20. He, W., Cowan, C. W. & Wensel, T. G. RGS9, a GTPase accelerator for phototransduction. *Neuron* **20,** 95–102 (1998).
21. Makino, E. R., Handy, J. W., Li, T. & Arshavsky, V. Y. The GTPase activating factor for transducin in rod photoreceptors is the complex between RGS9 and type 5 G protein β subunit. *Proc. Natl Acad. Sci. USA* **96,** 1947–1952 (1999).
22. Pugh, E. N. Jr & Lamb, T. D. Amplification and kinetics of the activation steps in phototransduction. *Biochim. Biophys. Acta* **1141,** 111–149 (1993).
23. Bönigk, W. *et al.* Rod and cone photoreceptor cells express distinct genes for cGMP-gated channels. *Neuron* **10,** 865–877 (1993).
24. Zoche, M., Bienert, M., Beyermann, M. & Koch, K.-W. Distinct molecular recognition of calmodulin-binding sites in the neuronal and macrophage nitric oxide synthases: A surface plasmon resonance study. *Biochemistry* **35,** 8742–8747 (1996).
25. Hsu, Y.-T. & Molday, R. S. Modulation of the cGMP-gated channel of rod photoreceptor cells by calmodulin. *Nature* **361,** 76–79 (1993).
26. Lambrecht, H.-G. & Koch, K.-W. A 26 kd calcium binding protein from bovine rod outer segments as modulator of photoreceptor guanylate cyclase. *EMBO J.* **10,** 793–798 (1991).
27. Wolfrum, U. Centrin in the photoreceptor cells of mammalian retinae. *Cell Motil. Cytoskel.* **32,** 55–64 (1995).
28. Wolfrum, U., Liu, X., Schmitt, A., Udovichenko, I. P. & Williams, D. S. Myosin VIIa as a common component of cilia and microvilli. *Cell Motil. Cytoskel.* **40,** 261–271 (1998).
29. Danscher, G. Localization of gold in biological tissue. A photochemical method for light and electron microscopy. *Histochemistry* **71,** 81–88 (1981).

Acknowledgements. We thank J. Beavo, J. Nathans, I. Weyand and D. Weitz for antibodies, A. Eckert for preparing the manuscript, I. Weyand and R. Seifert for critical reading of the manuscript, and E. Pugh and R. Cote for discussions. This work was supported by grants from the EU (U.B.K.), the Deutsche Forschungsgemeinschaft (M.B., K.P.H., K.-W.K., U.B.K., U.W.) and the FAUN-Stiftung (U.W.).

Correspondence and requests for materials should be addressed to U.B.K. (e-mail: a.eckert@fz-juelich.de).

Increased affiliative response to vasopressin in mice expressing the V_{1a} receptor from a monogamous vole

Larry J. Young*, Roger Nilsen*, Katrina G. Waymire†, Grant R. MacGregor† & Thomas R. Insel*

* *Department of Psychiatry and Behavioural Sciences and † Center for Molecular Medicine, Emory University, Atlanta, Georgia 30322, USA*

Arginine vasopressin influences male reproductive and social behaviours in several vertebrate taxa[1] through its actions at the V_{1a} receptor in the brain. The neuroanatomical distribution of vasopressin V_{1a} receptors varies greatly between species with different forms of social organization[2,3]. Here we show that centrally administered arginine vasopressin increases affiliative behaviour in the highly social, monogamous prairie vole, but not in the relatively asocial, promiscuous montane vole. Molecular analyses indicate that gene duplication and/or changes in promoter structure of the prairie vole receptor gene may contribute to the species differences in vasopressin-receptor expression. We further show that mice that are transgenic for the prairie vole receptor gene have a neuroanatomical pattern of receptor binding that is similar to that of the prairie vole, and exhibit increased affiliative behaviour after injection with arginine vasopressin. These data indicate that the pattern of V_{1a}-receptor gene expression in the brain may be functionally associated with species-typical social behaviours in male vertebrates.

Arginine vasopressin (AVP) and its non-mammalian homologue, arginine vasotocin (AVT), are involved in many species-typical, male social behaviours, including communication[4–7], aggression[4–6], sexual behaviour[7] and, in monogamous species, pairbonding and paternal care[8]. Pharmacological studies using selective antagonists indicate that the behavioural effects of AVP are mediated by the V_{1a} receptor[8,9]. The V_{1a} receptor is a member of a family of evolutionarily related receptors for the neurohypophysial peptides, arginine vasopressin and oxytocin, which includes the V_{1a}, V_{1b}, V_2 and oxytocin receptors, all of which are G-protein-coupled proteins comprising seven transmembrane domains[10]. The V_{1a} receptor is exceptional in that its pattern of expression in the brain is phylogenetically plastic, with the neuroanatomical distribution of this receptor being unique in virtually every species examined[10].

Voles provide a useful model to test the relationship between receptor expression patterns and social behaviour. Prairie voles (*Microtus ochrogaster*) are highly affiliative, biparental and monogamous; whereas montane voles (*Microtus montanus*) are relatively asocial, non-paternal, and promiscuous[11]. In the male prairie vole, cohabitation and/or mating with a female stimulates both the central release of AVP[12,13] and the development of a pairbond and paternal care[8,12]. Central blockade of the vasopressin receptors during mating with a selective V_{1a} antagonist prevents the normal development of pairbonds between mates and paternal care in male prairie voles[8,14]. Prairie and montane voles have strikingly different distributions of V_{1a}-receptor binding in the brain (Fig. 1a). We tested the hypothesis that these differences in V_{1a}-receptor binding patterns would confer species differences in behavioural response to AVP. Using an ovariectomized stimulus female placed in one side of a two-chambered arena, 2 ng of AVP delivered by intracerebroventricular (i.c.v.) injection to male prairie voles resulted in a significant increase in affiliative behaviour (olfactory investigation and grooming) toward the stimulus female ($P < 0.01$) (Fig. 1b). In contrast, the identical treatment had no effect on affiliative behaviour, compared with control injections, in male montane voles.

To investigate the mechanisms of the species differences in regional gene expression, we compared the structures of the prairie and montane vole V_{1a}-receptor genes, including the 5′ flanking regions (Fig. 2a). The V_{1a}-receptor gene encodes a protein of 420 amino-acid residues that is 99% homologous between the vole species. The binding kinetics of the receptor protein and its second messenger coupling are identical in both species[3]. Both 5′ and 3′ rapid amplification of complementary DNA ends (RACE) were used to identify the transcriptional boundaries of the gene (data not shown). The prairie vole V_{1a} messenger RNA is composed of a 232-base-pair (bp) 5′ untranslated region (UTR), a 1,260-bp coding region followed by a 36-bp 3′ UTR. No CAAT or TATA enhancer

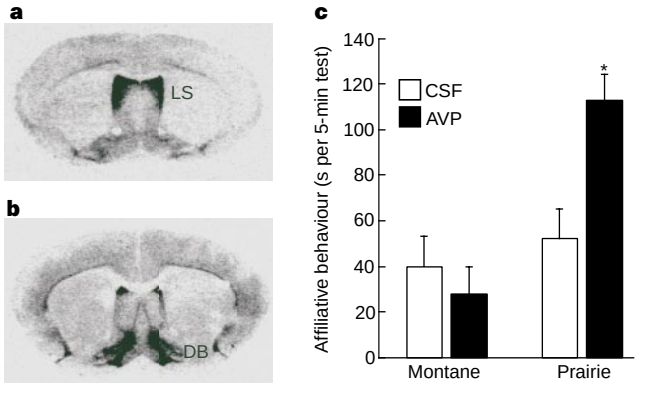

Figure 1 Montane and prairie voles differ both in V_{1a}-receptor binding pattern and behavioural response to arginine vasopressin. Receptor autoradiography[6] illustrates the different patterns of V_{1a}-receptor binding in the brains of **a**, the non-monogamous montane vole, and **b**, the monogamous prairie vole. Note the high intensity of binding in the lateral septum (LS) of the montane vole but not the prairie vole, and in the diagonal band (DB) of the prairie vole but not the montane vole. Similar differences exist throughout the brain. **c**, Male prairie but not montane voles exhibit elevated levels of affiliative behaviour after vasopressin is administered directly into the brain (two-way ANOVA: species effect, $F(1,27) = 10.3$, $P < 0.01$; * Fisher's LSD post-hoc test, $P < 0.01$ compared with CSF-treated prairie voles).

elements were found near the transcriptional initiation site. Northern analysis using poly $(A)^+$ RNA from brain demonstrate that the transcript length is similar in prairie and montane voles (data not shown). Both species have a 2.5-kilobase (kb) intron located between the sixth and seventh transmembrane domains. In contrast to the similarities in the coding sequences, the 5′ flanking region of the V_{1a} gene displays marked differences between the species. In the gene for the prairie vole V_{1a}-receptor, this region contains a 428-bp sequence that is rich in repetitive di- and tetra-nucleotide sequences, or microsatellite DNA, between −1,151 and −723 relative to the transcription start site. This sequence is not found in the montane vole V_{1a} gene and sequences on either side of the expansion are contiguous in the montane vole gene.

Using polymerase chain reaction (PCR) primers positioned on either side of the expansion, we found that the V_{1a}-receptor gene of the monogamous pine vole (*Microtus pinetorum*), which has a prairie vole-like pattern of V_{1a}-receptor expression[3], has a similar 5′ flanking sequence to that of the prairie vole gene (Fig. 2b). In contrast, the non-monogamous meadow vole (*Microtus pennsylvanicus*), which has a montane-vole-like pattern of receptors in the brain, did not have this sequence. In addition to the change in the 5′ flanking region, two distinct V_{1a} loci were isolated from prairie vole genomic libraries, indicating that the V_{1a}-receptor gene from the prairie vole has been duplicated (Fig. 2a). Southern blot analysis of genomic DNA from both prairie and montane voles confirms the duplication (not shown). The second V_{1a}-receptor locus has a 589-bp 5′ truncated fragment of an L1 long interspersed nuclear element (LINE) just upstream of the expansion. The LINE, a mobile genetic element or retrotransposon, inserted in the centre of a 10-bp palindrome (TTCTT/AAGAA). A deletion of a single base in the sequence encoding the third intracellular loop of the receptor results in a frame shift and premature stop.

Microsatellite DNA sequences in close proximity to transcription initiation sites can alter transcription[15,16], presumably by altering the structural environment of the region containing the *cis* regulatory sequences. Repetitive tetranucleotide repeats act as transcriptional enhancers in the human tyrosine hydroxylase gene[17]. In addition to the changes in the 5′ flanking region, gene duplication (that is, translocation to a new chromosomal environment) may contribute to the species differences in gene expression. The present data cannot distinguish between these mechanisms.

To determine whether the pattern of neural expression of V_{1a} receptors was sufficient to induce differences in social behaviour, we created transgenic mice using a prairie vole V_{1a}-receptor mini-gene containing 2.2 kb of the 5′ flanking region, both exons with the 2.5-kb intron, and 2.4 kb of 3′ flanking region. Expression of V_{1a} receptors in the central nervous system was detected by receptor autoradiography using the specific V_{1a}-receptor ligand, ^{125}I-Lin AVP[18] and *in situ* hybridization. In one of the four transgenic lines created, the neuroanatomical pattern of V_{1a}-receptor binding was remarkably similar to that of the prairie vole and was consistent for at least four generations (Fig. 3a, b, e). There were no differences in oxytocin-receptor binding between transgenic and non-transgenic mice (Fig. 3c, d). The pattern of V_{1a}-receptor mRNA in transgenic mouse brain was similar to that of ligand binding, demonstrating that the change in receptor binding was due to altered transgene expression in the brain (data not shown). The intensity of binding in the transgenic mouse was not elevated in brain regions that normally express V_{1a} receptor in the mouse, such as the diagonal band, lateral septum or the lateral hypothalamus (Fig. 3f). The level of expression of the prairie vole V_{1a} transgene in the mice was within the range of that found in prairie voles. Thus, any changes in response to AVP should be attributable to the expression of the receptor in new areas of the mouse brain and not simply to increased numbers of receptors in regions that are normally responsive to AVP in the mouse.

In a behavioural model similar to that used in the vole experiments, 2 ng of AVP increased the expression of affiliative behaviour, as measured by olfactory investigation and grooming, in transgenic but not non-transgenic mice ($P < 0.01$) (Fig. 4a), a response similar

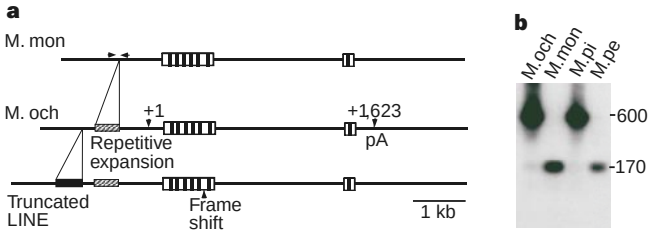

Figure 2 Structure of the V_{1a}-receptor gene in voles. V_{1a}-receptor genes from montane vole (M. mon) and prairie vole (M. och) were isolated from genomic DNA libraries. Transcription begins at +1 and polyadenylation (pA) occurs at +1,623 of the prairie vole gene. The boxed area within these sites represents the coding region and the vertical bars in the coding region represent the location of the transmembrane domains. Two distinct loci were isolated from prairie vole DNA. The sequences of the genomic clones have been deposited in GenBank (accession number AF069304). **b**, PCR amplification of genomic DNA using primers on either side of the expansion (arrows in **a**) demonstrates the presence of the expansion in monogamous prairie and pine (M. pi) voles (600-bp band), but not in promiscuous montane or meadow (M. pe) voles.

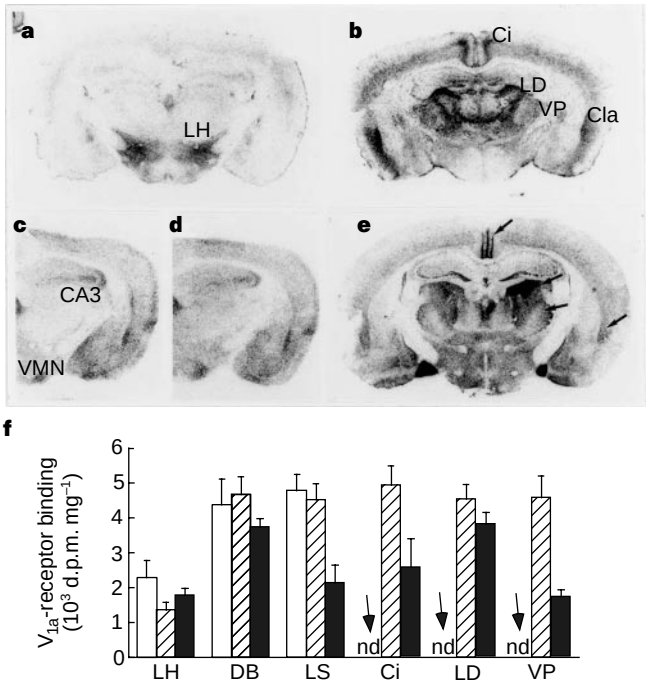

Figure 3 The pattern of V_{1a}-receptor binding of mice that are transgenic for the V_{1a}-receptor gene from the prairie vole is similar to that of the prairie vole. Receptor autoradiography[6] illustrates the distribution of V_{1a}-receptor binding in wild-type (**a**) and transgenic (**b**) mice. Although V_{1a}-receptor binding is altered, oxytocin-receptor binding is identical in wild-type (**c**) and transgenic (**d**) mice. Binding in the cingulate cortex (Ci), laterodorsal (LD) and ventroposterior (VP) thalamic nuclei, claustrum (Cla) and several other regions of transgenic mice is similar to that found in the brain of the prairie vole (**e**). **f**, Quantification of V_{1a}-receptor binding in wild-type mice (open bars), transgenic mice (striped bars) and prairie vole (filled bars) ($n = 6$–7 per group) demonstrates that receptor density in transgenic mice is not elevated in areas that normally express receptors in mice (for example, lateral hypothalamus (LH), diagonal band (DB) and lateral septum (LS)); nd indicates no detectable specific binding in wild-type mice.

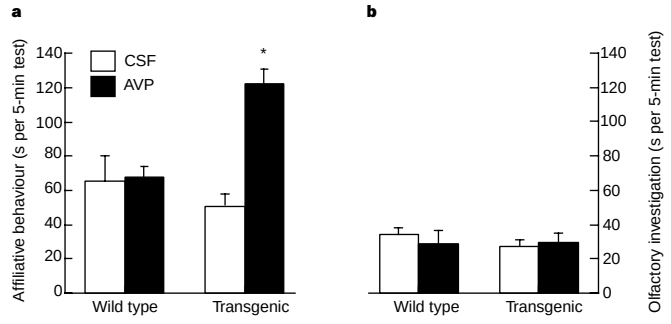

Figure 4 Behavioural response to AVD in transgenic mice. **a**, AVP increases the duration of affiliative behaviours directed towards an ovariectomized female in male transgenic mice with a prairie vole pattern of V_{1a}-receptor expression, but not in male wild-type mice (two-way ANOVA: genotype effect, $F(1,22) = 4.3$, $P < 0.05$; *Fisher's LSD post-hoc test, $P < 0.01$ compared with CSF-treated transgenic animals). **b**, AVP has no effect on olfactory investigation of cotton balls soiled with bedding from an ovariectomized female's cage in either transgenic or non-transgenic males.

to that observed in prairie voles (Fig. 1). This dose of AVP, identical to that used in the vole studies, did not change locomotor or autogrooming behaviour. When not in contact with the female, males of both genotypes actively explored the arena. To determine whether the increase in olfactory investigation was specific for a social stimulus, we tested another set of male mice with the same model but substituted cotton balls that had been scented with lemon extract. No genotype ($F = 0.129$, $P > 0.05$) or treatment ($F = 2.763$, $P > 0.05$) effects were found in olfactory exploration of this new stimulus. In a further test, we used cotton balls scented with bedding from ovariectomized females and still observed no effect of genotype ($F = 0.443$, $P > 0.05$) or treatment ($F = 0.038$, $P > 0.05$) (Fig. 4b). Thus, the increase in olfactory investigation and grooming after AVP injection in the transgenic mice seemed to indicate an increase in affiliative behaviour rather than a non-specific increase in investigation of new olfactory stimuli. At higher doses, transgenic mice, but not non-transgenic littermates, exhibited a dose-dependent increase in autogrooming behaviour after AVP injection (significant at 100 ng, data not shown).

Our results show that centrally administered AVP increases affiliative behaviour in the monogamous male prairie vole, but not in the montane vole. This acute effect of AVP may be essential for the long-lasting effects of AVP in monogamous mammals, such as the formation of pairbonds[8]. The species difference in response to AVP is likely to be due to differences in the distribution of V_{1a}-receptor expression in the brain as (1) vole species with similar social organization have similar receptor patterns[3], and (2) transgenic induction of the prairie vole pattern of V_{1a} receptors in mice results in a pro-social response to AVP. Although many genes are likely to be involved in the evolution of complex social behaviours such as monogamy, our data indicate that changes in the expression of a single gene can have an impact on the expression of components of these behaviours, such as affiliation. These observations, together with the species diversity in the neuroanatomical distribution of V_{1a} receptors in the brain, indicate that changes in the pattern of V_{1a}-receptor gene expression in the brain may be a common mechanism to alter the behavioural response to AVP, enabling adaptation to changing socioecological situations. □

Methods

Animals and behaviour tests. Prairie and montane voles used in this study were laboratory-reared animals derived from field-caught specimens. Transgenic mice were generated by pronuclear injection in FVB/N embryos.

The testing arena comprised two standard mouse cages connected by means of a tube to allow free movement between the cages. Ovariectomized, conspecific females were used in all tests to eliminate sexual attractivity as a possible confounding variable. In both vole and mouse experiments, male subjects were lightly anaesthetized with Metofane and injected i.c.v. with 4 μl of either artificial cerebrospinal fluid (CSF) or 2 ng arginine vasopressin dissolved in the same volume of CSF. After a 5-min recovery period, the subjects were placed in the empty cage of the testing arena. Behaviour was recorded for 5 min beginning at the moment when the subject first entered the cage containing the stimulus female. This time line was chosen based on preliminary observations of the duration of other AVP effects, such as autogrooming and locomotion. Affiliative behaviour was defined as olfactory investigation or grooming of the stimulus animal. In the experiments with voles, it was necessary to anaesthetize the female during the test as females typically show some aggressive behaviour during the first few minutes of contact with a male. In the experiments with mice, the females were not anaesthetized but were tethered to one side of the cage to restrict movement. In some experiments, female mice were replaced with cotton balls scented with either 10 μl of lemon extract or with soiled bedding from ovariectomized female cages. All experiments included 5–8 animals per treatment. Duration of affiliative behaviour was analysed using a two-way analysis of variance (ANOVA) with species or genotype and treatment as factors, followed by Fisher's least significant difference (LSD) post-hoc test.

Molecular analysis. Genomic clones were isolated from EMBL3 libraries derived for montane and prairie vole genomic DNA (Clontech), and sequenced on both strands. Amplification of the 5′ flanking region of the V_{1a} receptor was done using PCR from approximately 100 ng of genomic DNA isolated from the liver of prairie, montane, meadow and pine voles. Ten per cent of the reaction was electrophoresed on a 1.2% agarose gel, transferred onto nylon membrane, and probed with a ^{32}P-labelled fragment of the prairie vole V_{1a}-receptor gene containing the amplified region.

Received 7 May; accepted 20 May 1999.

1. Young, L. J., Wang, Z. & Insel, T. R. Neuroendocrine bases of monogamy. *Trends Neurosci.* **21,** 71–75 (1998).
2. Bester-Meredith, J. K., Young, L. J. & Marler, C. A. Species differences in paternal behavior and aggression in *Peromyscus* and their associations with vasopressin immunoreactivity and receptors. *Horm. Behav.* (in the press).
3. Insel, T. R., Wang, Z. & Ferris, C. F. Patterns of brain vasopressin receptor distribution associated with social organization in microtine rodents. *J. Neurosci.* **14,** 5381–5392 (1994).
4. Ritters, L. V. & Panksepp, J. Effects of vastotocin on aggressive behavior in male Japanese quail. *Ann. NY Acad. Sci.* **807,** 478–480 (1997).
5. Ferris, C. F. *et al.* Vasopressin/serotonin interactions in the anterior hypothalamus control aggressive behavior in gold hamsters. *J. Neurosci.* **17,** 4331–4340 (1997).
6. Young, L. J., Winslow, J. T., Nilsen, R. & Insel, T. R. Species differences in V_{1a} receptor gene expression in monogamous and non-monogamous voles: behavioral consequences. *Behav. Neurosci.* **111,** 599–605 (1997).
7. Moore, F. L. & Miller, L. J. Arginine vasotocin induces sexual behavior of newts by acting on cells in the brain. *Peptides* **4,** 97–102 (1983).
8. Winslow, J., Hastings, N., Carter, C. S., Harbaugh, C. & Insel, T. A role for central vasopressin in pair bonding in monogamous prairie voles. *Nature* **365,** 545–548 (1993).
9. Ferris, C. F. & Potegal, M. Vasopressin receptor blockade in the anterior hypothalamus suppresses aggression in hamsters. *Physiol. Behav.* **44,** 235–239 (1988).
10. Barberis, C. & Tribollet, E. Vasopressin and oxytocin receptors in the central nervous system. *Crit. Rev. Neurobiol.* **10,** 119–154 (1996).
11. Shapiro, L. E. & Dewsbury, D. A. Differences in affiliative behavior, pair bonding, and vaginal cytology in two species of vole (*Microtus ochrogaster* and *M. montanus*). *J. Comp. Psychol.* **104,** 268–274 (1990).
12. Bamshad, M., Novak, M. & DeVries, G. J. Cohabitation alters vasopressin innervation and paternal behavior in prairie voles. *Physiol. Behav.* **56,** 751–758 (1994).
13. Wang, Z., Smith, W., Major, D. E. & De Vries, G. J. Sex and species differences in the effects of cohabitation on vasopressin messenger RNA expression in the bed nucleus and stria terminalis in prairie voles (*Microtus orchogaster*) and meadow voles (*Microtus pennsylvanicus*). *Brain Res.* **650,** 212–218 (1994).
14. Wang, Z., Ferris, C. F. & De Vries, G. J. Role of septal vasopressin innervation in paternal behavior in prairie voles (*Microtus ochrogaster*). *Proc. Natl Acad. Sci. USA* **91,** 400–404 (1994).
15. Choong, C. S., Kemppainen, J. A., Zhou, Z. & Wilson, E. M. Reduced androgen receptor gene expression with first exon CAG repeat expansion. *Mol. Endrocrinol.* **10,** 1527–1535 (1997).
16. Klesert, T. R., Otten, A. D., Bird, T. D. & Tapscott, S. J. Trinucleotide repeat expansion at the myotonin dystrophy locus reduces expression of *DMAHP*. *Nature Genet.* **16,** 402–406 (1997).
17. Meloni, R., Albanése, V., Ravassard, P., Treilhou, F. & Mallet, J. A tetranucleotide polymoric microsatellite, located in the first intron of the tyrosine hydroxylase gene, acts as a transcription regulatory element *in vitro*. *Hum. Mol. Gen.* **7,** 423–428 (1998).
18. Barberis, C. *et al.* Characterization of a novel, linear radioiodinated vasopressin antagonist: an excellent radioligand for vasopressin V_{1a} receptors. *Neuroendocrinol.* **62,** 135–146 (1995).

Acknowledgements. This work was supported by grants from the NIMH and NCRR to L.J.Y. and T.R.I.

Correspondence and requests for materials should be addressed to L.J.Y. (e-mail: lyoun03@emory.edu).
The sequences of the genomic clones have been deposited in GenBank (AF069304).