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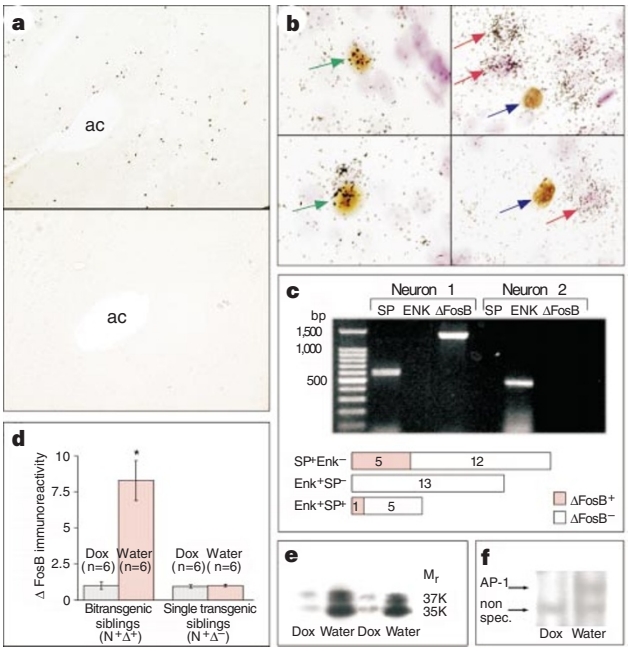

Figure 1 ΔFosB expression in NAc of bitransgenic NSE tTA × TetOp–Δ*fosB* ($N^+\Delta^+$) mice. **a**, Immunohistochemistry showing ΔFosB induction in bitransgenic but not single transgenic NSE-tTA ($N^+\Delta^-$), mice (original magnification 200×). ac, anterior commissure. **b**, Colocalization of ΔFosB with dynorphin, but not enkephalin, in $N^+\Delta^+$ mice. Left: green arrows, ΔFosB-positive, dynorphin-positive NAc neurons. Right: red arrows, enkephalin-positive, ΔFosB-negative NAc neurons; blue arrows, ΔFosB-positive, enkephalin-negative NAC neurons. **c**, Single-cell RT-PCR confirming ΔFosB expression exclusively in substance-P-positive neurons. Top, representative gel. Bottom, number of neurons double-positive for neuropeptide and ΔFosB. **d**, Bar graph and western blot (**e**) showing ΔFosB induction in $N^+\Delta^+$, but not $N^+\Delta^-$, mice switched to water (asterisk: $t = 5.27$, $P < 0.0005$, Student's *t*-test). **f**, Gel shift showing induction of AP-1 binding activity in ΔFosB-expressing mice. non spec., non-specific band. Dox, doxycycline.

Expression of the transcription factor ΔFosB in the brain controls sensitivity to cocaine

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Acute exposure to cocaine transiently induces several Fos family transcription factors in the nucleus accumbens[1], a region of the brain that is important for addiction[2,3]. In contrast, chronic exposure to cocaine does not induce these proteins, but instead causes the persistent expression of highly stable isoforms of ΔFosB[4–6]. ΔFosB is also induced in the nucleus accumbens by repeated exposure to other drugs of abuse, including amphetamine, morphine, nicotine and phencyclidine[7–10]. The sustained accumulation of ΔFosB in the nucleus accumbens indicates that this transcription factor may mediate some of the persistent neural and behavioural plasticity that accompanies chronic drug exposure[1]. Using transgenic mice in which ΔFosB can be induced in adults in the subset of nucleus accumbens neurons in which cocaine induces the protein, we show that ΔFosB expression increases the responsiveness of an animal to the rewarding and locomotor-activating effects of cocaine. These effects of ΔFosB appear to be mediated partly by induction of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) glutamate receptor subunit GluR2 in the nucleus accumbens. These results support a model in which ΔFosB, by altering gene expression, enhances sensitivity to cocaine and may thereby contribute to cocaine addiction.

We used the tetracycline gene-regulation system[11] to develop a mutant mouse that inducibly overexpresses ΔFosB[12]. In the absence of the tetracycline derivative doxycycline, the tetracycline transactivator tTA (encoded by one transgene) binds to the tetracycline responsive promoter (TetOp) and thereby activates transcription of *ΔfosB* (encoded by a second transgene) in bitransgenic animals. We used the neuron-specific enolase (NSE) promoter to restrict tTA (and hence ΔFosB) expression to the nervous system[12]. One line of NSE–tTA mice (line A), when crossed with TetOp–*ΔfosB* mice, showed robust and highly selective expression of ΔFosB throughout the striatum (including the core and shell of the nucleus accumbens (NAc), Fig. 1a), with very low expression elsewhere in brain or peripheral tissues[12]. The targeting of the striatum is presumably due to the insertion site of the NSE–tTA gene in this line. Moreover, only one subset of NAc medium spiny neuron is targeted in these animals. Two independent methods—dual-labelling immunohistochemistry-*in situ* hybridization (Fig. 1b) and single-cell polymerase chain reaction with reverse transcription (RT-PCR; Fig. 1c)—

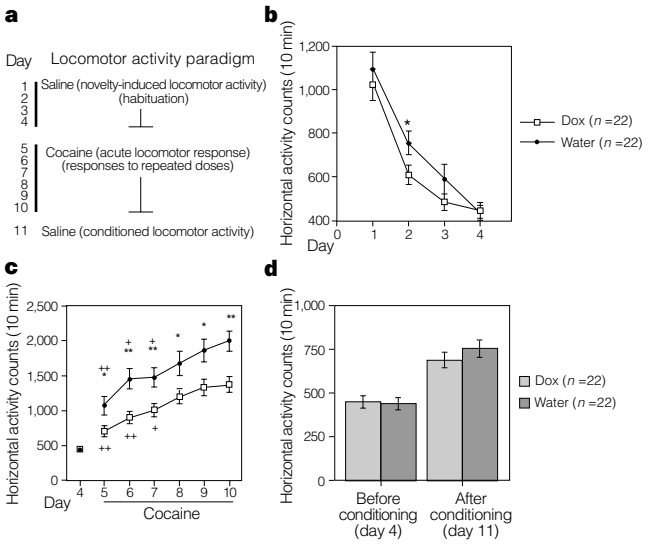

Figure 2 Effect of ΔFosB expression on locomotor activity. **a**, Experimental protocol. **b**, No differences in novelty-induced locomotion or habituation across all four days in ΔFosB-expressing mice, although they showed delayed habituation on day 2 (simple main effect (asterisk): $F_{1,35} = 4.22$, $P < 0.05$). **c**, ΔFosB-expressing mice were more active in response to the initial cocaine injection (10 mg kg^{-1}, i.p.) (day 5; $P < 0.01$), and maintained higher activity with repeated cocaine injections ($F_{1,35} = 11.8$, $P = 0.0015$). (Single and double asterisks denote significant differences between groups of $P < 0.05$ and $P < 0.01$, respectively. Single and double crosses denote significant within-group differences compared to day 10 of $P < 0.05$ and $P < 0.01$, respectively.) Dox and water groups both sensitized with repeated cocaine injections ($F_{5,32} = 31.7$, $P < 0.0001$). **d**, Both groups showed increased locomotor activity in response to a saline injection on day 11 compared to their activity on day 4 (Dox: $F_{1,42} = 18.6$, $P < 0.0001$; Water: $F_{1,28} = 28.9$, $P < 0.0001$), with no influence of ΔFosB on this effect (Day 4: $F_{1,35} = 0.082$, $P = 0.77$; Day 11: $F_{1,35} = 1.68$, $P = 0.20$).

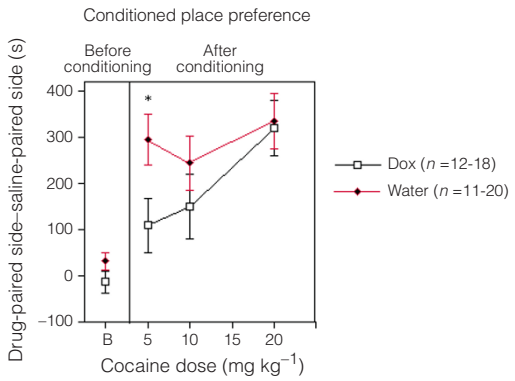

Figure 3 Effect of ΔFosB expression on place conditioning. Place conditioning to cocaine (5, 10 or 20 mg kg^{-1}) is shown. Three-way analysis of variance (water × dose × conditioning) indicated that ΔFosB-expressing mice spent significantly more time in the drug-paired compartment ($F_{1,238} = 4.01$, $P < 0.05$). At the lowest cocaine dose tested, ΔFosB-expressing mice spent ~3-fold more time in the drug-paired compartment (asterisk, $t = 3.71$, $P < 0.01$). Baseline scores (saline side) in dox and water mice were 475 and 462 s, respectively.

showed that ΔFosB expression colocalizes selectively with the neuropeptides dynorphin and substance P (markers of NAc neurons that project directly to the ventral tegmental area), but not with enkephalin (a marker of NAc neurons that project indirectly to the ventral tegmental area via the ventral pallidium). This is important because chronic cocaine administration selectively induces ΔFosB within this former subset of NAc (and striatal) neurons[7,13].

Removal of doxycycline from bitransgenic mice that had been conceived and raised on doxycycline (200 μg ml^{-1}) to prevent possible developmental consequences of transgene expression increased ΔFosB (~8-fold) in the NAc (Fig. 1d, e) and dorsal striatum (not shown). This ΔFosB induction is not an artifact of doxycycline exposure per se, as its removal from single transgenic NSE–tTA mice did not alter ΔFosB expression (Fig. 1d). Expression of the *ΔfosB* transgene is associated with the induction of an AP-1 complex (Fig. 1f), indicating that the ΔFosB is functional. ΔFosB expression did not affect the ability of acute cocaine exposure to induce c-Fos and several Fos-like proteins in the NAc, indicating that certain other indices of transcriptional regulation are normal in the ΔFosB mice (not shown).

We first studied the effects of ΔFosB induction in NAc and dorsal striatum on locomotor activity[5] (Fig. 2a). Once again, to prevent development complications, ΔFosB expression was suppressed by conceiving and raising all mice on doxycycline; subsequently, the transgene was activated in half of the mice by removing doxycycline. There were no obvious deficits in bitransgenic ΔFosB-expressing mice; ΔFosB expressors (water) and their doxycycline suppressed littermates (dox) also had similar weights. Locomotion associated with initial exposure to a novel environment was identical in the two groups. ΔFosB-expressing mice showed delayed habituation (day 2), but the two groups habituated similarly by day 4 (Fig. 2b).

However, ΔFosB expression markedly increased the locomotor responses of mice given cocaine (Fig. 2c). ΔFosB-expressing mice exhibited a ~50% greater increase in locomotor activity in response to initial cocaine exposure (10 mg kg^{-1}, i.p.) than wild-type mice. They also maintained higher levels of activity throughout the course of chronic cocaine administration (Fig. 2c), although ΔFosB did not alter the rate of locomotor sensitization (progressive increases in activity that develop with repeated drug exposure[14–16]). The enhanced responsiveness of ΔFosB-expressing mice to cocaine was not due to differences in cocaine absorption or metabolism, as serum cocaine levels were indistinguishable between ΔFosB-expressing mice and their control littermates (not shown). With repeated cocaine treatment, normal mice learn to associate the injection and the testing environment with cocaine; after this conditioning, an injection of saline causes a modest increase in activity (conditioned locomotion)[5]. Development of conditioned locomotion was normal in the ΔFosB-expressing mice (Fig. 2d).

Next, we assessed the effects of ΔFosB expression on the rewarding properties of cocaine using place conditioning, where animals associate the effects of repeated cocaine exposure with a particular environment[17]. Bitransgenic mice maintained on doxycycline exhibited a dose-dependent development of place conditioning to cocaine (Fig. 3). Bitransgenic littermates removed from doxycycline to induce ΔFosB showed a marked enhancement in sensitivity to the rewarding properties of cocaine, with maximal place conditioning observed at the lowest dose of cocaine used. The responses of single transgenic NSE–tTA mice on water were indistinguishable from those of the bitransgenic mice maintained on doxycycline (not shown), indicating that doxycycline exposure itself does not affect cocaine place conditioning. We also studied the mice in the Morris water maze, which provides a measure of sensory perception and spatial memory. Bitransgenic mice on doxycycline and their littermates on water showed equivalent learning curves and performance in this test, both during and after training (see Supplementary Information).

As a transcription factor, ΔFosB acts by altering gene expression. We considered glutamate receptor subunits as putative targets of ΔFosB for several reasons. First, NAc neurons are quiescent in their basal state and depend upon glutamatergic input for activity[18]; second, the NAc is subsensitive to glutamate after chronic cocaine

treatment[19]; third, glutamate receptor antagonists block the induction of certain behavioural adaptations to repeated drug exposure[14,16]; and finally, the genes for specific glutamate receptor subunits, including GluR2, contain AP-1 sites[20–23]. Expression of ΔFosB in bitransgenic mice significantly increased levels of GluR2 (an AMPA receptor subunit) by more than 50% in the NAc (Fig. 4a). Removal of doxycycline from single transgenic mice did not cause this effect. ΔFosB had no effect on levels of several other glutamate receptor subunits (Fig. 4b). Moreover, GluR2 induction was not observed in dorsal striatum (93 ± 19% of dox animals, $n = 6$) or other brain regions (not shown). Induction of GluR2 in NAc of ΔFosB-expressing mice could represent a direct effect of ΔFosB on the GluR2 gene, as a portion of the GluR2 gene promoter that contains a consensus AP-1 site[23] binds ΔFosB (Fig. 4c).

Unlike other AMPA receptor subunits, GluR2 undergoes RNA editing, which alters a genetically coded glutamine into an arginine located in the pore region of the molecule[24,25]. The Q → R change makes AMPA receptors that contain the GluR2 subunit virtually impermeable to calcium ions and decreases their overall current. Therefore, a selective increase in GluR2 in the NAc could have important functional consequences. To determine whether the ΔFosB-induced increase in GluR2 levels could account for the enhanced behavioural sensitivity to cocaine in the ΔFosB-expressing mice, we microinjected a recombinant herpes simplex virus vector encoding GluR2 (HSV–*gluR2*) directly into the NAc of rats and tested cocaine's rewarding properties using place conditioning. For comparison, we injected vehicle or viral vectors encoding GluR1 (HSV–*gluR1*), LacZ (HSV–*lacZ*, which encodes β-galactosidase, a control protein) or the unedited version of GluR2 (HSV–*gluR2(Q)*); GluR2Q retains permeability to calcium ions and consequently resembles GluR1 electrophysiologically. With bilateral injections of vehicle or HSV–*lacZ* into the NAc, a threshold dose of cocaine (1.25 mg kg^{-1}) failed to establish a place preference (Fig. 5a). In contrast, HSV–*gluR2*-mediated overexpression of GluR2 in the NAc markedly enhanced the rewarding effects of this low dose of cocaine. Overexpression of GluR1 had the opposite effect: rats injected with HSV–*gluR1* spent significantly less time in the drug-paired environment (that is, cocaine was aversive). Overexpression of GluR2(Q) also tended to make cocaine aversive. These HSV vectors mediate transgene overexpression in a moderate number of neurons in a highly localized area of about 1 mm in diameter around the injection site[17,26]. This is shown for GluR2 and LacZ in Fig. 5b and c, respectively. Moreover, these HSV treatments are not associated with any detectable toxicity[17,26] (for example, no gliosis, based on cresyl violet staining (Fig. 5d)).

Our data show that selective induction of ΔFosB in dynorphin/substance-P-containing neurons of the NAc and dorsal striatum in the adult is sufficient to increase an animal's responsiveness to the rewarding and locomotor-activating effects of cocaine. This finding is consistent with the observation that cocaine is also more reinforcing in 5HT$_{1B}$-receptor knockout mice, which show a compensatory increase in ΔFosB in the NAc[27]. Induction of ΔFosB by chronic cocaine exposure could, therefore, be a mechanism for relatively long-lived increases in sensitivity to the stimulant and rewarding effects of the drug. Such increases in sensitivity, which accompany repeated drug exposure, may contribute to human cocaine addiction[14–16]. FosB knockout mice are also more sensitive to some of the initial behavioural effects of cocaine (which may seem paradoxical given the results of this study), although the knockouts fail to sensitize to repeated cocaine exposures[5]. However, the interpretability of the knockout is limited in several ways. First, these mice lack not only ΔFosB but also full-length FosB, which is induced acutely by cocaine[4]. Second, as these are constitutive knockout mice,

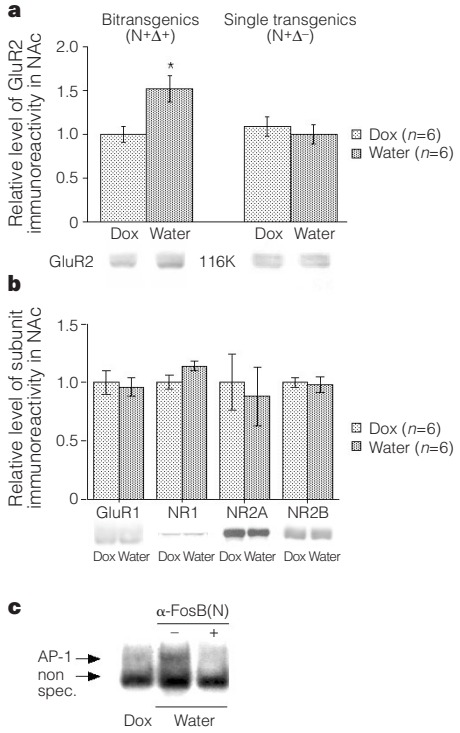

Figure 4 Effect of ΔFosB expression on levels of glutamate receptor subunits and AP-1 binding in NAc. **a**, **b**, Levels of glutamate receptor subunits in NAc, measured by western blot, relative to dox group. **a**, Increased GluR2 levels in bitransgenics (asterisk, $t = 2.82$, $P < 0.05$, Student's t-test), but not single transgenics, upon switching to water (that is, upon ΔFosB induction). **b**, No effect of ΔFosB on other glutamate receptor subunits. NR, NMDA receptor subunit. **c**, Gel shift of NAc extract showing increased binding to a GluR2 gene promoter fragment, which contains an AP-1 site, upon ΔFosB expression, and its disruption by an anti-FosB(N-terminal) antibody (α-FosB(N)).

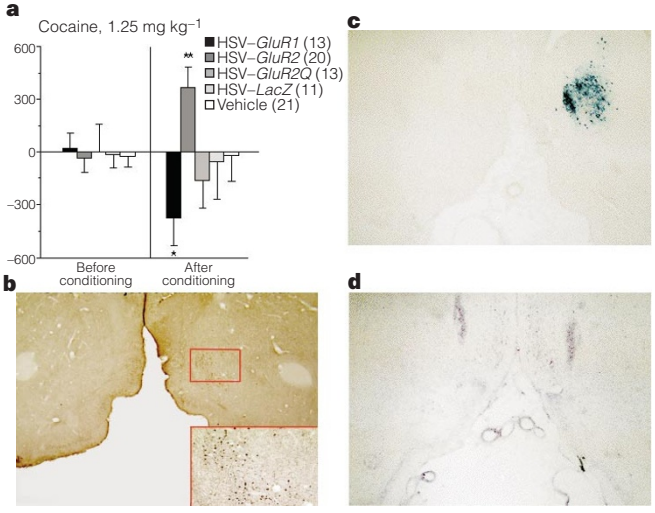

Figure 5 Effect of HSV vectors in NAc on place conditioning to cocaine. **a**, Effect of a threshold cocaine dose (1.25 mg kg^{-1}, i.p.) depended on vector treatment (treatment × sessions interaction: $F_{4,77} = 3.04$, $P < 0.05$). Cocaine had no effect in rats injected with vehicle or HSV–*lacZ*. Rats given HSV–*gluR2* injections spent more time in the cocaine-paired chamber, whereas rats given HSV–*gluR1* spent less time in the cocaine-paired chamber. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; Fisher's t-test. **b**, Immunohistochemistry showing GluR2 induction three days after unilateral HSV–*gluR2* injection into NAc (original magnification 25×, inset 200×). ac, anterior commissure. **c**, Expression of β-galactosidase three days after unilateral HSV–*lacZ* injection into NAc (25×). **d**, Adjacent cresyl-violet-stained section from **c** showing lack of gliosis after viral treatment (25×).

the loss of ΔFosB and FosB occurs from the earliest stages of development and in all tissues. Third, the knockouts show enhanced sensitivity to initial cocaine exposure; this is difficult to relate to ΔFosB, which is induced only in response to chronic cocaine exposure. These results underscore the caution that must be used in interpreting data from constitutive mutant mice and highlight the advantages of inducible, tissue-specific mutants like those used here.

We also show, by viral-mediated gene transfer, that increased GluR2 expression in the NAc can account for the enhanced sensitivity to cocaine's rewarding effects seen in the ΔFosB-expressing mice. While GluR2 is probably just one of many targets regulated by ΔFosB in the NAc, our results support a model in which chronic cocaine exposure results in the gradual accumulation of ΔFosB in this brain region, which then causes increased expression of GluR2. An important goal for future research is to understand how increased GluR2 expression in the NAc enhances reward mechanisms. The increase in GluR2 could account for the reduced sensitivity of NAc neurons to AMPA seen after chronic cocaine exposure, as well as for reductions in Ca^{2+} flux seen in these neurons[19]. Moreover, inhibition of NAc neurons has been related directly to drug reward[28]. Together, the results indicate that ΔFosB may mediate relatively long-lived changes in gene expression that increase sensitivity to cocaine and thereby contribute to the development of cocaine addiction. □

Methods

Mice

Male mice derived from NSE–tTA line A and TetOp–*ΔfoxB* line 11 (ref. 12) were maintained on an outbred background (50% ICR, 50% 57B16 × SJL). Unless otherwise indicated, all mice were conceived and raised on 200 μg ml^{-1} doxycycline (Sigma) to suppress ΔFosB expression during development. Littermates were divided at weaning: half remained on doxycycline and half were switched to water, and the animals were used 11 weeks later. All data are expressed as mean ± s.e.m.

Locomotor activity

Locomotor activity (Fig. 2a) was studied as described[5] except that mice were habituated for 4 days before receiving cocaine. As before, activity was measured for 10 min to avoid stereotypy[5].

Place conditioning

An unbiased conditioning protocol[5] was used, with the following modifications. On day 1, mice explored the three chambers freely for 20 min; they did not show a preference for any chamber before conditioning. During the next three days mice were confined to one large chamber for 20 min, where they received saline (1 ml kg^{-1}, i.p.). Four hours later, they were confined to the other side for 20 min, where they received cocaine. On day 5, mice were placed in the central chamber and allowed to move freely in all three chambers for 20 min. The time they spent in the cocaine-paired chamber minus the time in the saline-paired chamber provided a measure of place conditioning. Place conditioning of rats was as described[17,26].

HSV viral surgery

Complementary DNAs for *gluR1*, *gluR2*, *GluR2(Q)* and *lacZ* were inserted into the HSV amplicon HSVPrpUC and packaged into virus with the helper 5*dl*1.2 (ref. 17). Transgene expression was regulated by the constitutive promoter for the HSV immediate-early gene *IE4/5*. HSV vectors were injected into the medial NAc as described[17].

Western blotting and gel shifts

NAc and dorsal striatum were excised from 1-mm-thick coronal slices of mouse brain using 15- and 14-gauge syringe needles, respectively. Western blotting was performed as described[5] with the following primary antibodies, the specificity of which has been established previously: rabbit anti-Fra (Fos-related antigen) (1:4000), rabbit anti-GluR1 (1:1000, Chemicon AB 1504), mouse anti-GluR2/4 (1:3333, Pharmingen 60011A), mouse anti-NR1 (1:2000, Pharmingen 60021A), rabbit anti-NR2A (1:1000, Chemicon AB 1555P) and rabbit anti-NR2B (1:1000, Chemicon AB 1557P). Equal loading and transfer of proteins was confirmed by the following: (1) all blots were stained with amido black; (2) levels of actin (92 ± 21% of dox animals, $n = 6$) and of neurofilament-160 (99 ± 14%, $n = 6$) were not altered by ΔFosB expression; and (3) blots used to measure GluR2 (which was increased by ΔFosB) were also used to measure levels of NMDA receptor subunits (which were not affected by ΔFosB). Gel shifts were performed as described[4] with 20 μg of total protein and a ^{32}P-labelled AP-1 oligonucleotide. Gel shifts shown in Fig. 4c were performed in an identical manner, except that a 19mer (GACCCAGTGACTAAGGCAA) containing an AP-1 site (underlined) located ~1.2-kb 5′ of the transcription initiation region in the GluR2 gene was used[23]. The specificity of the AP-1 bands seen with both probes was established by cold competition and supershift studies[4–6].

Histology

Immunohistochemical analysis of ΔFosB and GluR2 was done as described[5,26]. Coronal sections (40 μm) were labelled with a rabbit polyclonal anti-FosB(amino-terminal) antibody (1:5000, Pharmigen 6011A), and then processed with standard techniques. β-Galactosidase and cresyl violet staining of HSV-injected sections was done as described[17,26].

Dual-label immunohistochemistry-*in situ* hybridization

ΔFosB expression was localized to a subset of NAc neuron using a dual-labelling procedure. 20-μm free-floating coronal sections were stained for the ΔFosB transgene (as described above). After mounting on slides, sections were prepared for *in situ* hybridization[5] using ^{35}S-labelled riboprobes for enkephalin (a 476-bp probe corresponding to nucleotides 314–790 (ref. 29)) or dynorphin (a 1714-bp probe corresponding to nucleotides 272–1986) (ref. 30). Following film autoradiography, tissue sections were dipped in emulsion, exposed for 2–5 weeks and counterstained with cresyl violet.

Single-cell RT-PCR

Striatal (including NAc) neurons from ~6-week-old mice were acutely dissociated, and single-cell RT-PCR was performed[29] to detect neuropeptide or ΔFosB transgene messenger RNA. The primer sequences used in these reactions have been published elsewhere[12,29].

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Function of Rieger syndrome gene in left–right asymmetry and craniofacial development

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Rieger syndrome, an autosomal dominant disorder, includes ocular, craniofacial and umbilical abnormalities. The *pitx2* homeobox gene, which is mutated in Rieger syndrome[1,2], has been proposed to be the effector molecule interpreting left–right axial information from the early embryonic trunk to each organ[3–7]. Here we have used gene targeting in mice to generate a loss-of-function allele that would be predicted to result in organ randomization or isomerization. Although *pitx2*$^{-/-}$ embryos had abnormal cardiac morphogenesis, mutant hearts looped in the normal direction. *Pitx2*$^{-/-}$ embryos had correctly oriented, but arrested, embryonic rotation and right pulmonary isomerism. They also had defective development of the mandibular and maxillary facial prominences, regression of the stomodeum and arrested tooth development. *Fgf8* expression was absent, and *Bmp4* expression was expanded in the branchial-arch ectoderm. These data reveal a critical role for *pitx2* in left–right asymmetry but indicate that *pitx2* may function at an intermediate step in cardiac morphogenesis and embryonic rotation.

Although Rieger syndrome results from haploinsufficiency[8], the *pitx2*^{hd} allele, which deleted exon 5 and part of exon 6, including the homeobox (Fig. 1a–c), did not result in an obvious haploinsufficient phenotype. Analysis of 239 embryos from F1 intercrosses revealed a loss of *pitx2*^{hd} mutants at embryonic day (E) 14.5, probably from haemodynamic compromise (see below). To investigate left–right axis determination, we focused on cardiac looping, embryonic rotation and individual organ *situs*[9,10].

At E8.5, *pitx2*$^{hd-/-}$ hearts had looped rightwards and expression of

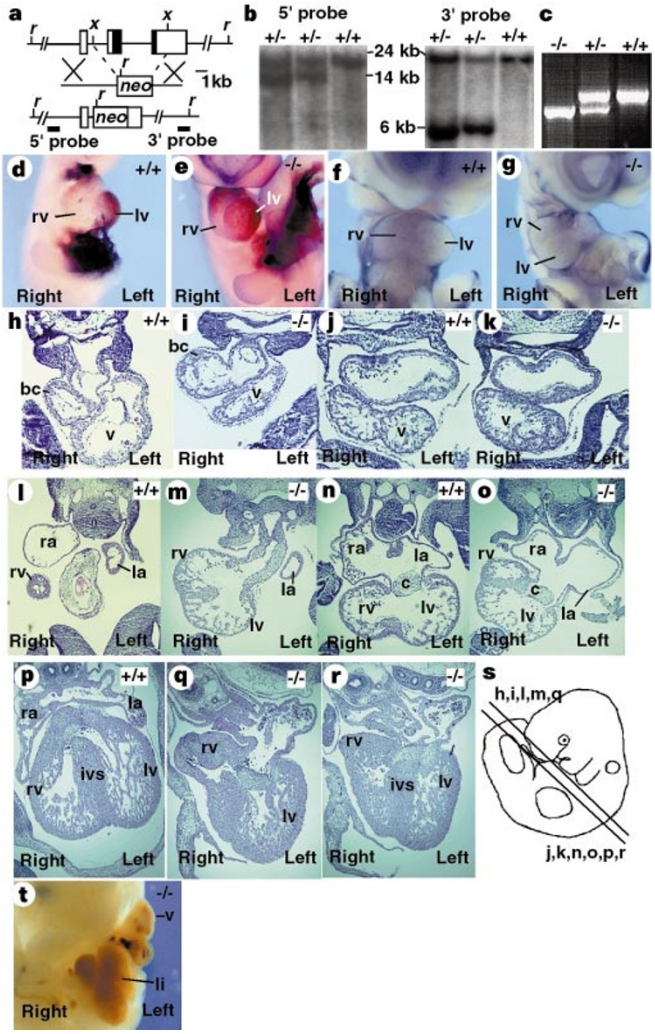

Figure 1 Targeting strategy and cardiac phenotype. **a**, Partial *pitx2* structure. Homeobox, black. Targeting vector (middle), *pitx2*^{hd} (bottom). r, *Eco*RV; x, *Xho*1. **b**, Southern blots. **c**, PCR genotyping. **d**–**g**, *eHAND* (**d**, **e**), *Bmp4* (**f**, **g**) whole-mount *in situ* hybridization. **h**–**r**, Rostral (**h**, **i**, **l**, **m**, **q**) and caudal (**j**, **k**, **n**, **o**, **p**, **r**) transverse sections: E9.5 (**h**–**k**), E10.5 (**l**–**o**), E12.5 (**p**–**r**). **s**, Planes of section for h–**r**. **t**, E12.5 embryo. bc, bulbus cordis; c, cushions; ivs, interventricular septum; la, lv left atrium, ventricle; li, liver; ra, right atrium; rv, right ventricle; v, ventricle.

eHAND, a left ventricle marker[11], was maintained (Fig. 1d, e). Although early stages of asymmetric cardiac morphogenesis were spared in *pitx2*$^{hd-/-}$ embryos, at E10.5, mutant ventricles were displaced rightwards (Fig. 1f, g). Sections of mutants showed rightwards deviation of the bulbus cordis at E9.5 (Fig. 1h, i, s) and a normal atrioventricular relationship (Fig. 1j, k, s). At E10.5, ventricular deviation was intensified, with rightward, dorsal distortion of the atrioventricular canal and outflow tract (Fig. 1l–o, s). Mutant ventricles formed on the same dorsal–ventral plane as the outflow tract (Fig. 1l, m). Despite these abnormalities, which may represent a delay in cardiac looping[11], *pitx2*$^{hd-/-}$ embryos formed distinct ventricles (Fig. 1p–s). At E12.5, *pitx2*$^{hd-/-}$ hearts developed ectopically in a ventral, left-sided location with the apex directed cranial and rightwards (Fig. 1t), perhaps secondarily to defective embryonic rotation (see below; Fig. 2f, g). Thus, *pitx2* acts after the ventricular region of the heart tube bends to the right.

Pitx2$^{hd-/-}$ embryos correctly initiated counterclockwise rotation; however, turning was arrested, suspending the mutant lower trunk