding [22], and individual recognition is required. The signaling effect may be induced by plasma OT release in response somatosensory stimulation, during suckling in mother–offspring interaction, and huddling, grooming and copulation. A mechanism where increased OT concentrations serve as a physiological marker could reinforce social attachment, whether experienced in terms of energy conservation or reduced anxiety and fear.

CONCLUSION
The present study shows that an endogenous anxiolytic mechanism may be induced in rats under specific social conditions by OT injections of a conspecific. The process involves an oxytocinergic mechanism at the level of the CeA. An oxytocinergic mechanism at the level of CeA is consistent with the complex sensory and cognitive involvement in social attachment.

REFERENCES
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