the number, order and duration of copulations, and determination of offspring paternity. Further studies of sperm competition involving taxa at different stages of divergence are needed to determine the frequency of conspecific sperm preference and the rapidity with which it arises. *Drosophila* is an ideal genus in which to pursue genetic and anatomic studies of conspecific sperm preference because there are mutant markers in many species, the fate of sperm can be followed easily within a female, and there are tools to produce males lacking sperm or various components of the seminal fluid[20]. □

Methods

All stocks were reared at 24 °C with a 12-h light–dark cycle. Stock information is available from the author on request. Males and females were collected as virgins under CO_2 anaesthesia and stored in 8-dram food vials. Each female's first mating took place on the fourth day after eclosion, and her second mating (if any) occurred two days later. All males were four days old at the time of mating. Flies were transferred without anaesthesia into a food vial for observation, and 10–60 vials were observed simultaneously. All mating observations began within 1 h of lights coming on in the incubator, and lasted from 45 min to 10 h depending on the ease with which mating occurred. All copulations were observed and timed (unless otherwise noted), and males were removed from the observation vial within 5 min of copulation ending. Females failing to mate on the first day were discarded. Of the females that did mate successfully, a random subset was never given the opportunity to remate. Females that refused to remate were discarded. Females were stored individually in food vials for the two days between the first and second mating. They were transferred to a fresh vial for the second mating, and thereafter transferred to fresh vials every three days until they stopped laying fertile eggs. All offspring from each of these vials were reared to adulthood and scored for paternity. Paternity was determined by the presence of a mutant marker, except in the case of hybrids between *D. simulans* and *D. mauritiana*, which were distinguished by the shape of the male genital arch. F_1 females were obtained by crossing *D. simulans ebony* females with *D. mauritiana singed* males. XO males were produced by crossing virgin *D. simulans* females to males from a *D. simulans* attached-X, attached-XY stock. All male offspring from this cross lack only a Y chromosome, and so should produce normal seminal fluids[21]. All matings were performed as described above with the following exceptions. Copulations between *D. simulans* females and *D. mauritiana* males are often abnormally brief, resulting in interruptions of sperm transfer[22]. To control for this, all matings known not to result in sperm transfer were excluded from the study. Insemination was determined directly for single matings and first matings by the presence of larvae in the female's food vial. Insemination could not be assessed directly for second matings, but all matings shorter than those resulting in insemination after a single mating (<8 min) were excluded from the study. Matings of *D. mauritiana* females with *D. simulans* males could not be observed owing to the extreme discriminatory behaviour of the females. Instead, 10 virgin males and 10 virgin females were crowded together on the third day after eclosion, and left for 24 h at 24 °C. Females were transferred under CO_2 anaesthesia to individual vials on the morning of the fourth day after eclosion. Insemination was determined by the presence of larvae in the vials. Inseminated females were aged and re-mated to *D. mauritiana* males as described above.

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Molecular evidence from retroposons that whales form a clade within even-toed ungulates

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The origin of whales and their transition from terrestrial life to a fully aquatic existence has been studied in depth. Palaeontological[1,2], morphological[3] and molecular studies[4–7] suggest that the order Cetacea (whales, dolphins and porpoises) is more closely related to the order Artiodactyla (even-toed ungulates, including cows, camels and pigs) than to other ungulate orders. The traditional view that the order Artiodactyla is monophyletic has been challenged by molecular analyses of variations in mitochondrial and nuclear DNA[5–7]. We have characterized two families of short interspersed elements (SINEs) that were present exclusively in the genomes of whales, ruminants and hippopotamuses, but not in those of camels and pigs. We made an extensive survey of retropositional events that might have occurred during the divergence of whales and even-toed ungulates. We have characterized nine retropositional events of a SINE unit, each of which provides phylogenetic resolution of the relationships among whales, ruminants,

hippopotamuses and pigs. Our data provide evidence that whales, ruminants and hippopotamuses form a monophyletic group.

We attempted to resolve the issue of whether the order Artiodactyla is monophyletic or paraphyletic by basing our analysis on the presence or absence of SINEs at particular orthologous loci of certain groups of species. SINEs are retroposons that have been amplified and integrated into genomes by retroposition[8–11], that is, by the integration of a reverse-transcribed copy of RNA. As a consequence of the nature of retroposons, SINEs can be found specifically within members of a particular clade[10–13]. It is generally believed that SINEs are not excised precisely and, moreover, that SINEs have not been inserted independently at orthologous loci within different evolutionary lineages. These features mean that SINEs are very useful for the reconstruction of phylogenetic relationships among closely related species[12,13].

We have characterized two new and different families of SINEs, designated the CHR-1 (for Cetacea, hippopotamus and Ruminantia) and CHR-2 family of repeats, from the genomes of several species of whales. The consensus sequences of these two families of SINEs are shown in Fig. 1a. The order Artiodactyla is traditionally divided into three suborders: Ruminantia (chevrotains, deer, cows, sheep), Tylopoda (camels) and Suiformes (pigs, peccaries and hippopotamuses). Dot-hybridization studies showed that these two families of SINEs are distributed extensively in the genomes of Cetacea, Ruminantia and hippopotamus, but were not detected in those of Tylopoda or of Suiformes other than the hippopotamus (Fig. 1b). These results suggest that whales, ruminants and hippopotamuses form a monophyletic group. This possibility prompted us to isolate specific genomic loci at which SINEs had been inserted.

The first approach to this involved random screening, to identify loci that contained a CHR-1 or CHR-2 SINE unit, followed by cloning and sequencing. Polymerase chain reactions (PCRs) were performed with genomic DNA from various cetacean and artiodactyl species to determine whether or not the locus might be informative from a phylogenetic perspective. Second, we performed a comprehensive survey of the protein-coding genes, in standard databases, in which an intron contained one unit of CHR-1 or CHR-2. When the length of the intron was short enough for generation of a PCR product from the entire intron, we designed one set of primers by reference to the sequences of exons. We used these two approaches to characterize seven different loci with a CHR-1 or CHR-2 SINE unit, as described below.

Our analysis indicates that a CHR-2 SINE had been integrated at the locus Pm52 in a common ancestor of cetaceans (Fig. 2A). The patterns of PCR products are shown in Fig. 2A, a. We performed hybridization experiments with the SINE sequence to confirm that the SINE unit had been integrated in a common ancestor of all cetaceans (Fig. 2A, b), and with the flanking sequence to confirm that the orthologous locus of each species had been amplified accurately (Fig. 2A, c). The presence of the SINE unit in longer fragments (about 620 base pairs in length) in cetaceans (lanes 1–7) and the absence of the SINE unit in shorter fragments (about 230 base pairs (bp) in length) in artiodactyls (lanes 8–15) were confirmed by sequencing. The small fluctuations in fragment length were due to insertions and deletions of several nucleotides (data not shown). The Pm72 locus yielded similar results (Fig. 2B). The presence of these two loci indicates that the order Cetacea forms a monophyletic group.

The locus pgha3, at which a CHR-1 SINE was integrated in intron C of the gene for the α-subunit of a pituitary glycoprotein hormone (Fig. 2C), and locus c21-352, at which a CHR-1 SINE was integrated in intron C of the gene for steroid 21-hydroxylase (Fig. 2D), demonstrate the monophyly of ruminants.

Locus Gm5 was isolated by random screening by using CHR-1 SINE as probe. The SINE unit seems to have been integrated in a common ancestor of cetaceans, ruminants and hippopotamuses, suggesting that these three evolutionary lineages are monophyletic (Fig. 2E). The sequences of the fragments from the short-finned pilot whale, cow, hippopotamus and Bactrian camel confirmed the presence of the SINE unit in the longer fragment, and its absence in the shorter fragment, respectively. In lane 15 for the pig, a longer band was detected, but sequencing showed that this was due to insertion of another SINE unit, PRE-1 (ref. 14) in another site of this locus (data not shown).

The loci aaa228 and aaa792 are both derived from the gene for the α-subunit of the F(0)F(1) ATP synthase (designated the *atpA1* gene) in the bovine genome. At locus 228, a CHR-1 SINE is present in the intron between exons 2 and 3, suggesting monophyly of cetaceans, ruminants and hippopotamuses (Fig. 2F, a). Hybridization experiments using two different kinds of probe (Fig. 2F, b and c) confirmed this conclusion.

Locus aaa792, between exons 10 and 11, is more complex. Three different families of SINEs (CHR-1, CHR-3 and Bov-tA[15]) became associated independently with this locus during the evolution of

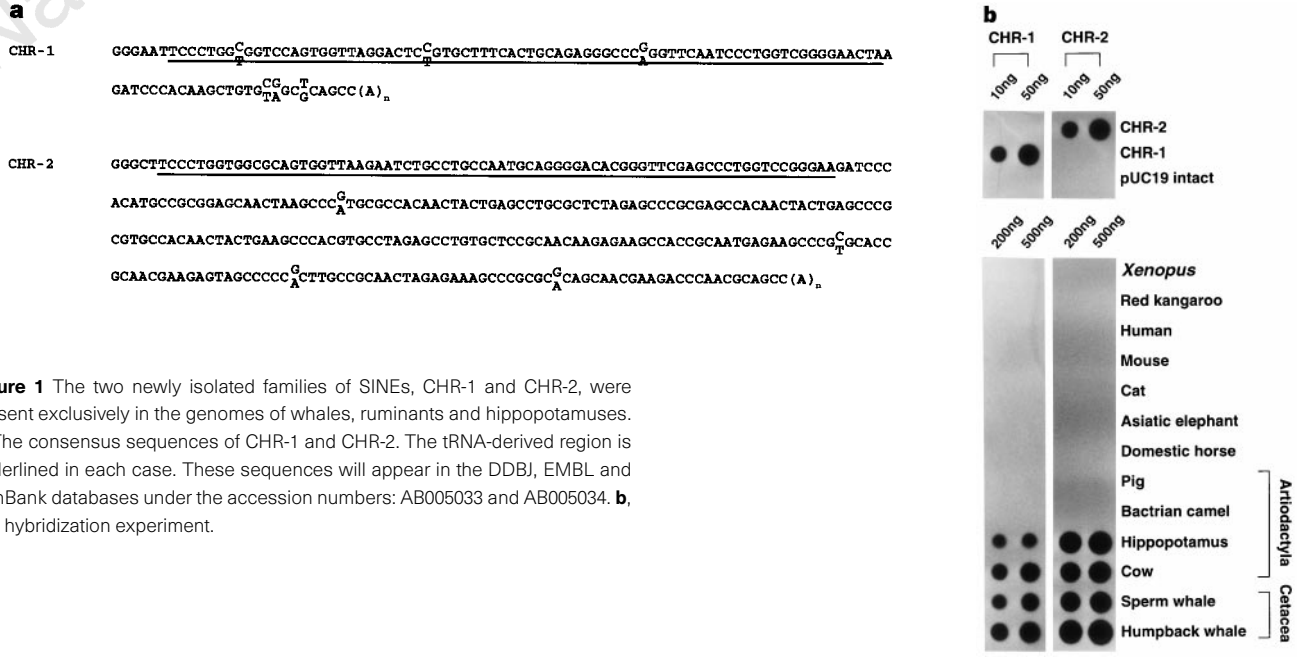

Figure 1 The two newly isolated families of SINEs, CHR-1 and CHR-2, were present exclusively in the genomes of whales, ruminants and hippopotamuses. **a**, The consensus sequences of CHR-1 and CHR-2. The tRNA-derived region is underlined in each case. These sequences will appear in the DDBJ, EMBL and GenBank databases under the accession numbers: AB005033 and AB005034. **b**, Dot hybridization experiment.

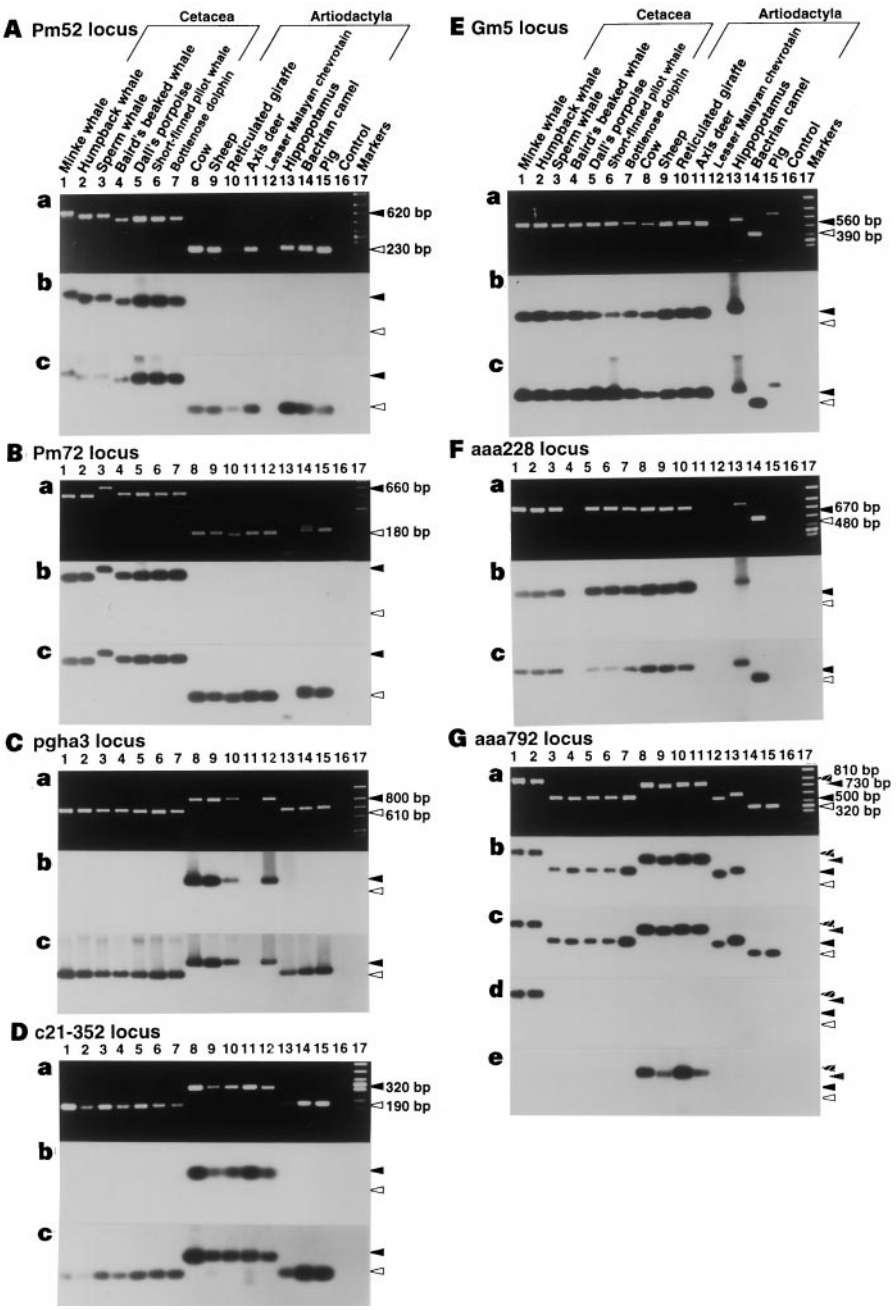

Figure 2 Analysis of the seven loci at which a SINE unit(s) was inserted during the evolution of cetaceans, ruminants and hippopotamuses: **A**, Pm52; **B**, Pm72; **C**, pgha3; **D**, c21-352; **E**, Gm5; **F**, aaa228; **G**, aaa792. **a**, Products of PCR; **b**, **c**, results of hybridization experiments with different kinds of probe, namely, a unit sequence of the SINE (**b**) and the flanking sequence (**c**), respectively. In **G**, **d** and **e** show results of hybridization experiments with two different SINE probes, the CHR-2 SINE and the Bov-tA SINE, respectively.

cetaceans and even-toed ungulates. The first integration event, involving a CHR-1 SINE, occurred in a common ancestor of cetaceans, ruminants and hippopotamuses (Fig. 2G, a–c). The pattern of PCR products is shown in Fig. 2G, a. Hybridization experiments with the CHR-1 sequence (Fig. 2G, b) and the flanking sequence (Fig. 2G, c) as probe, respectively, showed that the SINE unit was integrated at the orthologous loci of the species designated above lanes 1–13, but not at those of the camel (lane 14) or the pig (lane 15). These results confirm the monophyly of cetaceans, ruminants and hippopotamuses, excluding camels and pigs. However, the lengths of fragments generated by PCR (Fig. 2G, a) varied among species (lanes 1–13), but we deduced from the sequences of the main fragments that the other two different kinds of SINE were involved in this locus. Experiments using new probes confirmed that a CHR-2 SINE was integrated in the lineage of the minke and humpback whales (Fig. 2G, d, lanes 1 and 2), and that a Bov-tA SINE was integrated in the lineage of the pecora (cows, sheep, deer and giraffes), indicating that the lineage forms a monophyletic group (Fig. 2G, e, lanes 8–11). The sequences of major fragments are shown in Fig. 3.

All results for the seven loci are congruent (Fig. 4), and provide conclusive evidence for the paraphyly of the order Artiodactyla, which should include the order Cetacea, and for the paraphyly of the suborder Suiformes, from which hippopotamuses should be excluded. Hippopotamuses form a monophyletic group with cetaceans and ruminants.

The inclusion of cetaceans within the order Artiodactyla has been proposed previously[5], as the Ruminantia/Cetacea clade with the Suiformes (pigs and peccaries) as an outgroup. The possibility of clustering the hippopotamus with the Cetacea has also been suggested[6,7], even though hippopotamuses have traditionally been grouped with pigs and peccaries on morphological grounds[16]. However, careful reanalyses of available molecular data[17–19] indicate that the hypothesis of artiodactyl paraphyly was not supported convincingly from a statistical point of view. However, recent analyses of genes for milk casein[7] provided new, convincing support

Figure 3 An alignment of sequences of the aaa792 locus in the cow (BT, *Bos taurus*), minke whale (BA, *Balaenoptera acutorostrata*), hippopotamus (HA, *Hippopotamus amphibius*) and bactrian camel (CB, *Camelus bactrianus*). Boxed sequences indicate direct repeats arising from duplication upon retroinsertion. The underlined sequences show the sequences used for primers. Bars indicate deletions. Nucleotides identical to those in the cow are indicated by dots.

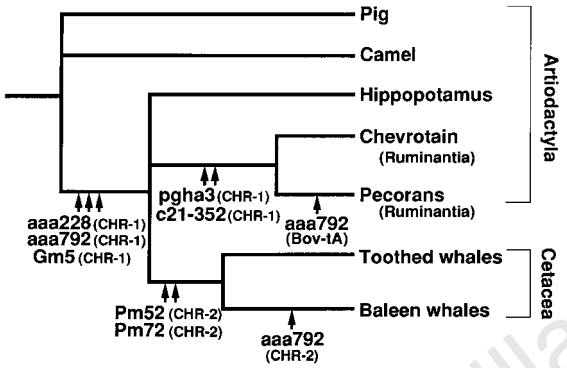

Figure 4 Phylogenetic relationships among cetaceans and artiodactyls, as deduced from the sites of insertion of SINEs. Arrows indicate the timing of insertion of SINEs. Types of SINE are shown in parentheses.

for the ((cetaceans, hippopotamuses), ruminants) tree, supporting a previous hypothesis[5]. Our analysis of SINE retrotranspositions seems to provide unambiguous support for that hypothesis.

The conclusions from our retropositional analysis are inconsistent with earlier morphologically based hypotheses[16,20,21]. Paleontological and morphological data suggest that modern whales originated from the Archaeocetes (primitive aquatic cetaceans), which first appeared in the early Eocene epoch[22]. The Archaeocetes are believed to have originated from mesonychians, which appeared before the Eocene[20]. However, the most primitive artiodactyls (Dichobunids) first appeared in the early Eocene, and the origin of nearly all the families of artiodactyls can only be traced back to the middle or the late Eocene[23,24]. Such a sequence of appearance of these animals is inconsistent with our molecular data. However, a recent calibration of molecular clocks suggests that divergences among orders of eutherian mammals can be traced back more than 100 Myr. Hence, diversification of avian and mammalian orders might not have been an adaptive radiation after the Cretaceous/Tertiary extinction event (65 Myr ago), but might have been correlated with the fragmentation of emergent land areas during the Cretaceous[25]. We believe that recent molecular data will lead to the reinterpretation by palaeontologists of many fossil records of Artiodactyla to match our conclusions. Extensive morphological reversals and convergences, as well as large gaps in the fossil record, will then have to be acknowledged. □

Methods

Polymerase chain reaction. PCR was performed in a 50-μl reaction mixture containing 0.2 mM dNTP, 200 ng of primer, Tth buffer (final Mg^{2+} concentration, 1.5 mM) and 1 unit of Tth DNA polymerase (Toyobo, Osaka). Annealing temperature was chosen from 49 °C to 58 °C. A portion of the PCR products was analysed by electroporesis in an agarose gel containing 2% (w/v) Nusieve GTG and 1% (w/v) Seakem GTG (FMC BioProducts, Rockland, ME). Hybridization and washing were performed as described[13].

Sequences of primers for PCR. Pm52 locus, 5′ primer, 5′-TCCTGATTCC (C/T)CTGAACAAA-3′, and 3′ primer, 5′-GGG(G/A)AAGACT(C/T)CCA(G/A)(C/T)TTTGAAAT-3′; Pm72 locus, 5′ primer, 5′-TTTAAAGCATGGCAGTTGGATTT(G/A)T-3′, and 3′ primer, 5′-GGATCTGTTTTTACTTTGACC-3′; pgha3 locus, 5′ primer, 5′-TCGGTGTGGTTCTC(G/C)AC(C/T)CT-3′, and 3′ primer, 5′-TGC(C/T)CCAATCTATCA(G/A)TG(C/T)ATG-3′; c21-352 locus, 5′ primer, 5′-GAGAATTCCTTCTG(G/A)AT(G/A)GT(G/C)AC-3′, and 3′ primer, 5′-(G/A)(C/T)CCGCAGCTCCATGGA(G/A)CC-3′; Gm5 locus, 5′ primer, 5′-GTAATGTGATTTGGCTTAGTGC-3′, and 3′ primer, 5′-TCAGCTCCTGGTGGCAGTCT-3′; aaa228 locus, 5′ primer, 5′-GCTTGATACCTACCACTATGAA-3′, and 3′ primer, 5′-CCTGG(A/C)(A/T)GTCT(G/C)AATTTGCAC-3′; and aaa792 locus, 5′ primer, 5′-TGTGGA(A/T)(G/T)NTG(G/C)CAGATTT(A/T)AAAG-3′, and 3′ primer, 5′-CAGCCACTTGCTCTTCAATAGC-3′.

Locus. Of the seven loci described, Pm52 and Pm72 were newly isolated by cloning and sequencing from a genomic library of sperm whales (*Physeter macrocephalus*), and Gm5 was isolated from that of short-finned pilot whale (*Globicephala macrorhynchus*). The other four loci were found in bovine genomic sequences in the EMBL database, as follows (accession numbers, locus name, SINE family): (X00004, pgha3, CHR1); (M11267 and M13545, c21-352, CHR-1); (X64565 and S48112, aaa228, CHR-1); (X64565 and S48112; aaa792; CHR-1, CHR-2 and Bov-tA).

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Formation of olfactory memories mediated by nitric oxide

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Sheep learn to recognize the odours of their lambs within two hours of giving birth, and this learning involves synaptic changes within the olfactory bulb[1,2]. Specifically, mitral cells become increasingly responsive to the learned odour, which stimulates release of both glutamate and GABA (γ-aminobutyric acid) neurotransmitters from the reciprocal synapses between the excitatory mitral cells and inhibitory granule cells[1]. Nitric oxide (NO) has been implicated in synaptic plasticity in other regions of the brain as a result of its modulation of cyclic GMP levels[3–7]. Here we investigate the possible role of NO in olfactory learning. We find that the neuronal enzyme nitric oxide synthase (nNOS) is expressed in both mitral and granule cells, whereas the guanylyl cyclase subunits that are required for NO stimulation of cGMP formation[8] are expressed only in mitral cells. Immediately after birth, glutamate levels rise, inducing formation of NO and cGMP, which potentiate glutamate release at the mitral-to-granule cell synapses. Inhibition of nNOS or guanylyl cyclase activity prevents both the potentiation of glutamate release and formation of the olfactory memory. The effects of nNOS inhibition can be reversed by infusion of NO into the olfactory bulb. Once memory has formed, however, inhibition of nNOS or guanylyl cyclase activity cannot impair either its recall or the neurochemical release evoked by the learned lamb odour. Nitric oxide therefore seems to act as a retrograde and/or intracellular messenger, being released from both mitral and granule cells to potentiate glutamate release from mitral cells by modulating cGMP contentrations. We propose that the resulting changes in the functional circuitry of the olfactory bulb underlie the formation of olfactory memories.

NO may mediate synaptic plasticity changes in brain regions such as the hippocampus in order to influence both long-term potentiation (LTP) and memory formation[3–7], but this idea is controversial[9,10]. In rodents, the inhibitory GABAergic granule and periglomerular cells of the primary sensory processing region for airborne odours, the olfactory bulb, contain large amounts of nNOS[11], which converts arginine to citrulline and NO[3,4]. We investigated whether this could also be the case in sheep, and whether the increased sensitivity of mitral-to-granule cell synapses that results from learning the odours of their lambs might involve potentiation of glutamate release by NO through modulation of cGMP.

We cloned and sequenced the ovine nNOS gene and found that it was 88% homologous with human[12], rat[13] and mouse[14] sequences (EMBL accession number, X99042). *In situ* hybridization and immunocytochemistry indicated that mitral as well as periglomerular and granule cells contained nNOS messenger RNA and protein (Fig. 1a). The dendrodendritic connections between the granule and mitral cells in particular contained large amounts of nNOS immunoreactivity. Only mitral cells expressed the mRNAs for the α_a and β_1 subunits of soluble guanylyl cyclase (Fig. 1a), which are both required for NO to cause cGMP formation[8]. The granule and periglomerular cells expressed low levels of only the β_1 subunit. Thus NO probably only stimulates cGMP formation in mitral cells.

In brain, NO is released by glutamate acting on both NMDA (*N*-methyl-D-aspartate) and AMPA ((2-aminomethyl)phenylacetic acid) receptors[3,4,15]. In the mouse accessory olfactory bulb, activation of both receptor types is required for the formation of pheromonal olfactory memory[16] and both are present on the main olfactory bulb mitral and granule cells[17]. We used *in vivo* microdialysis to determine whether glutamate acts on both types of receptors to evoke NO and cGMP release in the olfactory bulb. Local retrodialysis infusions of NMDA or AMPA dose-dependently increased glutamate, GABA, NO and cGMP, although NMDA was more potent. These actions were blocked by specific receptor antagonists (Fig. 1b) and the NOS inhibitor L-nitroarginine (L-NARG). A selective inhibitor of NO-induced guanylyl cyclase, 1H-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ)[18] also prevented stimulation of cGMP, but not NO release (Fig. 1b). Both L-NARG and ODQ significantly reduced agonist-induced increases in glutamate and GABA, showing that agonist effects are partly mediated by NO and cGMP. Infusions of an NO donor, *S*-nitrosoacetylpenicillamine (SNAP), dose-dependently increased cGMP, glutamate and GABA; these effects were blocked by ODQ (Fig. 1b). Thus NO can act as a retrograde or intracellular messenger in the olfactory bulb by stimulating glutamate release through modulation of cGMP.

The importance of NO release in the olfactory bulb for plasticity changes underlying olfactory memory was investigated in *post partum* animals given bilateral local infusions by microdialysis probes of drugs targeting the NO signalling pathway. In control animals receiving either no treatment or an inactive enantiomer of L-NARG (D-NARG), which did not inhibit NOS, it was found that glutamate, GABA, noradrenaline, NO and cGMP all increased during the first 30 min after birth (Figs 2 and 3). Behavioural tests showed that all these animals formed an olfactory memory that allowed selective recognition of lambs (Fig. 4a). However, all animals treated with the ionotropic glutamate receptor antagonist γ-D-glutamylglycine (DGG), and NOS inhibitor L-NARG, or the guanyl cyclase inhibitor ODQ, accepted their own and strange lambs equally, indicating that these agents completely prevented olfactory memory formation (Fig. 4a). The quality of maternal care shown by ewes towards their lambs during the two hours immediately *post partum* was unaffected by the drugs, confirming that