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THE VASOPRESSIN 1b RECEPTOR IS PROMINENT IN THE HIPPOCAMPAL AREA CA2 WHERE IT IS UNAFFECTED BY RESTRAINT STRESS OR ADRENALECTOMY

W. S. YOUNG,[a]* J. LI,[a1] S. R. WERSINGER[a1] AND M. PALKOVITS[a,b]

[a]*Section on Neural Gene Expression, National Institute of Mental Health, National Institutes of Health, 9000 Rockville Pike, Building 49, Room 5A56, Bethesda, MD 20892-4483, USA*

[b]*Laboratory of Neuromorphology, Hungarian Academy of Sciences and Semmelweis University, Budapest, Hungary 1094*

Abstract—The vasopressin 1b receptor (Avpr1b) is one of two principal receptors mediating the behavioral effects of vasopressin (Avp) in the brain. Avpr1b has recently been shown to strongly influence social forms of aggression in mice and hamsters. This receptor appears to play a role in social recognition and motivation as well as in regulating the hypothalamic–pituitary–adrenal axis. Most of these studies have been performed in knockout mice, a species in which the localization of the Avpr1b has not been described, thus precluding correlations with the behaviors. We performed *in situ* hybridization histochemistry (ISHH) with specific probes and found especially prominent expression within the CA2 pyramidal neurons of the hippocampus, with much lower expression in the hypothalamic paraventricular nucleus and amygdala. Reverse transcriptase–polymerase chain reaction (RT-PCR) confirmed expression in those as well other areas in which the ISHH was not sensitive enough to detect labeled cells (e.g. piriform cortex, septum, caudate-putamen and lower brainstem areas). Mouse Avpr1b transcript levels were not altered in the CA2 field by restraint stress or adrenalectomy. Finally, ISHH and RT-PCR showed expression of the *Avpr1b* gene in the rat and human hippocampi as well. We suggest that the CA2 field may form or retrieve associations (memories) between olfactory cues and social encounters.

Key words: social memory, aggression, corpora amylacea, paraventricular nucleus.

In species ranging from mollusks to mammals, the neuropeptide vasopressin (Avp) or its homologs participate in the regulation of social behavior (Grober and Sunobe, 1996; Albers and Bamshad, 1998; Dantzer, 1998; Godwin et al., 2000; Bester-Meredith and Marler, 2001; Goodson and Bass, 2001; Pitkow et al., 2001; Semsar et al., 2001). In mammals, aggression, affiliative behavior, and social recognition are regulated by Avp. Avp and related compounds have also been shown to play a role in memory (de Wied, 1965; Bohus et al., 1972; de Wied et al., 1991). Pharmacological (Ferris et al., 1985, 1988; Bluthe and Dantzer, 1992; Engelmann et al., 1992; Alescio-Lautier et al., 1995; Bamshad and Albers, 1996; Dantzer, 1998; Dluzen et al., 1998) and more recent genetic (Bielsky et al., 2004; Egashira et al., 2004) manipulations have implicated the vasopressin 1a receptor (Avpr1a) in the regulation of some of these behaviors.

There is, however, mounting evidence that the vasopressin 1b receptor (Avpr1b) is also an important regulator of social behavior. Analysis of the Avpr1b knockout mouse indicates that this receptor regulates aggression and social memory (Wersinger et al., 2002, 2004). Pharmacological antagonism of the Avpr1b produces reduced anxiety and aggression (Griebel et al., 2002; Blanchard et al., 2005; Stemmelin et al., 2005). To date, no radiolabeled ligand has been used to map the distribution of the Avpr1b in any species. Immunohistochemical studies in rats suggest that the Avpr1b has a limited regional distribution and is expressed at lower levels than the Avpr1a (Hernando et al., 2001). Interestingly, Avpr1b-immunoreactivity in rat is found in regions involved in the regulation of social behavior, including the olfactory tubercle, taenia tecta, cingulate cortex, amygdala and medial preoptic area (Hernando et al., 2001).

In situ hybridization histochemistry (ISHH) has been used to map the distribution of Avpr1b transcripts in the rat pituitary (Lolait et al., 1995) and brain (Vaccari et al., 1998), but that probe has significant sequence identities with the Avpr1a and oxytocin receptor (Oxtr). Two recent studies using oligonucleotide probes showed significant expression in Avp magnocellular neurons in the rat (Hurbin et al., 1998, 2002). However, the wash conditions, given the lengths of the probes, were not appropriately stringent. These shortcomings open the possibility of locations inadvertently assigned as sites of Avpr1b expression. For this reason, we generated more specific Avpr1b probes for the mouse (and rat and human) to examine the distribution of this receptor's transcripts more accurately by both ISHH and reverse transcriptase–polymerase chain reaction (RT-PCR).

EXPERIMENTAL PROCEDURES

Animals

Adult C57BL/6J and 129/P3J strains of mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Avpr1b +/+ and −/−

[1] Present address: Graduate program in Molecular, Cell, Genetics and Development, Yale University, New Haven, CT 06511, USA (J. Li); Department of Psychology, The University at Buffalo, SUNY, Buffalo, NY 14260, USA (S. R. Wersinger).
*Corresponding author. Tel: +1-301-496-8767.
E-mail address: wsy@mail.nih.gov (W. S. Young).
Abbreviations: Avp, vasopressin; Avpr1a, vasopressin 1a receptor; Avpr1b, vasopressin 1b receptor; Crh, corticotropin-releasing factor; ISHH, *in situ* hybridization histochemistry; Oxtr, oxytocin receptor; PVN, paraventricular nucleus of the hypothalamus; RT-PCR, reverse transcriptase-polymerase chain reaction.

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mice of mixed background (C57BL/6J and 129/SvJ) were created as described previously (Wersinger et al., 2002) and bred in our own colony. Six adrenalectomized and six sham-operated C57BL/6 mice were obtained from Taconic (Germantown, NY, USA). Their brains were removed 7 days after surgery. Adult Sprague–Dawley rats were also obtained from Taconic.

All animals were given food and water *ad libitum*. The adrenalectomized mice were also supplied with 0.9% saline. All animal procedures were approved by the NIMH Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996). Every effort was made to minimize the number of animals used and their suffering.

For ISHH (see below), three C57BL/6J, two 129/P3J and three mixed C57BL/6J-129/SvJ mice were sectioned through the entire extent of the neuraxis from the medulla to olfactory bulbs. Another C57BL/6J and a mixed mouse were examined throughout the extent of the hippocampus. These series sampled sections every 100 μm. Over 20 C57BL/6 mice were examined at the level of the dorsal hippocampus, from approximately 1–2.5 mm behind the bregma. Two Sprague–Dawley rats were serially sectioned from the medulla to the olfactory bulbs as well.

Human tissues

Hippocampal tissue blocks and samples were obtained from the Human Brain Tissue Bank (Budapest, Hungary). One was from a 36 year-old female and the other from a 50 year-old male. An isolated punch sample for RT-PCR was obtained from a 35 year-old male. None of these subjects had a known neurological disease and the tissues were obtained within 2 h of death. After being removed from the skull, brains were rapidly frozen, dissected and the samples were stored at −70 °C until use. The protocol of tissue sampling and retrospective assessments was approved by the institutional review board of the Semmelweis University, Budapest. A pituitary from a 63 year-old male with a 16 h postmortem interval was obtained from the NIMH Human Brain Collection. The patient was an alcoholic with no known neuropathology.

ISSH

ISHH was performed as described (Bradley et al., 1992; Young and Mezey, 2006). Briefly, rodent brains and pituitaries were removed and frozen on powdered dry ice. They were then stored at −80 °C until 12 μm sections were cut at −15 °C and thaw-mounted onto SuperFrost Plus slides (VWR, Batavia, IL, USA). Sections from human samples were cut at a thickness of 16 μm. They were then stored at −80 °C until warmed to room temperature, fixed in 4% formaldehyde in phosphate-buffered saline, treated with acetic anhydride and defatted through a series of alcohols. Radiolabeled riboprobe was applied to the sections for 20–24 h at 55 °C before treating with RNase and stringent washes at 65 °C.

The sections were apposed to phosphorimaging plates for up to 2 weeks before scanning in a Cyclone system (Perkin-Elmer, Boston, MA, USA). Sections for quantitation were analyzed using the Cyclone software. Image intensities were well within the linear range of the phosphorimaging system. Results were analyzed using unpaired *t*-tests (InStat, GraphPad Software, San Diego, CA, USA). Most slides were subsequently coated with Ilford K.5D (Polysciences, Warrington, PA, USA) or Kodak NTB (Rochester, NY, USA) nuclear emulsions and exposed for up to 4 months before developing and staining with 0.2% Toluidine Blue.

Riboprobes

Two separate riboprobes were designed for use in mice to avoid any cross-reactivity with related receptor sequences, especially the Avpr1a and Oxtr. A 5′ probe that targeted bases 628–825 of the mouse Avpr1b mRNA (GenBank accession #NM_011924) was used initially and then a longer 3′ probe was made and used for the majority of ISHH that targeted bases 1635–2057. Analogous 3′ probes targeted bases 1549–1982 of the rat receptor (GenBank accession #U27322) and 1318–1824 of the human receptor (GenBank accession #D31833).

A rat corticotropin-releasing factor (Crh) probe that targeted bases 223–720 of the mouse mRNA (97% sequence identity, GenBank accession #NM_205769) was used in adjacent sections from the mice that underwent restraint stress or adrenalectomy to gauge the effectiveness of the treatments. All probes, including sense versions, were labeled using ^{35}S-UTP and the appropriate RNA polymerases.

RT-PCR

Total RNA was isolated from tissues using the RNeasy Kit (Qiagen, Valencia, CA, USA) and stored frozen in water at −20 °C until used. One hundred nanograms of RNA was used in each assay. The mouse Avpr1b primers were GCTGGCCCAAGTCCTCATCTTCTG and GCGGTGACTCAGGGAACGT producing a 322 bp product from mRNA (and 2095 bp from genomic DNA). To control for RNA loading, the mouse samples were checked using primers against b-actin (CCAGGTCATCACTATTGGCAACG and CTCAGGAGGAGCAATGATCTTGA) that produced a 266 bp product from mRNA (and 343 bp from genomic DNA). This was done by distributing the samples among a set of wells in 10 μl (containing 200 μg) and then removing 5 μl for another set of wells in the same plate. An RT-PCR reaction mix was made, divided in two and, after the appropriate primer pairs were added, aliquoted to the RNA samples. The reactions were subsequently run on the same gel from parallel wells. The rat primers were GGCGGTGCTCACAGCTTGCTACGG and CTGTTGAAGCCCATGTAGATCCAG and produced 384 bp and 9083 bp products from mRNA and genomic DNA, respectively. The human primers were GGCCCTCACCTTCCACCTTAGCTG and CTGTTGAAGCCCATGTAGATCCAG and produced 283 bp and 5721 bp products from mRNA and genomic DNA, respectively. The reactions were run using the Invitrogen One-Step RT-PCR system (Carlsbad, CA, USA) at 50 °C×30 min, 94 °C×2 min, (94 °C×45 s, 55 °C×45 s, 72 °C×30 s) for 40 cycles, and 72 °C×5 min. The samples were then run on 1.5% agarose gels or 2% NuSieve (FMC BioProducts, Rockland, ME, USA) gels.

Restraint stress

Six C57BL/6J mice were restrained in DecapiCones (Braintree Scientific, Braintree, MA, USA) for one hour. They were then returned to their cages for another three hours at which time their brains were rapidly removed. Five control C57BL/6J mice were left in their cages until their brains were removed.

RESULTS

The mouse, rat and human Avpr1b riboprobes were tested first on the respective pituitaries and showed good cellular labeling (Fig. 1). The brain distributions of Avpr1b transcripts were the same in C57BL/6J and 129/P3J strains or in the mixed backgrounds that the Avpr1b −/− mice were maintained on, with either the 5′ or 3′ probe. In the brain, labeling of the dorsal hippocampal CA2 pyramidal cells was the highest (Fig. 2) at 20–40 grains per 100 μm^2 after a 4 month exposure. In fact, at the level of resolution of the phosphorimager, the only labeling within the brain that was seen is in this portion of hippocampus. In the most rostral coronal sections of the hippocampus, pyramidal cell neurons of the medial portion of the CA2 that is separated by