of a pair can breed, one or more members of each larger coalition may best be viewed as non-breeding helpers. Their presence increases the reproductive success of their companions (through augmenting coalition size), but they have little personal reproductive success. Such behaviour could only enhance their genetic representation in subsequent generations if it increased the reproductive success of close relatives[11]. A relatively low proportion of males in trios lack any kinship connections with their companions (Fig. 4*a*). Many trios containing non-relatives consist of a related pair with an unrelated third. The paternity data include one such trio and the non-breeder in this group was closely related to one of the breeders (Fig. 4*b*). His failure to breed was ameliorated by his relative's success.

Within-coalition variance in male reproductive success results from two components. First, males typically father entire litters. A male will attempt to monopolize a female throughout her 2–4-day oestrus by remaining nearby and preventing his coalition partners from mating with her[5,14]. As in other species[25], this behaviour ensures that the consorting male fathers her entire litter: mixed paternity occurred in only one of 24 litters containing two or more cubs[19]. Second, within-coalition variance in the number of litters fathered by each male increases significantly with increasing coalition size ($P = 0.0084$, $n = 44$ litters; probability determined as described in legend to Fig. 4*b*). Breeding within each pride is typically synchronous so that several females are often in oestrus at once[2,26]. But larger coalitions do not preferentially reside in larger prides[8], thus the number of males in a large coalition more often exceeds the number of available oestrous females.

Solitary males spend long periods selecting potential companions[7] and behavioural studies indicate that mating success is more disparate within coalitions where partners are less closely matched in age or vigour[5]. The risk of becoming a non-breeder is greater in a trio, and males that form unrelated trios seem to be more sensitive to potential disparities in mating success than males that form pairs: the age difference between unrelated partners is significantly lower in trios (0.75 years) than in pairs (2.5 years, $P < 0.05$).

Previous explanations for cooperation among male lions did not require kinship to maintain the behaviour. Each male was assumed erroneously to father an equal proportion of offspring in all coalition sizes, and therefore to gain equally as per capita reproductive success increased with coalition size[4,5,8]. But it is now clear that this is only true for small coalitions, kinship is essential for the maintenance of larger coalitions where reproduction is highly skewed.

In the Serengeti population, the DNA band-sharing technique allowed us to measure whether companions, neighbours or mating partners were even distantly related. Although these data suggest that similar analyses could be conducted in populations that had not previously been studied in such detail, the relationship between relatedness and band sharing differed between the Serengeti and the nearby Ngorongoro Crater. Thus the degree of band sharing should be calibrated against an independent measure of kinship[19,27]. □

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Mitochondrial DNA analysis implying extensive hybridization of the endangered red wolf *Canis rufus*

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THE red wolf, previously endemic to the southeastern United States, declined precipitously in numbers after 1900 because of habitat destruction, predator control programmes, and hybridization with coyotes[1,2]. Hybridization with coyotes probably occurred as these animals, which adjust well to agriculture, became numerous and moved eastwards[1–4]. By 1970, red wolves existed only in extreme southeastern Texas and southwestern Louisana (Fig. 1)[2]. In 1967, red wolves were classified as endangered and a captive breeding programme was begun in 1974 after passage of the Endangered Species Act, about a year before they became extinct in the wild. Protein electrophoresis and morphometrics have been used to try to discriminate red wolves from hybrids and coyotes[1,4,5]. But because the average substitution rate of mitochondrial DNA in mammals is much greater than that of nuclear genes[6], mtDNA analysis is a more useful way of distinguishing closely related species. We have now analysed mtDNA restriction-enzyme sites and cytochrome *b* gene sequence variation in captive red wolves and in 77 canids sampled during the capture period. We also used the polymerase chain reaction to amplify and then sequenced mtDNA from red wolf skins collected before substantial hybridization of red wolves with coyotes is thought to have occurred. Phylogenetic analysis indicates that red wolves have either a grey wolf or coyote mtDNA genotype, demonstrating hybridization among these species. Thus, the red wolf is entirely a hybrid form or a distinct taxon that hybridized with coyotes and grey wolves over much of its previous geographical range. Our findings, however, do not argue against the continued protection of the red wolf.

We analysed red wolf mtDNA from three sources: the captive colony, blood samples collected between 1974 and 1976 in Texas and Louisiana, and skins collected between 1905 and 1930 (Fig. 1).

The four matrilines represented in the captive red wolf population displayed a single mtDNA genotype. This genotype was

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TABLE 1 Genotype frequencies

	No. of restriction enzymes used				
	1	2	3	4	Total
Coyote-type	22	28	15	—	65 (84%)
Gray wolf-type (Northern)	—	1	—	4	5 (7%)
Gray wolf-type (Mexican)	4	1	—	2	7 (9%)
Total	26	30	15	6	77

Frequency of coyote-type and grey wolf-type genotypes in Texas and Louisiana canids from 1974 to 1976 (Fig. 1). Entries indicate the number of individuals classified as having a coyote or grey wolf genotype on the basis of restriction fragment patterns for one to four diagnostic restriction enzymes. Methods. DNA preparation and analysis follows our protocol described in refs 7 and 9. DNA samples were surveyed for restriction site variation using the following 4 restriction endonucleases: *Bcl*I, *Bgl*II, *Eco*RV and *Hin*cII. These enzymes produce diagnostic restriction fragment patterns unique to grey wolves (grey-wolf type) or coyotes (coyote-type). *Hin*cII digestion produces a restriction fragment pattern unique to captive red wolves and two wild-caught coyotes from Louisiana[8]. *Bgl*II digestion produces a restriction fragment pattern found only in Mexican grey wolves.

compared with those found in a sample of 327 coyotes and 276 grey wolves from throughout North America[7,8]. To do this we used 21 restriction enzymes which revealed 138 independent restriction sites representing 670 nucleotides or roughly 4.0% of the canid mtDNA genome[7,9]. A phylogenetic tree relating a subset of these genotypes by maximum parsimony suggests that the genotype of the captive red wolf is grouped in a monophyletic clade including only coyote genotypes, and is indistinguishable from coyote genotype 32 found in two recent coyotes from Louisiana (Fig. 2*a*). Thus, the captive red wolves have a mtDNA genotype phylogenetically grouped with those of living coyotes.

Owing to sampling error or stochastic lineage extinction, however, the captive red wolf genotype may not be representative of those in the source population. Thus, we examined mtDNA from blood samples of 77 wild canids captured during the initial selection period (Fig. 1). These canids were classified on morphologic criteria as coyotes (58%), red wolf/coyote hybrids (31%), or red wolves (11%). Because the mtDNA yield in these samples was small we used only one to four diagnostic restriction enzymes[7,8] and found that 84% of the samples had a coyote genotype, 7% had a northern grey wolf genotype, and 9% had a polymorphism found only in Mexican grey wolves (Table 1, Fig. 1). We were able to type 30 of the 77 canids with the enzyme *Hin*cII, and found that 23 (77%) had the restriction-fragment pattern that is diagnostic of captive red wolves (Table 1). Thus, the coyote-like genotype of captive red wolves was common in wild canids from the source population.

Morphologic and mtDNA classifications of the 1974–76 canids often do not correspond. For example, of the 12 canids with a grey wolf mtDNA genotype, one was identified as a red wolf, four as coyotes, and six as red wolf/coyote hybrids, and one as unknown. The high frequency of morphologic hybrids, the poor correspondence between mtDNA and morphologic classifications, and the presence of only coyote and grey wolf genotypes, suggest hybridization occurred between these two species in the source population before 1974. A less advanced hybrid zone between grey wolves and coyotes now exists in the Great Lakes region[7]. Our data also indicate that northern and Mexican grey wolf genotypes persisted in Texas canids a long time after the grey wolf had become extinct in the southeastern United States[1,3].

The apparent hybridization among Texas and Louisiana canids compelled us to examine samples from museum pelts of red wolves collected from 1905 to 1930, before extensive hybridization with coyotes is thought to have occurred (Fig. 1)[1]. We amplified 398 base pairs of the cytochrome *b* gene from six pelts from five southeastern states using universal primers and the polymerase chain reaction (Fig. 1)[10]. Sequences were compared with those obtained by the same method from recent blood samples of five coyotes, three grey wolves, a captive red wolf, a dog and a golden jackal (Table 2). The results confirm the conclusions suggested by restriction-site data[7]. Grey wolves and coyotes are very distinct, differing by 21–28 nucleotides or roughly 5.3–7.0% of their cytochrome *b* sequence. Within each species, no more than seven substitutions separate genotypes. The six historical red wolf sequences are similar or identical to those of the grey wolf or coyote. A phylogenetic analysis of the cytochrome *b* sequence data shows two significant monophyletic clades found in all 1,000 bootstrap replicates of our data[11]: one clade containing grey wolf and three red wolf genotypes and the other containing coyote and two red wolf genotypes (Fig. 2*b*). Thus, red wolf genotypes are classified with those of either the coyote or grey wolf.

Two situations could account for our results. First, the red wolf could have been a distinct species with unique mtDNA genotypes that were missed in our survey or had become extinct through genetic drift. This species then hybridized with both coyotes and grey wolves over much of its geographical range. Second, the red wolf phenotype could have derived entirely from hybridization between coyotes and grey wolves[12]. Red wolves are intermediate in size and may be difficult to distinguish morphologically from the other two species[1,2,4]. The red wolf could have been a southeastern subspecies of the grey wolf that was morphologically but not genetically (by our criteria) distinct from other grey wolves. This subspecies then hybridized with coyotes that were numerically increasing throughout much of the red wolf's geographical range. Arguing against red wolves' constituting a distinct subspecies, are the probable high rates of gene flow from adjacent populations of grey wolves living in similar habitats that would tend to obliterate the distinctive morphology of the red wolf subspecies. In fact, gene flow has apparently stifled genetic differentiation among even very distant

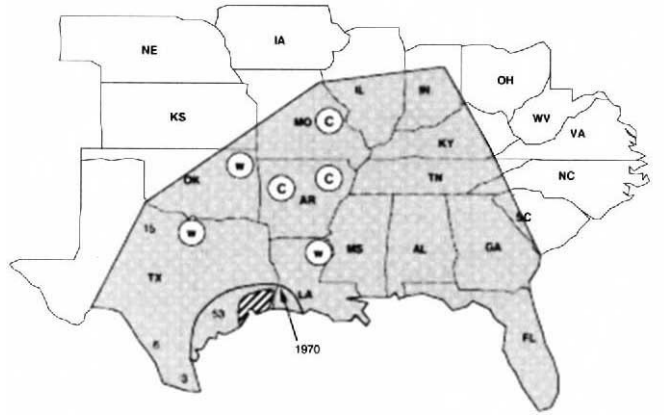

FIG. 1 Geographical range and sampling localities for red wolves. The shaded area indicates the distribution of red wolves *circa* 1700 (ref. 16) and the smaller bounded area in southeastern Texas the geographical range *circa* 1970, near the initiation time of the red wolf recovery programme. Map numbers indicate the location and number of Texas and Louisiana canids from 1974 to 1976 analysed; circled letters indicate historical samples and their genotype (C, coyote; W, grey wolf). The striped area in extreme southeastern Texas includes locations of 12 individuals with northern or Mexican grey wolf genotypes (Table 1). Specimens examined: 1974–76 samples from Texas (Chambers Co. [7], Brazoria Co. [27], Cameron Co. [3], Harris Co. [2], Jefferson Co. [12], Liberty Co. [2], Montague Co. [15], Webb Co. [6]) and Louisiana (Calcasieu Co. [3]), and historical samples from Texas (Parker Co., 1930 (NMNH 249694)), Louisiana, (Talluala, 1905 (NMNH 136106)), Arkansas, (Stillwater, 1921 (NMNH 236542)) and (Onyx, 1922 (NMNH 242564)), Olkahoma, (Red Fork, 1905 (NMNH 135747)), Missouri, (Barren, 1924 (NMNH 244246)). NE, Nebraska; KS, Kansas; OK, Oklahoma; TX, Texas; MO, Missouri; AR, Arkansas; LA, Louisiana; IA, Iowa; IL, Illinois; IN, Indiana; KY, Kentucky; TN, Tennessee; MS, Mississippi; Al, Alabama; OH, Ohio; WV, West Virginia; VA, Virginia; NC, North Carolina; SC, South Carolina; GA, Georgia; FL, Florida.

TABLE 2 Sequence comparisons

	Coyotes					Grey wolves			Dog	Red wolves					
	3	1	7	14, 24	22	1	4	Mex		Cap	Ark1	Ark2	Mo	La,Ok,Tx	Cau
CLA-3	—	0.3	0.8	1.3	1.0	5.8	6.0	5.5	6.8	0.2	0.2	1.0	0.1	5.8	7.3
CLA-1	1	—	1.0	1.0	1.3	6.0	6.1	5.8	7.0	1.0	1.5	1.2	1.0	6.0	7.0
CLA-7	3	4	—	0.5	0.3	5.5	5.8	5.3	6.5	1.0	1.5	0.5	0.5	5.5	7.5
CLA-14,24	5	4	2	—	0.8	5.5	5.8	5.3	6.5	0.5	1.5	1.0	1.0	5.5	7.0
CLA-22	4	5	1	3	—	5.8	6.0	5.5	6.8	0.5	1.5	0.8	1.0	5.8	7.8
CLU-1	23	24	22	22	23	—	0.3	0.3	1.5	5.0	5.0	4.5	5.0	0.0	6.5
CLU-4	24	25	23	23	24	1	—	0.5	1.8	5.3	5.3	4.8	5.3	0.3	6.5
CLU-MEX	22	23	21	21	22	1	2	—	1.8	4.8	4.8	4.3	4.8	0.3	6.3
DOG	27	28	26	26	27	6	7	7	—	6.0	6.0	5.5	6.0	1.5	7.5
CRU-CAP	7	6	4	2	5	20	21	19	24	—	1.0	0.8	1.5	5.0	6.5
CRU-ARK1	7	6	6	6	7	20	21	19	24	4	—	0.8	1.5	5.0	7.0
CRU-ARK2	4	5	2	4	3	18	19	17	22	3	3	—	0.3	4.5	7.3
CRU-MO	3	4	2	4	3	20	21	19	24	6	6	1	—	5.0	7.0
CRU-LA,OK,TX	23	24	22	22	23	0	1	1	6	20	20	18	20	—	6.5
CAU	29	28	30	28	31	26	26	25	30	26	28	26	28	26	—

Percentage sequence divergence (above diagonal) and number of nucleotide substitutions (below diagonal) in 398 bp of the cytochrome *b* gene from samples of coyotes (CLA-), grey wolves (CLU-), captive (CRU-CAP) and historic red wolves (CRU-), a dog, and the golden jackal (CAU). See Fig. 1 for locality information. Methods. Two universal primers for cytochrome *b*, H1549 and L14725 (ref. 10), were synthesized and used to amplify 398 bp of mtDNA sequence. Double-stranded sequence was amplified first and used in a second PCR reaction to generate single-stranded template by the unbalanced primer method[15]. Each PCR reaction mixture contains ~10 ng genomic DNA; 10 mM dNTP mix in a reaction buffer of 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.3), and 2.5 U *Taq* DNA polymerase in 50 μl. Each primer (25 pmol) was used for the double-stranded amplification. For single-stranded amplification we used an unequal ratio of 25 to 0.25 pmol. Thirty-five to forty cycles of amplification were run in a programmable Perkin-Elmer Cetus DNA thermal cycler as follows: denaturation at 94 °C for 1 min, annealing at 50–55 °C for historical samples, or 60–65 °C for recent samples both for 1 min, extension at 72 °C for 1 min. Double-stranded reaction products (15 μl) were separated in a 3% Nusieve (FMC Corporation, Rockland, Maryland) agarose gel, the appropriate band was excised and resuspended in 10–100 μl H_2O. The double-stranded product (1–5 μl) was used to produce single-stranded template. After concentration with Centricon 100 microconcentrators (Amicon), 7 μl single-stranded product was sequenced using the limiting primer in the second PCR and a Sequenase kit (USB). Because of the sensitivity of PCR amplification, several controls were designed to detect contamination. Tissue obtained from museum samples was extracted using sterile materials and filtered pipette tips. Four historical samples were extracted in R.K.W.'s laboratory and two by S.M.J. at the University of California at San Francisco. All were then amplified and sequenced in a laboratory in which canine DNA had not previously been handled. As a control, tissues from two individuals were extracted in both laboratories, and the duplicate samples were sequenced. In each case the resulting sequence was identical in the duplicates. Finally, extraction controls and no-template PCR controls were also used.

populations of coyotes[8]. The least our data show is that extensive hybridization has occurred among red wolves, coyotes and grey wolves.

Both empirical and theoretical studies indicate that a hybrid zone even as large as the past geographical range of the red wolf is possible[7,8,13]. The present area of the hybrid zone between grey wolves and coyotes in Minnesota and Canada is about half the geographical range hypothesized for red wolves[7]. Furthermore, hybrid zone width can be 50 times the average dispersal distance[13]. Grey wolves and coyotes are large mobile predators and the distance moved in a single generation often exceeds 100 kilometres[14]. Thus with time, a hybrid zone several thousand kilometres in width could develop. Further research, especially on nuclear genes, is needed to define better the temporal and spatial extent of the red wolf hybrid zone, but our results suggest that, relative to the past geographical range of the red wolf, it is the largest yet described for a terrestrial vertebrate.

The management policy of the US Fish and Wildlife Service does not grant protection to hybrids[18]. Nevertheless, protection of red wolves should be continued. Even if the red wolf is entirely a hybrid, it filled the role as top predator throughout its former geographic range and was thus an integral part of the ecosystem. The captive population of red wolves seems to be morphologically and genetically representative of the canid that

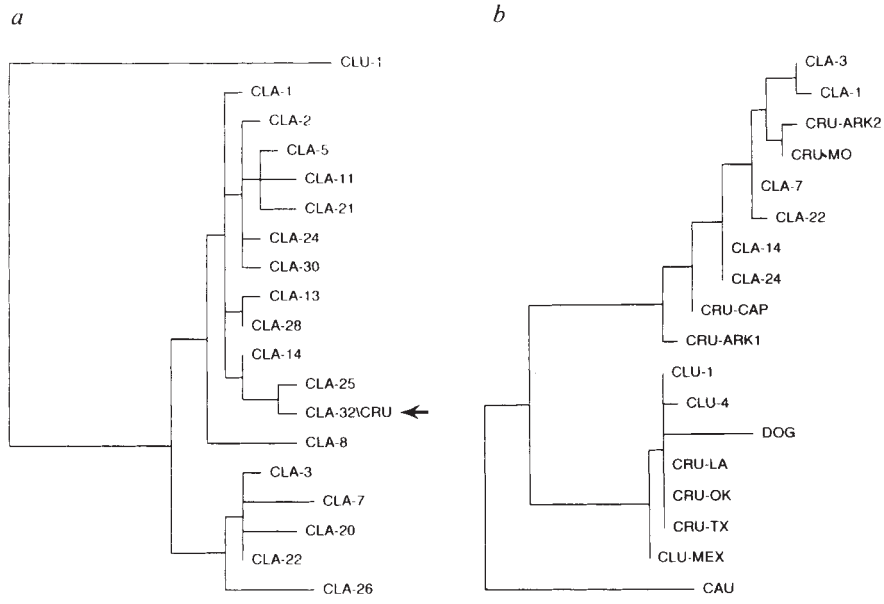

FIG. 2 *a*, A most parsimonious phylogenetic tree of coyote, *Canis latrans* (CLA), and captive red wolf (CRU) genotypes based on analysis of restriction site data[7,8]. The arrow points to the single genotype found in a sample of eight red wolves representing the four known matrilines in the captive population of red wolves. Genotype numbers correspond to those reported previously[7,8]. The tree was generated using the branch-and-bound option of the PAUP program (version 2.4, D. Swofford, Illinois Natural History Survey, Champaign) and rooted by a genotype of the grey wolf, *Canis lupus* (CLU-1)[7]. Tree length, 70; overall consistency index, 0.85. *b*, The most parsimonious tree of recent and historical DNA samples of canids on the basis of 398 bp of sequence from cytochrome *b*. Species designations as above. Locality of historical specimens: MO, Missouri; ARK, Arkansas; LA, Louisiana; OK, Oklahoma; TX, Texas (see Fig. 1). Genotypes CLU-1 and CLU-4 are from wolves found in Minnesota and Alaska, respectively, and CLU-MEX is from a Mexican wolf. The PAUP-generated tree is rooted by sequence data from the golden jackal *Canis aureus* (CAU). Tree length, 56; overall consistency index, 0.86.

existed in the southeastern United States, and so its reintroduction there would restore an essential component of the fauna. Moreover, protection should continue unless loss of the hybrid form is judged to be reversible. The absence of red wolf-like individuals in the present hybrid zone in Minnesota and Canada suggests that unique environmental and genetic conditions are required for the genesis of a red wolf phenotype. □

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Disruption of retinogeniculate afferent segregation by antagonists to NMDA receptors

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AFFERENT activity has an important role in the formation of connections in the developing mammalian visual system[1,2]. But the extent to which the activity of target neurons shapes patterns of afferent termination and synaptic contact is not known. In the ferret's visual pathway, retinal ganglion cell axons from each eye segregate early in development into eye-specific laminae in the lateral geniculate nucleus (LGN)[3]. The dorsal laminae (termed laminae A and A1) then segregate further into inner and outer sublaminae that retain input from on-centre and off-centre retinal axons, respectively[4,5]. Thus, individual retinogeniculate axons form terminal arbors within laminae A and A1 that are restricted to one inner or outer sublamina[6]. We report here that blockade of *N*-methyl-D-aspartate (NMDA) receptors on LGN cells with specific antagonists during the period of sublamina formation prevents retinal afferents from segregating into 'On' and 'Off' sublaminae. Retinogeniculate axons have arbors that are not restricted appropriately, or are restricted in size but inappropriately positioned within the eye-specific laminae. NMDA receptor antagonists may specifically disrupt a mechanism by which LGN neurons detect correlated afferent and target activity[7], and have been shown to reduce retinogeniculate transmission more generally[8–10], causing LGN cells to have markedly reduced levels of activity. These results therefore indicate that the activity of postsynaptic cells can significantly influence the patterning of inputs and the structure of presynaptic afferents during development.

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TABLE 1 Axons in normal and D-APV-treated animals

	No. of animals	No. of axons	Mean arbor area in μm^2 (s.e.)	Mean sublamina index (s.e.)
Normal 2-week-old	3	12	5250 (601)	0.70 (0.01)
Normal 3-week-old	3	16	8155 (653)	0.88 (0.05)
D-APV-treated, 3-week-old	3	12	7959 (807)	0.66 (0.03)

The arbors that were examined lay within the central two-thirds of the LGN, where the thickness of the A-laminae is roughly uniform, and their locations were judged to be between 0 and 60 degrees in azimuth and ±30 degrees in elevation[24]. In this region, in each animal, every arbor that could be fully reconstructed was drawn; we did not sample or select arbors (this procedure led to 2–7 reconstructed axons per animal in each group). The arbor reconstructions were made without knowledge of laminar borders or sublaminar halves. The axons in each of the three groups spanned similar ranges of LGN locations. In each group, there were nearly equal numbers of arbors in the binocular and monocular portions of the LGN, and there were no differences between these axons. The area of an arbor was measured as the area covered by the outermost branch tips in the two-dimensional reconstruction of the arbor. The 'sublamina index' was obtained from the position of the arbor with respect to a line bisecting lamina A or A1 parallel to laminar borders. The bisected laminar halves closely approximate sublaminae; the sublamina index varies between 0.5 and 1, being 1 for an arbor that was entirely confined to one half of lamina A or A1 and 0.5 for an arbor that was located exactly midway between laminar borders. Statistical comparisons employed the Mann–Whitney *U*-test and were made by: (1) pooling the axons from each animal and treating each animal as a single datum, (2) treating each axon as a single datum.

Experiments were done on 32 ferret kits. Timed pregnant ferrets were either purchased from Marshall Research Animals or bred in our colony. We first examined, in normal ferret kits, the time course of segregation of retinogeniculate afferents into eye-specific laminae and 'On' and 'Off' sublaminae. At birth (embryonic day 41) axons from the two eyes overlap extensively in the LGN[3]. Axon arbors start to segregate into eye-specific laminae by the first postnatal week, and laminar segregation is essentially complete by two weeks (Fig. 1*a*; $n = 2$). Further division of laminae A and A1 into sublaminae occurs during the third postnatal week, and sublaminae can be clearly identified by three weeks (Fig. 1*b*; $n = 3$).

We next examined the effect on sublaminar development of blocking NMDA receptors on LGN cells between two and three weeks of age. Antagonists were delivered using osmotic minipumps either directly into the thalamus, near the LGN, or systemically using subcutaneous infusion. We first assayed the effect of the antagonists using intraocular injections of the anterograde tracer wheat-germ agglutinin conjugated to horseradish peroxidase. Blockade of NMDA receptors in the LGN with D-APV (D-2-amino-5-phosphonovaleric acid) delivered into the posterior thalamus, or with MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate) delivered subcutaneously, blocks the formation of On and Off sublaminae (Fig. 1*c*, *d*). These effects are dose-dependent. Although D-APV delivered at a minipump concentration of 0.8 mM ($n = 3$) blocks sublamina formation (Fig. 1*c*), a concentration of 0.08 mM does not ($n = 2$). MK-801 blocks sublamina formation at a concentration of 4.75 mM (Fig. 1*d*; $n = 2$) but not at 1.2 mM ($n = 2$). In contrast, thalamic infusion of 0.8 mM L-APV (the inactive isomer of APV) has no effect on sublaminae ($n = 2$). Similarly, thalamic ($n = 2$) or subcutaneous ($n = 1$) infusion of the saline vehicle has no effect on the formation of sublaminae.

We next examined the morphology of single retinal axon arbors in the LGN by filling optic tract axons with horseradish