Hormones and Behavior 79 (2016) 18–27

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Antagonism of V1b receptors promotes maternal motivation to retrieve pups in the MPOA and impairs pup-directed behavior during maternal defense in the mpBNST of lactating rats

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ARTICLE INFO

Article history:
Received 18 August 2015
Revised 26 December 2015
Accepted 29 December 2015
Available online 30 December 2015

Keywords:
Arginine-vasopressin
Maternal aggression
Maternal care
Maternal defense test
Pup retrieval test
SSR149415
V1a receptor
V1b receptor

ABSTRACT

Recent studies using V1b receptor (V1bR) knockout mice or central pharmacological manipulations in lactating rats highlighted the influence of this receptor for maternal behavior. However, its role in specific brain sites known to be important for maternal behavior has not been investigated to date. In the present study, we reveal that V1bR mRNA (qPCR) and protein levels (Western blot) within either the medial preoptic area (MPOA) or the medial-posterior part of the bed nucleus of the stria terminalis (mpBNST) did not differ between virgin and lactating rats. Furthermore, we characterized the effects of V1bR blockade via bilateral injections of the receptor subtype-specific antagonist SSR149415 within the MPOA or the mpBNST on maternal behavior (maternal care under non-stress and stress conditions, maternal motivation to retrieve pups in a novel environment, maternal aggression) and anxiety-related behavior in lactating rats. Blocking V1bR within the MPOA increased pup retrieval, whereas within the mpBNST it decreased pup-directed behavior, specifically licking/grooming the pups, during the maternal defense test. In addition, immediately after termination of the maternal defense test, V1bR antagonism in both brain regions reduced nursing, particularly arched back nursing. Anxiety-related behavior was not affected by V1bR antagonism in either brain region. In conclusion our data indicate that V1bR antagonism significantly modulates different aspects of maternal behavior in a brain region-dependent manner.

Introduction

Maternal behavior serves to increase the probability that the offspring survive to maturity (for review see Numan and Insel, 2003). This behavior is comprised of different activities directed towards the young like nursing, licking/grooming, retrieving the pups into the nest but also defending them from any potential threat. Numerous studies have shown the importance of arginine-vasopressin (AVP) and its V1a receptor (V1aR) in the regulation of maternal behavior of rats (Bosch and Neumann, 2008; Nephew and Bridges, 2008; for review see Bosch and Neumann, 2012). Shortly before parturition, the activity/expression of AVP and V1aR is up-regulated (Bosch et al., 2007; Caldwell et al., 1987; Caughey et al., 2011; Landgraf et al., 1991) in preparation for the forthcoming challenge of motherhood. This up-regulation continues into lactation (Bosch and Neumann, 2008; Van Tol et al., 1988; Walker et al., 2001), where the AVP system facilitates maternal behavior as demonstrated by central (Pedersen and Prange, 1979; Pedersen et al., 1982) and local manipulations within the medial preoptic area (MPOA; Bosch and Neumann, 2008; Pedersen et al., 1994) and the bed nucleus of the stria terminalis (BNST; Bosch et al., 2010). Both brain regions are important regulators of maternal behavior and form the so-called maternal "super-region" (for review see Numan and Insel, 2003). Specifically, the MPOA plays a distinct role in maternal motivation as measured by pup retrieval behavior (Kalinichev et al., 2000; Neumann et al., 2005; Numan, 1990; Pedersen et al., 1994) whereas the BNST is involved in maternal aggression (Bosch et al., 2010; Consiglio et al., 2005; Klampfl et al., 2014). Accordingly, in the MPOA and BNST endogenous AVP release increases during mother-pup interaction (Bosch et al., 2010). Inhibition of the V1aR in the MPOA of the lactating mother via local infusion of a V1aR antagonist or receptor down-regulation by antisense oligodeoxynucleotides decreases pup retrieval (Bosch and Neumann, 2008; Pedersen et al., 1994). Blocking V1aR within the BNST decreases aggression against a virgin female intruder rat during the maternal defense test without affecting maternal care (Bosch et al., 2010).

To date, most studies investigating the function of the AVP system in maternal behavior have focused on the V1aR. Considering the

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http://dx.doi.org/10.1016/j.yhbeh.2015.12.003
0018-506X/

expression of the second brain V1 receptor, the V1bR, in the maternal super-region (Hernando et al., 2001), it is very likely that the effects of AVP on maternal behavior are brought about by the two different AVP receptor subtypes. However, studies investigating the role of V1bR have been hindered due to a lack of specific pharmacological tools. An initial study in V1bR knockout mice demonstrated reduced maternal aggression in knockout compared to wild-type mothers (Wersinger et al., 2007). Recently, using the receptor subtype-specific V1bR antagonist SSR149415 (Serradeil-Le Gal et al., 2002), we revealed that in Wistar rats central blockade of the receptor reduced nursing and mother-pup interaction, whereas it did not affect pup retrieval or maternal aggression (Bayerl et al., 2014).

In the present study, we investigated the role of V1bR in various aspects of maternal behavior in more detail by focusing on the two maternal brain regions MPOA and BNST. Firstly, we assessed whether lactation is associated with altered V1bR mRNA (experiment 1) and/or protein level (experiment 2) by comparing lactating and virgin rats. Secondly, we studied the behavioral effects of acute local infusion of the V1bR antagonist SSR149415 within the MPOA (experiment 3) or the medial-posterior BNST (mpBNST; experiment 4) on maternal care under basal condition (non-stress) and following exposure to the maternal defense test (stress condition), as well as on maternal motivation to retrieve pups, maternal aggression and anxiety-related behavior.

Material & methods

Animals

Female Wistar rats (12–14 weeks, 220–250 g, Charles River Laboratories, Sulzfeld, Germany) were kept under standard laboratory conditions (12 h/12 h light–dark cycle, with lights on at 07:00 h; 22 ± 1 °C; 55 ± 5% relative humidity; free access to water and standard rat chow).

In experiments 1 and 2, half of the rats were kept as virgins, whereas the other half was mated with sexually experienced stud Wistar males. Pregnancy was confirmed the next day by the presence of sperm in vaginal smears (assigned as pregnancy day (PD) 1). Rats were kept in mixed groups (pregnant/virgin) of 3 to 4 animals before they were single-housed on PD 18 (or equivalent in virgins) to ensure undisturbed parturition.

For experiments 3 and 4, virgin female Wistar rats were mated as described above. Pregnant rats were group-housed up to four animals per cage until surgery on PD 18. After surgery, pregnant rats were single-housed in plexiglass observation cages (38 cm × 22 cm × 35 cm) to ensure undisturbed parturition. On the day of birth, offspring were culled to eight pups of mixed sexes and half of the bedding was replaced by new bedding. Dams were randomly assigned to one of the treatment groups on lactation day (LD) 1 and received the same treatment throughout the experiment.

For the maternal defense test, naïve virgin female Wistar rats (10 weeks, 180–220 g; Charles River) at random stages of their estrous cycle were used as intruders. They were kept group-housed in a separate room until behavioral testing to avoid olfactory recognition by the lactating mothers (for review see Bosch, 2013).

All experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local government of the Oberpfalz, Bavaria, Germany.

Experiment 1: Quantitative real-time PCR (qPCR) for V1bR mRNA in the MPOA and BNST

Non-manipulated, undisturbed rats were sacrificed on LD 4 (or equivalent in virgins at random stages of the estrous cycle; n = 3–5 per group). Brains were removed, snap-frozen in n-methylbutane on dry ice and stored at −80 °C until further processing. Next, brains were cut into 2 × 200 μm slices at the regions of interest, which were identified with the aid of a rat brain atlas (Paxinos and Watson, 2007). The MPOA and BNST were dissected with a puncher (inner diameter: 1 mm) and side-pooled; thus, a total of 4 punches of each brain region were taken per rat. Total RNA was extracted using RNeasy Micro Kit (Qiagen, Hilden, Germany) in combination with QIAshredder columns (Qiagen) and RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. RNA content was determined with the aid of a NanoDrop photospectrometer. Next, 100 ng of RNA were reverse transcribed into cDNA by use of SuperScript III according to manufacturer's instructions (Invitrogen, Darmstadt, Germany). SYBR Green-based (Qiagen) qPCR for V1bR (forward primer: 5′-CAT ACC TCC ATC CAC CTT CC-3′; reverse primer: 5′-TCT TCA TCC CTA CCT AGC CA-3′; Metabion, Planegg/Steinkirchen, Germany) relative to ribosomal protein L13A (Rpl13A; NM_173340; forward primer: 5′-ACA AGA AAA AGC GGA TGG TG-3′; reverse primer: 5′-TTC CGG TAA TGG ATC TTT GC-3′; Metabion) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (Ywhaz; NM_013011; forward primer: 5′-TTG AGC AGA AGA CGG AAG GT-3′; reverse primer: 5′-GAA GCA TTG GGG ATC AAG AA-3′; Metabion) as reference genes (Bonefeld et al., 2008) was performed on the 7500 Fast Real Time PCR Systems v2.0.6 (Applied Biosystems, Darmstadt, Germany) and mRNA levels were quantified with the comparative C_T method ($\Delta\Delta C_T$). Specificity of the primers was assured by omitting reverse transcription and by using ddH_2O as template. The PCR protocol consisted of an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 45 s. At the end of the protocol a melting curve was generated and PCR products were analyzed by agarose gel electrophoresis to confirm the specificity of the primers. All samples were run in duplicate.

Experiment 2: Western blot analysis for V1bR protein in the MPOA and BNST

Rats were treated and brain punches were taken as described for experiment 1 (n = 3–5 per group). Proteins were extracted from punches in 50 μl lysis buffer (containing 0.5 mM of 0.02% EDTA (Sigma Life Science, Steinheim, Germany), 250 mM of NaCl (powder; VWR Chemicals, Leuven, Belgium), 50 mM of 1 M HEPES (Sigma Life Science), 0.5% of Igepal (Sigma-Aldrich, Steinheim, Germany), 10% of Halt-Protease & Phosphatase Inhibitor 10× Cocktail (Thermo Scientific, Rockford, USA)) in which the probes were homogenized with a sterile handheld pestle. After gentle rotation (1 h, 4 °C) and centrifugation (15 min, 13.000 rpm, 4 °C) the supernatant containing the protein-fraction was collected on ice. The protein concentrations were determined using the BCA Protein Assay Kit (Pierce/Thermo Scientific) and the Optima plate reader (BMG Labtech GmbH, Ortenberg, Germany). Fifteen μg of each protein sample were electrophoretically separated on a 12% SDS-gel (Criterion TGX Stain Free Precast Gel, Bio-Rad, Munich, Germany) and subsequently transferred onto a 0.2 μm nitrocellulose membrane (Bio-Rad) by means of the Turbo Trans Blot unit (Bio-Rad). Equal loading of protein samples was verified using the stain free technology provided by Bio-Rad, which quantifies the amount of total protein loaded in one lane by staining tryptophans, instead of using a single band of one reference protein. This technique was preferred over traditional methods of loading control, since pregnancy and lactation are known to induce substantial changes in protein levels in the brain, which might bias relative gene expression (Gilda and Gomes, 2013; Rivero-Gutiérrez et al., 2014). Non-specific binding sites on the nitrocellulose membranes were blocked with 5% milk-powder (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) in TBST for 1 h at room temperature and incubated with a specific antibody against the rat V1bR (1:5000, Alpha Diagnostic, San Antonio, USA; for verification of specificity see Supplemental Information and Fig. S1) in TBS-T overnight at 4 °C. Bands were visualized by a secondary HRP-conjugated anti-rabbit IgG antibody (1:2000 in 5% milk-powder, 1 h at room temperature; Cell Signaling Technology) and Clarity Western ECL Substrate (Bio-Rad). Images were taken with the ChemiDocTM XRS+ system (Bio-Rad).