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Dynamic changes in extracellular release of GABA and glutamate in the lateral septum during social play behavior in juvenile rats: Implications for sex-specific regulation of social play behavior

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Abstract

Social play is a motivated and rewarding behavior that is displayed by nearly all mammals and peaks in the juvenile period. Moreover, social play is essential for the development of social skills and is impaired in social disorders like autism. We recently showed that the lateral septum (LS) is involved in the regulation of social play behavior in juvenile male and female rats. The LS is largely modulated by GABA and glutamate neurotransmission, but their role in social play behavior is unknown. Here, we determined whether social play behavior is associated with changes in the extracellular release of GABA and glutamate in the LS and to what extent such changes modulate social play behavior in male and female juvenile rats. Using intracerebral microdialysis in freely behaving rats, we found no sex difference in extracellular GABA concentrations, but extracellular glutamate concentrations are higher in males than in females under baseline condition and during social play. This resulted in a higher glutamate/GABA concentration ratio in males versus females and thus, an excitatory predominance in the LS of males. Furthermore, social play behavior in both sexes is associated with significant increases in extracellular release of GABA and glutamate in the LS. Pharmacological blockade of GABA-A receptors in the LS with bicuculline (100 ng/0.5 μ l, 250 ng/0.5 μ l) dose-dependently decreased the duration of social play behavior in both sexes. In contrast, pharmacological blockade of ionotropic glutamate receptors (NMDA and AMPA/kainate receptors) in the LS with AP-5 + CNQX (2 mM +0.4 mM/0.5 μ l, 30 mM+3 mM/0.5 μ l) dose-dependently decreased the duration of social play behavior in females, but did not alter social play behavior in males. Together, these data suggest a role for GABA neurotransmission in the LS in the regulation of juvenile social play behavior in both sexes, while glutamate neurotransmission in the LS is involved in the sex-specific regulation of juvenile social play behavior.

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Keywords

GABA; glutamate; lateral septum; microdialysis; sex difference; social play

INTRODUCTION

Social play is a highly motivated and rewarding behavior (Trezza et al., 2010, 2011) predominantly displayed between juveniles of both sexes across many mammalian species (Bekoff & Byers, 1998; Pellis & Iwaniuk, 2000; Burghardt, 2005). Studies in humans, non-human primates, and rodents suggest that social play activities among peers contribute to the development of social and emotional competence (Pellegrini, 1988; Suomi & Harlow, 1972; Sigman & Ruskin, 1999; Hol et al., 1999; Guralnick et al., 2006; Cordoni & Palagi, 2011). Social play deficits are observed in children diagnosed with neurodevelopmental disorders such as autism spectrum disorders (ASD), early-onset schizophrenia, and attention-deficit/hyperactivity disorder (Alessandri, 1992; Moller & Husby, 2000; Jordan, 2003). Therefore, a better understanding of the neurobiological regulation of social play behavior may help to gain insights into normal as well as impaired expression of this behavior.

The lateral septum (LS) plays a critical role in modulating social, motivational, and rewarding behaviors (Clarke & File, 1982; Goodson et al., 1997; Beiderbeck et al., 2007; Scotti et al., 2011; Luo et al., 2012; McDonald et al., 2012; Veenema et al., 2012; Lukas et al., 2013; Harasta et al., 2015). This is likely mediated through extensive connections of the LS with regions important for social information processing (i.e., the bed nucleus of the stria terminalis and medial amygdala) as well as with regions that play a role in motivation and reward (i.e., the ventral tegmental area and nucleus accumbens; Sheehan et al., 2004). As such, the LS has been proposed to be a key node in the social decision-making network (O'Connell & Hofmann, 2011). Not surprisingly then, the LS has been shown to be involved in the regulation of social play behavior in juvenile rats and juvenile hamsters (Beatty et al., 1982; Cheng & Delville, 2009; Veenema et al., 2013; Bredewold et al., 2014). Interestingly, while lesioning of various brain areas resulted in decreased expression of social play behavior (Meaney et al., 1981; Beatty & Costello, 1983; Bell et al., 2009), electrolytic lesioning of the septum increased social play behavior in juvenile male and female rats (Beatty et al., 1982). This increase could be due to the disinhibition of output regions of the LS, since LS output is mainly GABAergic (Risold & Swanson, 1997a; Zhao et al., 2013).

GABA and glutamate, the principle inhibitory and excitatory neurotransmitters in the brain, largely modulate LS neuronal activity (Joëls & Urban, 1984a,b; Allaman-Exertier et al., 2007). GABA released in the LS likely derives from local LS neurons sending recurrent axon collaterals to neighboring neurons (Onteniente et al., 1987; Phelan et al., 1989; Jakab & Leranth, 1990; Risold & Swanson, 1997b), thereby inhibiting intraseptal neuronal activity. GABAergic input may also originate from sources projecting to the LS such as the nucleus accumbens (Zahm et al., 2013). Glutamate released in the LS derives from multiple brain regions (Leranth et al., 1999; Riedel et al., 2008, 2013; Chee et al., 2015) with the most prominent source to the LS coming from the hippocampus (Zaczek et al., 1979; Gallagher et al., 1995). Given the modulatory role of GABA and glutamate on LS neuronal

activity, we hypothesized that GABA and glutamate released in the LS are involved in the regulation of social play behavior.

The current study aimed to characterize the role of GABA and glutamate neurotransmission in the LS in the regulation of social play behavior. We used intracerebral microdialysis in freely behaving male and female juvenile rats to determine whether extracellular release of GABA and glutamate in the LS are altered during social play behavior. Using acute pharmacological manipulations, we then determined the involvement of GABAergic and glutamatergic signaling in the regulation of male and female social play behavior. Because the expression of social play behaviors is similar between male and female juvenile rats when tested in dyads (Veenema et al., 2013, Bredewold et al., 2014), we predicted that both sexes will show similar changes in extracellular GABA and/or glutamate release during the display of social play behavior. Since electrolytic lesioning of the septum ablates any LS output and increased social play behavior (Beatty et al., 1982) likely by eliminating inhibitory LS output, we further predict that blocking LS-GABA receptors will decrease, while blocking LS-glutamate receptors will increase social play behavior in both sexes.

EXPERIMENTAL PROCEDURES

Animals

Male and female Wistar rats (23 days of age) were obtained from Charles River (Raleigh, NC) and maintained under standard laboratory conditions (12 h light/dark cycle, lights off at 14:00 h, 22 °C, 50% humidity, food and water *ad libitum*). Rats were housed in same-sex groups of four in standard rat cages (48 × 27 × 20 cm) unless otherwise mentioned. The experiments were conducted in accordance with the guidelines of the NIH and approved by Boston College Institutional Animal Care and Use Committee.

Social play test

During the beginning of the dark phase (between 14:00 h and 15:00 h) and under red light conditions, each experimental rat was exposed to an age- and sex-matched unfamiliar rat for a period of 10 min. All tests were videotaped for subsequent analysis of behavior by a researcher blind to the treatment condition using JWatcher (<http://www.jwatcher.ucla.edu/>). The duration of social play was scored according to Veenema & Neumann (2009) and consisted of the total amount of time spent in playful social interactions including nape attacks (the experimental rat displays nose attacks or nose contacts toward the nape of the neck of the unfamiliar rat), pinning (the experimental rat holds the unfamiliar rat on its back in a supine position), and supine poses (the experimental rat is pinned by the unfamiliar rat). In addition, the number of nape attacks, pins, and supine poses as well as the duration of social investigation (the experimental rat is sniffing the anogenital and head/neck regions of the unfamiliar rat), the duration of allogrooming (the experimental rat is grooming the unfamiliar rat), and the duration of non-social exploration were scored.

Probe implantation and microdialysis procedure

Experimental rats were housed individually at 28 days of age and were exposed to the social play test one day thereafter to familiarize the rats to the test procedure. At 30 days of age,

rats were anesthetized with isoflurane (Butler Schein Animal Health, Dublin, OH) and mounted on a stereotaxic frame with the tooth bar set at -4.5 mm. The u-shaped microdialysis probes (Brainlink, Groningen, the Netherlands) were unilaterally implanted into the LS according to Lukas et al. (2011) at coordinates 0.4 mm caudal to bregma, -1.5 mm lateral to the midline, 5.6 mm beneath the surface of the skull with an angle of 10° to avoid damage to the sagittal sinus. The probes were filled with sterile Lactated Ringer's solution (pH 7.4) and fixed to the skull with three stainless steel screws and dental cement. Two 5 cm long pieces of polyethylene tubing (PE 20, Plastics One, Roanoke, VA) filled with Ringer's solution were connected to the inflow and the outflow of the probe and fixed with dental cement. One day after surgery, probes were flushed with Ringer's and rats were familiarized to the microdialysis sampling procedure while undergoing a social play test. Two days after surgery, probes were connected via PE20 tubing to a syringe mounted onto a microinfusion pump and perfused with Ringer's (3.0 $\mu\text{l}/\text{min}$, pH 7.4) to establish equilibrium between the inside and outside of the microdialysis membrane. Two hours later, five consecutive 10-min dialysates were collected: dialysates 1 and 2 were taken under baseline (undisturbed) conditions, dialysate 3 was taken during the 10-min social play test, and dialysates 4 and 5 were taken thereafter. Microdialysates were collected in 0.5 ml Eppendorf tubes and were immediately frozen on dry ice and subsequently stored at -45°C until quantification for glutamate and GABA using LC-MS/MS. Rats were killed with CO_2 and proper probe placement was checked histologically on Nissl-stained cryocut coronal brain sections (see Fig. 1A). Rats with incorrect probe placement were removed from the analysis, resulting in final groups of $n=11$ males and $n=7$ females.

LC-MS/MS

Concentrations of GABA and glutamate in dialysates were quantified using an integrated HPLC-MS/MS system. 10 μl dialysate samples were mixed with 10 μl 0.02 M formic acid in water containing 0.04% ascorbic and 4 μl stable labelled isotope mixture containing GABA labelled with 6 deuterium and glutamate labelled with 5 deuterium. The mixtures were then placed in an autosampler (SIL-20 HT with pre-treatment option, Shimadzu, Japan) and 30 μl of derivatization mixture, containing 5 mg/ml Symdaq reagent in 0.5M NaHCO_3 buffer (Brainlink, the Netherlands), was automatically added by the autosampler. After 2 min reaction time, 45 μl of the mixture was injected onto the HPLC Column. Chromatographic separation was performed on a reversed phase column (100×3.0 mm Synergi MAX-RP C12, particle size 2.5 μm , Phenomenex, Torrance, CA, USA) held on a temperature of 35° Celsius in a column oven (CTO-20, Shimadzu, Japan). Samples were eluted using a linear gradient of 0.2 – 70% of acetonitrile with 0.1% formic in ultrapure water with 0.1% formic acid for 5.3 min at a flow rate of 0.3 ml/min (LC-20 AD, Shimadzu, Japan). The flow was mixed (postcolumn) with a makeup flow of 0.15 ml/min of the mobile phase (acetonitrile with 0.1% formic acid in ultrapure water with 0.1% formic acid) and directed to the triple-quadrupole mass spectrometer (API 4000, Applied Biosystems, Foster City, CA, USA). The acquisition was run in multi-reaction mode (MRM) for 3 – 7 min. The following multi reaction monitoring (MRM) transitions were monitored; $349.3 \rightarrow 261.0$ for GABA-SymDaq complex and $355.1 \rightarrow 267.1$ for GABA- d_6 -SymDaq complex, $393.3 \rightarrow 305.1$ for glutamate-SymDaQ complex, $398.3 \rightarrow 310.1$ for glutamate- d_5 -SymDaQ complex. The absolute concentrations of GABA and glutamate were calculated using Analyst 1.4.2

software (Applied biosystems, Foster City, CA, USA), using analyte/IS ratio versus concentration, the calibrations curve was linear with $1/\times$ weighing function. Lower limit of quantitation was 0.8 nM on column for GABA and 0.02 μ M on column for glutamate and inter-day variation were 6.68 %CV for GABA and 3.77 %CV for glutamate.

Cannulation and microinjection procedure

After five days of daily handling to familiarize them with the injection procedure, a separate group of experimental rats (30 days of age) were anesthetized with isoflurane (Butler Schein Animal Health, Dublin, OH) and mounted on a stereotaxic frame with the tooth bar set at – 4.5 mm. Guide cannulae (22 gauge; Plastics One, Roanoke, VA) were implanted 2 mm dorsal to the medial part of the LS (see Fig. 1B) according to Veenema et al. (2013) at coordinates 0.4 mm caudal to bregma, – 1.0 mm lateral to the midline and 3.6 mm ventral to the surface of the skull under an angle of 10° from the midsagittal plane to avoid damage to the sagittal sinus. Cannulae were fixed to the skull with three stainless steel screws and dental cement and closed with a dummy cannula (Plastics One, Roanoke, VA). After surgery, rats were housed individually. Two days later, rats received a 0.5 μ l injection of either Ringer's solution (vehicle), the GABA-A receptor antagonist bicuculline (Sigma, either 100 ng or 250 ng), or a cocktail of the NMDA, and AMPA/kainate glutamate receptor antagonists AP-5 and CNQX (Sigma, either 2 mM/0.4 mM or 30 mM/3 mM) and were tested for social play 20 min later. GABA-A receptors (Araki et al., 1992a, 1992b; Hartig et al., 1995; Chen et al., 1998) and NMDA and AMPA/kainate receptors (Unnerstall & Wamsley, 1983; Monaghan et al., 1984; Jacobson & Cottrell, 1993) were chosen because of their abundant expression in the LS. Drug doses were chosen based on previous microinjections studies showing behavioral effects in rats (Yuen et al., 2012; Bast et al., 2005; Yousefi et al., 2012; Covelo et al., 2014). After the experiments, rats were killed with CO_2 , and charcoal was injected as a marker to check proper cannula placement histologically on Nissl-stained cryocut coronal brain sections (see Fig. 1B). Rats with incorrect probe placement were removed from the analysis. The final number per group is indicated in the bars of Fig. 3.

Statistics

Social play behaviors during microdialysis were analyzed using one-way ANOVAs. Glutamate and GABA release (percentage release; absolute levels) were analyzed using two-way ANOVAs for repeated measures (sex $\times$ dialysate). When main effects for dialysate were found, one-way ANOVAs for repeated measures were run separately for sex. For percentage release, this was followed by an LSD posthoc test. When main effects for sex were found, one-way ANOVAs for repeated measures were run separately for dialysate. The glutamate/GABA concentration ratio under baseline condition (average of dialysates 1 and 2 per rat) and during social play (dialysate 3) was analyzed using a one-way ANOVA for repeated measures (sex $\times$ dialysate). When a main effect for sex was found, one-way ANOVAs were run separately for dialysate. Effects of drug treatment were analyzed using two-way ANOVAs (sex $\times$ drug treatment). When a main interaction effect was found, a Bonferroni posthoc test was run to test for differences between groups. When main effects for drug treatment were found, one-way ANOVAs were run separately for sex to test for

differences between treatments. Data are presented as mean + SEM. Significance was set at $p < 0.05$.

RESULTS

Social play behavior during in vivo microdialysis (Video)

Male and female juvenile rats showed similar durations of social play behavior (males: 11.4 ± 2.2 % time; females: 8.56 ± 2.2 % time), number of nape attacks (males: 37.7 ± 6.5 ; females: 29.1 ± 5.6), pins (males: 9.3 ± 2.6 ; females: 7.7 ± 3.0), and supine poses (males: 1.5 ± 0.5 ; females: 0.7 ± 0.4). The duration and frequencies of these social play behaviors during ongoing microdialysis were very similar to those seen after microinjections (See Fig. 3 and Tables 1 and 2).

GABA release in the LS during social play behavior of male and female juvenile rats

Extracellular GABA concentrations—No sex difference was found for extracellular GABA concentrations. However, extracellular GABA concentrations are higher during social play compared to baseline ($F_{(1,16)} = 25.2$; $p < 0.001$) reaching significance in both males ($p < 0.005$) and females ($p < 0.05$; Fig 2A).

Percentage GABA release—The percentage release of GABA ($F_{(4,64)} = 9.69$; $p < 0.001$) increases during social play behavior in both male and female juvenile rats (Fig 2A). Specifically, in males, GABA release is significantly higher during social play (dialysate 3) compared to baseline GABA release (dialysate 1 and 2: $p < 0.01$) and GABA release after social play (dialysate 4: $p < 0.01$; dialysate 5: $p < 0.05$; Fig 2A). In females, GABA release is significantly higher during social play compared to baseline GABA release (dialysate 1 and 2: $p < 0.05$) and GABA release after social play (dialysate 5: $p < 0.05$; Fig 2A).

Glutamate release in the LS during social play behavior of male and female juvenile rats

Extracellular glutamate concentrations—A sex difference was found for extracellular glutamate concentrations ($F_{(1,16)} = 10.1$; $p < 0.01$), with males having higher extracellular glutamate under baseline condition ($p < 0.05$) and during social play ($p < 0.01$; Fig 2B). Moreover, extracellular glutamate concentrations are higher during social play compared to baseline ($F_{(1,16)} = 32.5$; $p < 0.001$) reaching significance in both males ($p < 0.05$) and females ($p < 0.01$; Fig 2B).

Percentage glutamate release—The percentage release of glutamate ($F_{(4,64)} = 12.2$; $p < 0.001$) increases during social play behavior in both male and female juvenile rats (Fig 2B). Specifically, in males, glutamate release is significantly higher during social play compared to baseline glutamate release (dialysate 1 and 2: $p < 0.05$) and glutamate release after social play (dialysate 4 and 5: $p < 0.05$; Fig 2B). In females, glutamate release is significantly higher during social play compared to baseline glutamate release (dialysate 1 and 2: $p < 0.005$) and glutamate release after social play (dialysate 4: $p < 0.05$; Fig 2B).

Glutamate/GABA concentration ratio: Because the extracellular concentration of glutamate was significantly higher in males than in females, the ratio of glutamate/GABA

concentrations was calculated as measure of glutamate excitation/GABA inhibition balance. Males have a higher glutamate/GABA concentration ratio compared to females ($F_{(1,16)} = 5.09$; $p < 0.05$) reaching significance under baseline condition ($p < 0.05$) and trending during social play ($p = 0.096$; Fig 3).

Effects of blockade of GABA-A receptors in the LS on social play behavior

Blockade of GABA-A receptors in the LS with the higher dose of bicuculline (250 ng/0.5 μ l; Fig 4A) but not with the lower dose of bicuculline (100 ng/0.5 μ l; Fig. 4B) reduced the duration of social play behavior ($F_{(1,37)} = 30.5$; $p < 0.001$) in males ($p < 0.01$) and in females ($p < 0.001$). Bicuculline (250 ng/0.5 μ l) also reduced the number of nape attacks ($F_{(1,37)} = 39.1$; $p < 0.001$), pins ($F_{(1,37)} = 9.19$; $p < 0.005$), and supine poses ($F_{(1,37)} = 9.99$; $p < 0.005$) in both sexes (Table 1). As a result, bicuculline (250 ng/0.5 μ l) increased cage exploration ($F_{(1,37)} = 9.89$; $p < 0.005$) in both sexes (Table 1). Neither dose of bicuculline altered social investigation or allogrooming (Table 1).

Effects of blockade of ionotropic glutamate receptors in the LS on social play behavior

Blockade of ionotropic glutamate receptors in the LS with the higher dose of AP-5 and CNQX (30+3 mM/0.5 μ l) but not with the lower dose of AP-5 and CNQX (2+0.4 mM/0.5 μ l) reduced the duration of social play behavior (sex $\times$ drug treatment effect: $F_{(1,38)} = 30.5$; $p < 0.05$), however, this reached significance in females ($p < 0.001$) but not in males ($p = 0.31$; Fig. 4C+D). AP-5 and CNQX (30+3 mM /0.5 μ l) also reduced the number of nape attacks ($F_{(1,38)} = 11.1$; $p < 0.005$) and pins ($F_{(1,38)} = 5.07$; $p < 0.05$) reaching significance in females only (nape attacks: $p < 0.005$; pins: $p < 0.05$; Table 2). Neither dose of AP-5 and CNQX altered social investigation, allogrooming, or cage exploration (Table 2).

DISCUSSION

This is the first study to show that the extracellular concentration of glutamate, but not GABA, in the LS is higher in juvenile male rats than in juvenile female rats, suggesting a sex difference in excitatory tone of the LS. Furthermore, increases in the extracellular release of GABA and glutamate were seen during the display of social play behavior in juvenile male and female rats. Blockade of GABA-A receptors in the LS dose-dependently decreased social play behaviors in both sexes. In contrast, concurrent blockade of NMDA, AMPA, and kainate receptors in the LS dose-dependently decreased social play behaviors in females, but did not alter social play behaviors in males. This suggests a role for GABA acting on GABA-A receptors in the LS in facilitating social play in both sexes, while glutamate acting on ionotropic glutamate receptors facilitates social play in females only.

GABA released in the LS facilitates social play behavior in juvenile male and female rats

Although the GABA system in the LS and other brain regions has been extensively studied (Sheehan et al., 2004; Watanabe et al., 2010), almost nothing is known about local *in vivo* release of GABA during social behavior. One study reported increased extracellular GABA release in the hypothalamic supraoptic nucleus during social defeat in adult male rats (Engelmann et al., 2004), but the behavioral relevance was not tested. Here, we show that extracellular release of GABA in the LS increased significantly during social play behavior

in both juvenile male and female rats and that this increase likely facilitated social play behavior in both sexes.

Extracellular GABA concentrations are similar between males and females under baseline condition and during the display of social play behavior. This extracellular GABA likely originated in the LS itself, because the LS consists mainly of GABAergic projection neurons (Kohler & Chan-Palay, 1983; Alonso & Frotscher, 1989; Risold & Swanson, 1997b) whose axons give rise to intrinsic collaterals (Onteniente et al., 1987, Phelan et al., 1989, Risold & Swanson, 1997a, Staiger & Nurnberger, 1991). Additionally, extracellular GABA may have been released in the LS from GABAergic sources outside the LS that are projecting to the LS, such as the nucleus accumbens (Zahm et al., 2013). However, it should be noted that these above-mentioned studies were carried out exclusively in adult male rats. Whether the same features are found in the juvenile male and female rat brain is unknown. Regardless, it is likely that higher levels of extracellular GABA in the LS during social play behavior contributed to the inhibition of LS neuronal activity, which in turn, may have affected the display of social play behavior.

To determine the functional significance of this increased release of GABA in the LS in the regulation of social play behavior, ionotropic GABA-A receptors were locally blocked with bicuculline prior to exposure to the social play test. GABA-A receptor blockade induced a dose-dependent decrease in social play behavior in both males and females, thereby confirming our prediction. This suggests that GABA, through activation of GABA-A receptors, facilitates social play behavior in both sexes. Interestingly, electrolytic lesioning of the septum increased social play behavior in both sexes (Beatty et al., 1982), while we show that bicuculline decreased social play behavior in both sexes. This indicates that electrolytic lesioning and bicuculline have opposite effects on LS neuronal output activity. Indeed, electrolytic lesioning eliminates all LS cells and therefore prevents any LS output, while blocking GABA-A receptors with bicuculline prevents GABA-mediated local inhibition and thus allows for enhanced LS output. Together, these findings suggest that the LS has an inhibitory effect on social play behavior. This seems in line with an inhibitory role of the LS on other behaviors including lordosis in female hamsters (Zasorin et al., 1975) and attack and defense behaviors in rats and other species (reviewed in Albert & Chew, 1980). This behavioral inhibition may be mediated through projections from the LS to regions such as the lateral hypothalamus, ventral tegmental area, and nucleus accumbens (Swanson & Cowan, 1979; Risold & Swanson, 1997b; Sheehan et al., 2004). LS projections to the latter two regions might be of particular interest for the regulation of social play behavior, a highly rewarding behavior, because this pathway has been shown to be involved in rewarding behaviors (Luo et al., 2011; Mahler & Aston-Jones, 2012). The precise mechanism and pathways by which GABA-A receptors in the LS facilitate social play behavior awaits further research.

Extracellular glutamate concentrations in the LS is higher in males than in females

One striking finding was that males have higher extracellular concentrations of glutamate in the LS compared to females under baseline condition and during the display of social play behavior. Because extracellular GABA concentrations in the LS were similar between males

and females, this resulted in a higher glutamate/GABA concentration ratio in males compared to females, which is a measure of the glutamate excitation/GABA inhibition balance. Accordingly, males show an excitatory predominance in the LS compared to females. Although extracellular glutamate is not a direct index of synaptic activity, the sex difference in extracellular glutamate may cause a sex difference in LS neuronal excitability, which in turn, could contribute to a sex-specific role of the LS in the regulation of social behavior.

Glutamate in the brain is synthesized *de novo* by astrocytes (Hertz et al., 1999). In astrocytes, glutamate is converted to glutamine before it is released in the extracellular space. Glutamine is taken up by neurons and converted back to glutamate. Once released into the synaptic cleft, glutamate can be removed by reuptake through glutamate transporters present on astrocytes and neurons. Thus, a sex difference in glutamate synthesis or reuptake in astrocytes and/or in neurons could account for the sex difference in extracellular glutamate concentrations in the LS. Notably, astrocytes in male rats have more complex morphology and greater astrocytic process length and number than those in female rats (Amateau & McCarthy, 2002; Mong & McCarthy, 2002), but whether this morphological sex difference is associated with a sex difference in glutamate synthesis is unclear.

Interestingly, individual variations in the glutamate excitation/GABA inhibition balance in cortical regions affect behavioral performance and executive function in healthy men (Jocham et al. 2012; de la Vega et al., 2014). Moreover, alterations in the glutamate/GABA ratio in the cortex may contribute to the pathophysiology of various neurological and psychiatric disorders (Sherwin, 1999; Sanacora et al., 2004; Kugaya & Sanacora, 2005). These findings in humans provide support that the sex difference in the glutamate/GABA ratio in the LS of rats might have behavioral implications. Furthermore, given our findings in rats, it may be relevant to explore in humans possible sex differences in the glutamate/GABA ratio in the cortex as well as in additional brain regions.

Sex differences in brain tissue glutamate (and GABA) content have been reported in the neonatal rat, with higher content in males in micro-dissected hypothalamic and limbic areas (the LS was not included), but these differences were temporary and disappeared after the first week of life (Davis et al., 1999). Even so, this temporal sex difference in glutamate and GABA content plays a role in the organizational component of sexual differentiation resulting in the expression of sexually dimorphic reproductive behaviors (Reviewed in McCarthy, 2010). Importantly, our data demonstrate that there may be longer lasting sex differences in the *in vivo* glutamate release and subsequently in the glutamate/GABA concentration ratio. Since our study is the first to compare amino acid neurotransmitter release between males and females, it would be of interest to determine whether the sex difference in extracellular glutamate is restricted to the juvenile period or maintained throughout adulthood and whether the sex difference in extracellular glutamate is specific to the LS or seen in additional brain regions.

Extracellular glutamate in the LS could have been released from local glutamatergic neurons (Garrido Sanabria et al., 2006), from local astrocytes, and/or from glutamatergic neurons located outside the LS and projecting to the LS. Astrocytes can release glutamate to

modulate activity in nearby neurons (Malarkey & Parpura, 2008) and may participate in rapid brain communication (Santello et al., 2012). However, it was recently argued that glutamate release from astrocytes regulates steady-state or homeostatic processes in the brain (Vardjan & Zorec, 2015). As such, dynamic increases in extracellular glutamate as seen during social play behavior less likely reflect glutamate released from astrocytes. Moreover, the sex difference in extracellular glutamate concentration is seen under baseline condition and during social play, suggesting a similar source of glutamate under both conditions. It may therefore be expected that the extracellular glutamate is released from axon terminals whose cell bodies are located in the hippocampus and projecting to the LS through the fimbrial pathway (Gallagher et al., 1995) or are located in other areas such as the lateral hypothalamus (Chee et al., 2015). Further studies will be required to address the source of extracellular glutamate in the LS and whether this differs between males and females.

Sex-specific involvement of glutamate released in the LS in the regulation of social play behavior

Similar to GABA, extracellular glutamate release in the LS increased during social play behavior in both sexes. To test the functional significance of this increase in extracellular glutamate, ionotropic glutamate receptors in the LS were blocked prior to exposure to the social play test. Interestingly, blockade of the three main types of ionotropic receptors for glutamate, namely NMDA, AMPA, and kainate receptors, dose-dependently reduced social play behavior in females, but did not alter social play behavior in males. The effect in females is in the opposite direction as we predicted. To clarify, activation of NMDA, AMPA and kainate receptor causes excitation of cells on which they are expressed, and thus, most likely will enhance LS neuronal output. Accordingly, blockade of these receptors with AP-5 and CNQX reduced LS neuronal output. However, a reduction in LS output is opposite to the predicted effect of bicuculline (i.e., increased LS neuronal output), yet both drug treatments reduced social play behavior in females. A recent study provides evidence for an alternative mechanism of action of glutamate, suggesting that glutamate inhibits LS neuronal output (Chee et al., 2015). In detail, glutamate released from lateral hypothalamic neurons evoked a delayed release of GABA in the LS, resulting in a net response of depressed LS firing. In this scenario, blockade of ionotropic glutamate receptors might allow for enhanced LS neuronal output, similar to the expected effect of bicuculline. As a result, blockade of ionotropic glutamate receptors or GABA-A receptors likely reduces social play behavior in females by enhancing LS output. This hypothesis would need to be confirmed with future studies.

Our findings suggest a lack of involvement of glutamate released in the LS in the modulation of social play behavior in males. However, extracellular glutamate concentration in the LS is higher in males than in females, suggesting that the doses of AP-5 and CNQX may not have been high enough to efficiently block the NMDA, AMPA, and kainate receptors. It is also possible that, in males, the subtypes of ionotropic glutamate receptors have opposing roles, e.g., via expression on different types of LS neurons, and blockade of these receptors may lead to an overall null effect. Indeed, NMDA receptors were found to inhibit while AMPA receptors were found to excite prefrontal cortex pyramidal neurons in

male rats (Takahata & Moghaddam, 1998; Del Arco & Mora, 1999). A recent study suggests that this action of NMDA receptors is sex-specific (Locklear et al., 2014). Finally, glutamate may modulate social play behavior in males via the activation of metabotropic glutamate receptors rather than ionotropic glutamate receptors. Subtypes of these G-protein coupled receptors are expressed in the LS and have been shown to be involved in social behavior (Mesic et al., 2015; Zhao & Gammie, 2015). Further studies are needed to prove the lack of involvement of glutamate in the regulation of male social play behavior.

Bicuculline (250 ng) as well as AP-5 + CNQX (30+3 mM) significantly decreased the duration of social play behavior as well as the number of nape attacks and pins in females. This indicates that both drug treatments decrease the motivation to play in females. In males, bicuculline (250 ng) significantly decreased the duration of social play behavior and the number of nape attacks, but not the number of pins. Because pins typically arise from attacks (Pellis & Pellis, 1987), it is expected that a decrease in the number of attacks corresponds with a proportional decrease in the number of pins. Since this is not the case in males, this may indicate that bicuculline not only decreased the motivation to play in males, but also may have altered the tactics of play. One possibility could be that bicuculline alters the responses of males to the play behavior of the intruder rat. Follow-up studies may further address this.

In addition to the glutamate system, our previous research has identified the vasopressin system as being involved in the sex-specific regulation of social play behavior (Veenema et al., 2013; Bredewold et al., 2014). In those studies, we found that blocking the vasopressin V1a receptor in the LS enhanced social play behavior in males, but reduced social play behavior in females. It is tempting to speculate that the vasopressin and glutamate systems interact to regulate social play behavior in sex-specific ways. For example, V1aR activation by vasopressin may alter glutamate release in the LS, which in turn, modulates social play behavior differently in males compared to females. Notably, both vasopressin and glutamate have also been implicated in the pathophysiology of autism (Meyer-Lindenberg et al., 2011; Choudhury et al., 2012; Rojas, 2014; Yang & Chang, 2014), a male-biased neurodevelopmental disorders showing impairments in social play behavior (Jordan, 2003; Rutter et al., 2003). Further research into the interactions between vasopressin and glutamate during social play may yield novel insights into the sex-specific regulation of social play behavior.

Conclusions

Our data demonstrate a role for GABA released in the LS in facilitating social play behavior in both male and female juvenile rats, while glutamate released in the LS facilitates social play behavior in females only. Higher concentrations of extracellular glutamate in the LS of males compared with females further suggest the involvement of glutamate in the sex-specific modulation of LS neuronal activity which may provide a mechanism by which social play behavior is regulated differently in male and female juvenile rats.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Juvenile male rats have higher extracellular glutamate concentrations in the lateral septum than juvenile female rats

Both sexes show increased GABA and glutamate release in the lateral septum (LS) during social play behavior

Blockade of GABA-A receptors in the LS decreased social play behavior in both sexes

Blockade of ionotropic glutamate receptors in the LS decreased social play behaviors in females, but not in males

GABA in the LS facilitates social play in both sexes, while glutamate may facilitate social play in females only

Fig. 1. Schematic drawing of the LS (bregma 0.20 mm; adapted from Paxinos and Watson, 1998) and representative enlargements of photomicrographs of Nissl-stained coronal sections of the rat brain indicating with a bracket the location and extend of the semipermeable membrane of a microdialysis probe placement unilaterally in the LS (left) and indicating with an arrow the microinjection location in the medial part of the LS using charcoal as marker (right). Correct placements were considered at bregma 0.20 mm $\pm$ 0.2 mm using the brain atlas by Paxinos and Watson (1998). The microinjection location was different compared to the microdialysis probe placement to allow the drugs to reach both sides of the LS.

Fig. 2.

Extracellular GABA (A) and glutamate (B) released in the LS of male and female juvenile rats. Five consecutive dialysates were taken: Dialysates 1 and 2 are taken under baseline condition, dialysate 3 includes exposure to the 10-min social play test (play), and dialysates 4 and 5 are taken after exposure to the social play test under undisturbed conditions. Data represent mean + SEM. Extracellular concentrations: * $p < 0.05$, # $p < 0.05$ vs. baseline, two-way ANOVA followed by one-way ANOVA. Percentage release: * $p < 0.05$ vs. dialysates 1, 2, 4, 5 (males), vs. dialysates 1, 2, 5 (females in A), vs. dialysates 1, 2, 4 (females in B), two-way ANOVA for repeated measures followed by one-way ANOVA for repeated measures and LSD posthoc test.

Fig. 3. Glutamate/GABA concentration ratio in the LS of male and female juvenile rats under baseline condition and during social play. Data represent mean + SEM. * $p < 0.05$, # $p = 0.098$, two-way ANOVA for repeated measures followed by one-way ANOVA.

Fig. 4.

Effects of pharmacological blockade of GABA-A receptors (A) or ionotropic glutamate receptors (B) in the LS on social play behavior in male and female juvenile rats. The GABA-A receptor antagonist bicuculline was injected in a dose of 100 ng/0.5 μ l or 250 ng/0.5 μ l. The NMDA receptor antagonist AP-5 and the AMPA/kainate receptor antagonist CNQX were concurrently injected in a dose of 2 mM+0.4 mM/0.5 μ l or 30 mM+3 mM/0.5 μ l. The duration of social play behavior is expressed as percentage of total time. Data represent mean + SEM. * $p < 0.01$, two-way ANOVA followed by one-way ANOVA (A) or Bonferroni posthoc test (B).

Effects of pharmacological blockade of GABA-A receptors in the LS on additional behaviors in the social play test of male and female juvenile rats. The GABA-A receptor antagonist bicuculline was injected in a dose of 100 ng/0.5 μ l (Bic 100) or 250 ng/0.5 μ l (Bic 250). The duration of social play behavior is expressed as percentage of total time.

Table 1

		Vehicle	Bic 100	Vehicle	Bic 250
Nape attacks	Males	18.6 $\pm$ 5.1	22.1 $\pm$ 3.7	20.8 $\pm$ 4.2	6.00 $\pm$ 2.9 *
	Females	18.0 $\pm$ 4.2	13.1 $\pm$ 1.9	21.6 $\pm$ 2.8	2.00 $\pm$ 1.4 *
Pins	Males	8.44 $\pm$ 1.8	8.38 $\pm$ 3.1	8.88 $\pm$ 2.9	3.11 $\pm$ 1.7
	Females	8.80 $\pm$ 1.5	5.67 $\pm$ 1.6	8.00 $\pm$ 2.0	1.77 $\pm$ 1.4 *
Supine	Males	2.89 $\pm$ 0.8	3.25 $\pm$ 1.1	3.63 $\pm$ 1.2	0.67 $\pm$ 0.3 *
	Females	3.50 $\pm$ 1.2	2.67 $\pm$ 0.6	3.27 $\pm$ 1.0	0.85 $\pm$ 0.6 *
Social investigation	Males	4.87 $\pm$ 0.8	3.59 $\pm$ 0.8	3.60 $\pm$ 0.9	3.11 $\pm$ 0.5
	Females	4.07 $\pm$ 0.8	3.24 $\pm$ 0.6	4.63 $\pm$ 0.8	2.5 $\pm$ 0.4
Allogrooming	Males	0.92 $\pm$ 0.4	1.72 $\pm$ 0.5	0.87 $\pm$ 0.5	0.46 $\pm$ 0.2
	Females	2.00 $\pm$ 1.0	0.40 $\pm$ 0.3	1.64 $\pm$ 0.6	0.96 $\pm$ 0.6
Cage exploration	Males	75.2 $\pm$ 1.4	74.7 $\pm$ 1.8	80.7 $\pm$ 2.6	87.5 $\pm$ 1.6 *
	Females	78.2 $\pm$ 1.6	85.3 $\pm$ 1.7	78.2 $\pm$ 1.7	85.4 $\pm$ 2.5 *

Data represent means $\pm$ SEM of number of nape attack, pins, and supine poses or duration (i) (in percentage of time) of social investigation, allogrooming, and cage exploration.

* $p < 0.05$ vs. vehicle, two-way ANOVA followed by one-way ANOVA.

Table 2

Effects of pharmacological blockade of ionotropic glutamate receptors in the LS on additional behaviors in the social play test of male and female juvenile rats. The NMDA receptor antagonist AP-5 was injected concomitantly with the AMPA/kainate receptor antagonist CNQX in a dose of 4 mM +0.4 mM/0.5 μ l (AC 4+0.4) or 30 mM+3 mM/0.5 μ l (AC 30+3). The duration of social play behavior is expressed as percentage of total time.

		Vehicle	AC 4+0.4	Vehicle	AC 30+3
Nape attacks	Males	28.8 $\pm$ 3.5	22.6 $\pm$ 3.6	26.7 $\pm$ 2.7	20.6 $\pm$ 5.3
	Females	19.8 $\pm$ 3.5	22.6 $\pm$ 3.4	31.1 $\pm$ 5.3	9.00 $\pm$ 2.6*
Pins	Males	4.88 $\pm$ 1.2	3.14 $\pm$ 1.2	6.33 $\pm$ 1.5	4.60 $\pm$ 1.7
	Females	6.25 $\pm$ 1.1	7.43 $\pm$ 1.4	9.55 $\pm$ 2.5	3.11 $\pm$ 1.0*
Supine	Males	3.25 $\pm$ 0.8	1.14 $\pm$ 0.5	2.67 $\pm$ 0.8	2.70 $\pm$ 0.9
	Females	2.63 $\pm$ 0.6	3.14 $\pm$ 0.7	3.00 $\pm$ 0.7	1.22 $\pm$ 0.5
Social investigation	Males	3.16 $\pm$ 0.6	3.01 $\pm$ 0.3	4.07 $\pm$ 0.6	5.42 $\pm$ 0.7
	Females	3.92 $\pm$ 0.9	2.49 $\pm$ 0.6	4.10 $\pm$ 0.6	5.06 $\pm$ 0.6
Allogrooming	Males	0.63 $\pm$ 0.3	2.16 $\pm$ 1.5	0.91 $\pm$ 0.4	1.43 $\pm$ 0.5
	Females	1.85 $\pm$ 1.0	7.43 $\pm$ 3.0	2.36 $\pm$ 0.8	1.10 $\pm$ 0.7
Cage exploration	Males	80.4 $\pm$ 2.0	84.0 $\pm$ 1.8	80.1 $\pm$ 1.2	82.0 $\pm$ 1.5
	Females	82.5 $\pm$ 2.5	76.5 $\pm$ 1.7	80.0 $\pm$ 2.4	85.1 $\pm$ 1.7

Data represent means $\pm$ SEM of number of nape attack, pins, and supine poses or duration (i) (in percentage of time) of social investigation, allogrooming, and cage exploration.

* $p < 0.05$ vs. vehicle, two-way ANOVA followed by one-way ANOVA.