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Supplementary Information accompanies the paper on www.nature.com/nature.

Acknowledgements We thank A. Kirincich for ADCP analyses, S. Oda and A. Walters for assistance and A. Pazar for crab fishery data. The Partnership for Interdisciplinary Studies of Coastal Oceans, funded by the David and Lucile Packard Foundation, and the US GLOBEC program, jointly funded by NSF and NOAA, both contributed to this work. J.A.B. acknowledges additional funding from NSF. ODFW ROV survey work was funded in part by the Oregon Department of Land Conservation and Development through NOAA.

Competing interests statement The authors declare that they have no competing financial interests.

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Enhanced partner preference in a promiscuous species by manipulating the expression of a single gene

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The molecular mechanisms underlying the evolution of complex behaviour are poorly understood. The mammalian genus *Microtus* provides an excellent model for investigating the evolution of social behaviour. Prairie voles (*Microtus ochrogaster*) exhibit a monogamous social structure in nature, whereas closely related meadow voles (*Microtus pennsylvanicus*) are solitary and polygamous¹. In male prairie voles, both vasopressin and dopamine act in the ventral forebrain to regulate selective affiliation between adult mates, known as pair bond formation, as assessed by partner preference in the laboratory^{2–4}. The vasopressin V1a receptor (V1aR) is expressed at higher levels in the ventral forebrain of monogamous than in promiscuous vole species⁵, whereas dopamine receptor distribution is relatively conserved between species. Here we substantially increase partner preference formation in the socially promiscuous meadow vole by using viral vector V1aR gene transfer into the ventral forebrain. We show that a change in the expression of a single gene in the larger context of pre-existing genetic and neural circuits can profoundly alter social behaviour, providing a potential molecular mechanism for the rapid evolution of complex social behaviour.

Fewer than 5% of mammalian species have a monogamous social structure, which typically includes pair bond formation between

adult mates and the biparental care of offspring⁶. Central administration of vasopressin facilitates each of these monogamous-typical behaviours in male prairie voles through V1aRs in the brain^{7–9}. The distribution of V1aRs in the brain varies considerably between monogamous and promiscuous vole species⁵ (Fig. 1).

Species-specific patterns of V1aR expression have provided insight into the neural mechanisms underlying pair bond formation. In particular, the ventral pallidum, located within the ventral forebrain and the mesolimbic dopamine reward pathway, highly expresses V1aRs in monogamous prairie and pine voles (*Microtus pinetorum*), but not in promiscuous meadow or montane voles (*Microtus montanus*)^{5,10}. Site-specific infusion of a selective V1aR antagonist into the ventral pallidum blocks pair bond formation in prairie voles⁴. Ventral forebrain V1aR expression is also higher in both the monogamous *Peromyscus* California mouse and the monogamous marmoset monkey than in promiscuous *Peromyscus* or primate species^{11–14}. Thus, V1aRs in the ventral forebrain are crucial for pair bond formation, and this V1aR pattern seems to be correlated with monogamous social organization across diverse taxa.

Although genetic analysis of V1aR shows more than 99% conservation of gene sequence between vole species, monogamous prairie and pine voles have an expansion of repetitive microsatellite DNA in the 5' regulatory region of the gene, whereas promiscuous montane and meadow voles do not⁹. Furthermore, species-specific V1aR expression patterns seem to be determined by proximate regulatory sequences of the gene, because mice transgenic for the prairie vole V1aR coding sequence and its flanking regions display prairie-like patterns of V1aR binding in the brain⁹. Because microsatellite DNA is highly unstable, it is possible that instability in the V1aR regulatory region could result in altered V1aR expression in different brain regions, leading to differences in social behaviour. We proposed that increased V1aRs in the ventral pallidum, in particular, could shift some individuals within a species to form pair bonds and ultimately result in the stable selection of monogamous social organization. To test this hypothesis directly, we used viral vector-mediated gene transfer to overexpress V1aR in the ventral pallidum in the socially promiscuous meadow vole, in essence recreating a singular evolutionary event in the laboratory.

The study consisted of three groups of meadow voles. The experimental group (V1aR-vp) overexpressed V1aR bilaterally in the ventral pallidum ($n = 11$). The first control group (Ctrl-vp) received ventral pallidal infusions of a vector expressing the *lacZ* gene ($n = 11$). The second control group (Ctrl-other) consisted of animals whose viral injections were inadvertently placed outside the ventral pallidum ($n = 9$); these animals were regressed, *ex post facto*, in the analysis after the completion of behavioural testing. V1aR autoradiography revealed a significant elevation of V1aR binding in the ventral pallidum of the V1aR-vp animals to about threefold that in the two control groups (Fig. 2). This is comparable to the degree of V1aR binding observed in the prairie vole (Fig. 1e).

All animals were paired with a behaviourally receptive female for 24 h, and subsequently placed in a partner preference test in which the animal could access both the partner and a novel female of comparable stimulus value. During the 3-h test, the time spent in side-by-side contact ('huddling') with each female was recorded.

The V1aR-vp group spent significantly more time huddling with the partner than the stranger ($P < 0.01$, Student's *t*-test), whereas the Ctrl-vp and Ctrl-other groups did not ($P > 0.05$, Student's *t*-test) (Fig. 3a). In addition, all the animals in the V1aR-vp group spent more time huddling with the partner than the stranger (11 of 11), whereas control animals were uniformly distributed across a wide range and did not prefer the partner significantly more than expected by random chance (12 of 20; $P > 0.05$, χ^2 test) (Fig. 3b). V1aR-vp animals also spent significantly more time in total side-by-side contact with the partner than the control animals ($P < 0.01$,

Student's *t*-test), but not significantly more time with the stranger than the control animals ($P > 0.05$, Student's *t*-test), indicating that their partner preference was not a result of a generalized, non-selective increase in affiliative behaviour.

Although several control animals did seem to display selective affiliation with the mate, none of them were placed within the top five strongest pair bonds, as defined by the total time spent huddling with the partner. However, this illustrates that individual variation does exist within the meadow vole population, indicating that the potential for pair bond formation might be present in promiscuous vole species. Meadow voles do not in fact completely lack V1aRs in the ventral pallidum, and thus the potential to engage affiliative neural circuits is in place. One recent study found that a subpopulation of meadow voles formed selective affiliations under certain laboratory conditions, although this phenomenon has not been reported in other laboratories or in nature¹⁵. Our results show that overexpression of V1aRs to a degree beyond naturally occurring levels in meadow voles can shift every individual in the species to form pair bonds.

To determine whether similar proximate neural mechanisms underlie pair bond formation in V1aR-*vp* meadow voles and prairie voles, we next examined the role of dopaminergic neurotransmission. Before pairing with the same receptive female for 24 h, all animals were pretreated with 50 mg kg⁻¹ intraperitoneal eticlopride, a D2-receptor antagonist. This dose of eticlopride blocks partner preference formation in prairie voles¹⁶. Subsequent partner preference testing showed that V1aR-*vp* animals, like control animals, did not significantly prefer the partner over the stranger ($P > 0.05$, Student's *t*-test) (Fig. 4a). In addition, there were no significant group differences in the total time spent huddling with the partner ($P > 0.05$, Student's *t*-test). Analysis of the distribution of individuals within each group indicates that the distribution of control animals was unaffected by pretreatment with eticlopride, whereas the distribution of V1aR-*vp* animals was shifted back to the

normal continuum of control meadow voles, with no significant preference for the partner over that expected from random chance ($P > 0.05$, χ^2 analysis) (Fig. 4b). This shows that pair bond formation in the transgenic meadow voles, as in prairie voles, depends on dopaminergic neurotransmission.

Because monogamous-typical behaviours typically include biparental care in addition to pair bond formation and increased sociality, we proposed that V1aR overexpression in the ventral pallidum might result in increased paternal care by the males. There were no significant differences between the groups in 'classic' paternal behaviours, such as the latency to retrieval or the total time spent in physical contact (namely licking, grooming or huddling) with the pups. However, V1aR-*vp* animals did display increased social approach behaviours towards the pups, including a shorter latency to approach ($P < 0.05$, Student's *t*-test), more time near the pups ($P < 0.05$, Student's *t*-test), and less time self-grooming ($P < 0.005$, Student's *t*-test) than control animals, which is consistent with the finding of increased total social contact observed during the partner preference test. These results indicate that, although both behaviours are regulated by central V1aR, the underlying mechanisms of paternal care and pair bond formation probably use different neural circuits. In prairie voles, the administration of V1aR antagonist into the ventral pallidum selectively blocks partner preference⁴ but not paternal care; similarly, V1aR antagonist in the medial amygdala blocks paternal care but not partner preference (M.M.L. and L.J.Y., unpublished data). A similar behavioural dissociation between pair bond formation and paternal care has been reported in wild subpopulations of prairie voles: Illinois and Kansas prairie voles both form partner preferences, but only Illinois voles display high levels of paternal care¹⁷. Thus, the same V1aR gene that regulates the suite of monogamous-typical behaviours can have different effects on behaviours, depending on the specific neural circuits that are engaged.

What proximate mechanisms might underlie pair bond for-

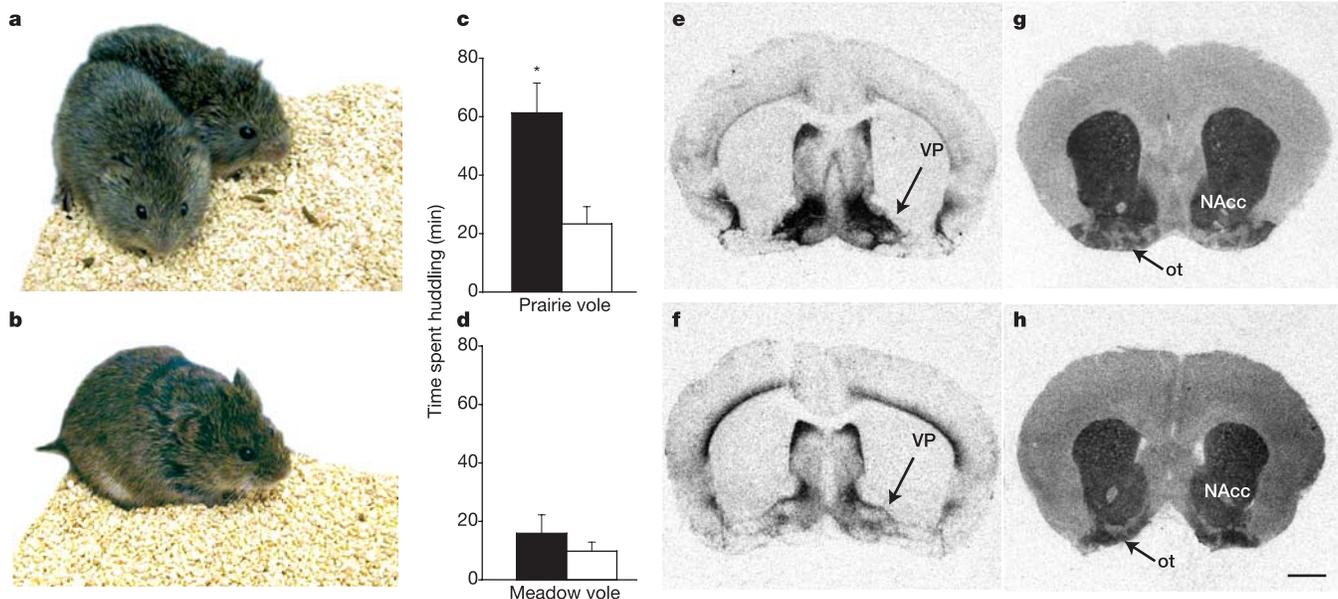


Figure 1 Comparison of brain neurochemistry and behaviour in prairie and meadow voles. **a, b**, Although prairie voles and meadow voles are similar in physical appearance, prairie voles are highly affiliative as depicted here in 'huddling' side by side (**a**), whereas meadow voles are solitary (**b**). **c, d**, Partner preference test. After mating and cohabitating with a female, a male prairie vole tended to spend significantly more time in contact with the partner (filled columns) than the stranger (open columns) ($P < 0.05$, Student's *t*-test) (**c**), whereas meadow voles do not form partner preferences and spent relatively little time

huddling with either female (**d**). Error bars, standard error. **e, f**, Autoradiograms of the ventral forebrain illustrating the typical prairie vole (**e**) and meadow vole (**f**) expression pattern of V1aR as shown by V1aR autoradiography. **g, h**, Despite considerable species differences in V1aR pattern, D2 receptor distribution was broadly similar in prairie voles (**g**) and meadow voles (**h**), as shown by D2 receptor binding in the nucleus accumbens (NAcc) and the olfactory tubercle (ot). Scale bar, 1 mm.

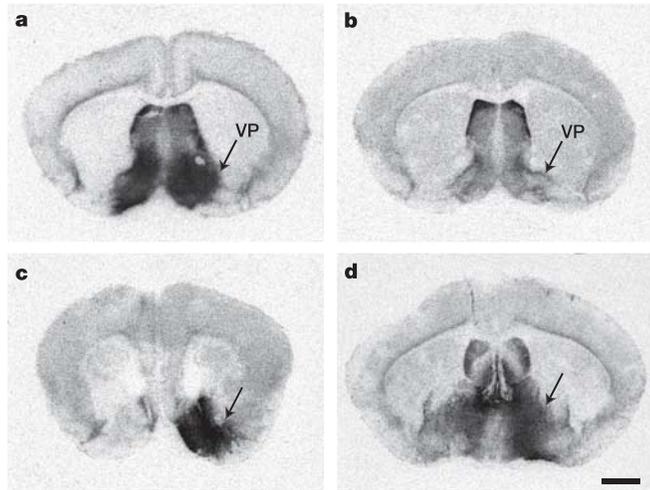


Figure 2 V1aR autoradiography at the level of the ventral pallidum. **a**, Meadow vole overexpressing the *V1aR* gene in the ventral pallidum by AAV-mediated gene transfer (V1aR-vp). **b**, Meadow vole infused with the AAV control vector expressing the *lacZ* gene into the ventral pallidum (Ctrl-vp). **c**, A stereotaxic injection inadvertently placed too rostral to the ventral pallidum, in this case located just ventral to the nucleus accumbens. **d**, A stereotaxic injection placed too caudal to the ventral pallidum, in this case just ventral to the fornix. Arrows depict ectopic AAV-mediated *V1aR* expression in **c** and **d**. Animals with AAV vector placement outside the ventral pallidum were placed in a second control group (Ctrl-other). Scale bar, 1 mm.

mation? V1aRs are also crucially involved in social memory formation. V1aR knockout mice display deficits in individual discrimination, whereas overexpression of *V1aR* in the rat brain by viral vector gene transfer increases the duration of social memory^{18,19}. We propose that, during pair bond formation, the concurrent activation of individual recognition and reward pathways results in convergent V1aR and D2 receptor activation in the ventral forebrain, leading to an association between the rewarding nature of sex and the olfactory signature of the partner, and thus the development of a conditioned partner preference. Similarly, the lack of V1aR in the ventral forebrain in promiscuous species might

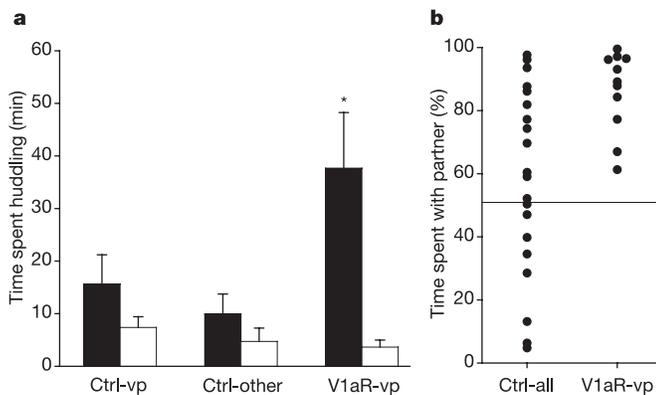


Figure 3 Partner preference test. **a**, V1aR-vp meadow voles spent significantly more time huddling with the partner (filled column) than the stranger (open column), whereas control animals (Ctrl-vp) and stereotaxic misses (Ctrl-other) did not ($P < 0.01$, Student's *t*-test). Error bars, standard error. **b**, A plot of the percentage of time spent with the partner for each subject indicates a shift from randomly distributed preferences in the control groups to 100% of animals preferring the partner in the V1aR-vp group ($P < 0.001$, χ^2 analysis). The y axis was calculated as the time spent huddling with the partner divided by the total time spent huddling with the partner and stranger, multiplied by 100.

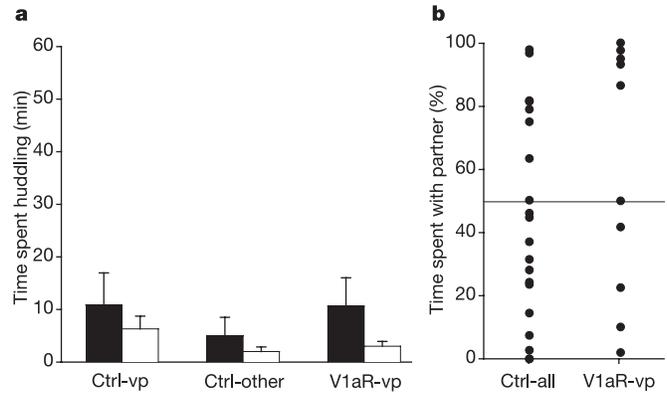


Figure 4 Partner preference test after eticlopride pretreatment. **a**, Eticlopride blocked partner preference in the V1aR-vp group. V1aR-vp, Ctrl-vp and Ctrl-other animals did not spend significantly more time with either the partner (filled columns) or the stranger (open columns) ($P > 0.05$, Student's *t*-test). Error bars, standard error. **b**, A plot of the percentage of time spent with the partner for each subject shows a similar distribution between control and V1aR-vp groups, with no significant difference from that expected by random chance ($P > 0.05$, χ^2 analysis).

lead to a lack of this selective association of reward with the partner's olfactory signature. In prairie voles, D1 receptors are upregulated after mating; the administration of a D1 receptor agonist inhibits pair bond formation, providing a potential mechanism for preventing the formation of subsequent pair bonds with novel individuals²⁰.

Although D2 receptor activation is required for pair bond formation in prairie voles, we did not observe any obvious species differences in D2 receptor patterns in the ventral forebrain (Fig. 1g, h). Thus, monogamous social organization might be the result of the insertion of the V1aR system into this ancient pre-existing reward circuit. Because our manipulation was performed on adult animals, this suggests that the pre-existing genetic complement and neural circuits underlying behaviour are fundamentally similar among vole species, and thus a simple alteration in the expression of a single gene can have a profound impact on behaviour.

The hypothesis that the vast majority of organismal complexity arises from differences in gene regulation, rather than from mutations in the coding regions themselves, has received much attention recently²¹. In prairie and pine voles, the 5' regulatory region of *V1aR* contains a microsatellite region that is virtually absent from montane and meadow voles⁹. This species-specific microsatellite polymorphism modulates gene expression in a cell-type-dependent manner²². Microsatellite DNA can rapidly contract and expand in length, providing a molecular mechanism for the generation of individual variability in brain V1aR patterns within the prairie vole species and between vole species^{23,24}. *V1aR* gene expression is exceptional in its phylogenetic plasticity in that no two species studied have identical V1aR distributions^{23,25}. Thus, the exceptional plasticity of V1aR patterns could result in an altered behavioural response to vasopressin, creating diversity in complex social behaviours and enabling adaptation to changing socioecological factors. It is noteworthy that similar genetic polymorphisms in the human *V1aR* promoter region exist, and one such allelic variant has been linked to autism²⁶, raising the possibility that human *V1aR* polymorphisms might also contribute to human variability in social behaviour.

We have shown that changes in the regional expression of a single gene can have a profound effect on the social behaviour of individuals within a species. Given a population of voles with variability in V1aR patterns and selection pressure for pair bond formation, one can see how monogamous social organization could

have evolved rapidly in voles. However, in a larger context, a single gene does not act alone in the control of complex social behaviour; it must ultimately be placed within pre-existing biological pathways that then interact with socioecological factors, developmental pathways and stochastic events in the lives of organisms. □

Methods

Animals and treatment

Meadow and prairie voles were laboratory-reared animals derived from field-caught specimens. Subjects were 2–6-month-old sexually naive male voles, 40–70 g, housed with one or two same-sex littermates in a 14:10 light:dark cycle and were provided with rabbit chow and water *ad libitum*. Experiments were performed at Emory University in compliance with the rules and oversight of the Emory Institutional Use and Care of Animals Committee.

The V1aR-vp group of male meadow voles (*n* = 16) received bilateral infusions into the ventral pallidum of the adeno-associated viral (AAV) vector containing the prairie vole V1aR gene and under the control of a neuron-specific enolase promoter as described previously²⁷. The Ctrl-vp group (control; *n* = 15) received bilateral infusions into the ventral forebrain of the control AAV vector expressing the lacZ gene under a cytomegalovirus promoter. The prairie vole V1aR and *Escherichia coli lacZ* recombinant viral vectors were cloned and packaged as described previously²⁷. AAV infusions were performed under isoflurane anaesthesia in a Kopf stereotax fitted with an Ultra Micro Pump II (World Precision Instruments) and 26-gauge Hamilton syringe. Stereotaxic coordinates were determined beforehand with dye injections, and varied depending on the weight of the animal (rostral, 1.5 mm; bilateral, 0.9 mm; ventral, 5.8 mm relative to Bregma). Once the syringe had been lowered to the injection site, the AAV vector was infused at a rate of 3–5 nl s⁻¹ for a total of 1 µl per side, at a viral titre of 10⁸ infectious units µl⁻¹, as described previously²⁷. Animals woke from the anaesthesia within 5 min of surgery and were allowed to recover for 2 weeks before behavioural testing.

In advance of cohabitation before the third partner preference test, all animals were pretreated with an intraperitoneal injection of 50 mg kg⁻¹ eticlopride, a selective D2-receptor antagonist, dissolved in 0.1 ml lactated Ringer's solution (Sigma). All animals were observed to mate during the 24-h cohabitation.

The animals used in Fig. 1 were sexually naive male prairie voles (*n* = 19) and sexually naive male meadow voles (*n* = 10) who were cohabitated with a sexually receptive conspecific female for 24 h and then immediately tested for partner preference.

Behavioural testing

Partner preference tests were performed immediately after a 24-h cohabitation with a behaviourally receptive conspecific female. Behaviour during the first hour of cohabitation was recorded for the number of mating bouts; V1aR-vp and Ctrl-vp animals did not differ significantly in the total number of mating bouts in any of the cohabitation periods. During the partner preference test, the experimental male was placed in the centre, neutral chamber of a three-chambered apparatus in which the partner female was tethered in one chamber and a novel stranger female was tethered in the second. The experimental animal was free to move throughout the chambers through Plexiglas (Perspex) connecting tubes. The time spent in each cage and the time spent huddling with each female were recorded by an experimenter blind to the treatment groups.

To show that the animals could be tested multiple times in the partner preference test, we separated the animals for 2 weeks, then paired them with the same receptive female for 24 h, and subsequently tested them again in the partner preference test. The results from the first partner preference test were replicated; thus, there was no significant effect of retesting the animals. In total, three sets of partner preference tests were conducted. All sets of partner preference tests were conducted 2 weeks apart. The third partner preference test consisted of pretreatment with eticlopride before the 24 h cohabitation with a receptive female.

For the paternal care test, because of limitations in the availability of meadow vole pups within the proper age window, only six subjects in each experimental group were chosen at random and tested. In brief, two pups between 2 and 5 days of age were placed in the opposite corner of the subject's home cage for 10 min. An experimenter, blind to the treatment group of each animal, recorded behaviours for latency to approach, latency to retrieval, time spent near the pups (within 5 cm), time spent in contact with the pups (licking, grooming, crouching), and time spent self-grooming.

Receptor autoradiography

V1aR expression in the ventral forebrain was revealed by receptor autoradiography with ¹²⁵I-linear-AVP antagonist (PerkinElmer/NEN) as described previously²⁸.

D2 receptor expression in the forebrain was detected by receptor autoradiography with ¹²⁵I-iodospiperone (PerkinElmer/NEN). In brief, brains were removed, snap-frozen on dry ice, and sliced on a cryostat at 20 µm on Superfrost Plus slides (Fisher Scientific). Slides were washed twice for 10 min each in 50 mM Tris-Cl buffer pH 7.4, then incubated for 1 h in 50 pM radiolabelled ligand dissolved in 5.7 mM ascorbic acid and Tris-ions buffer (0.7% NaCl, 0.04% KCl, 0.02% CaCl₂, 0.01% MgCl₂ in Tris buffer, pH 7.4). Slides were then washed four times (5 min each) in Tris-ions buffer followed by a final stirring, 30 min wash, and dipped in distilled water. After being dried with a stream of cool air, slides were apposed to Kodak Biomax MR film for 24 h.

Received 29 January; accepted 2 April 2004; doi:10.1038/nature02539.

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Acknowledgements We thank C. A. Sharer for contributing to the initial viral vector work, L. Miller for assistance with meadow vole breeding, and A. Z. Murphy and E. A. D. Hammock for their critical readings of the manuscript. Funding for this work was provided by grants from the NIH to M.M.L., Z.X.W., L.J.Y. and the Yerkes Center, and by a NSF STC grant to the Center for Behavioral Neuroscience.

Authors' contributions M.M.L. performed the experiments and wrote the manuscript. Z.X.W. provided the animals and behavioural testing equipment for the pilot studies, the D2 autoradiography protocol and scientific input. D.E.O. assisted with the paternal behaviour testing. X.H.R. and E.E.T. generated the viral vectors. L.J.Y. conceived the idea and co-wrote the manuscript.

Competing interests statement The authors declare that they have no competing financial interests.

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