not included in our analysis; the morphological characters most useful for discriminating *S. guntheri* were length of longest neck spine, snout-vent length, and width of brow ridge. Morphologically, the species of tuatara are cryptic.

Differentiation and taxonomy of *S. p. reischeki* were not assessed, because no surviving tuatara from its only population, Little Barrier Island, is known. However, the taxon *S. p. reischeki* must be considered valid until shown not to be differentiated from other populations of *S. punctatus.*

The tuatara is 'of extraordinary zoological interest'[13] as the most distinctive surviving reptilian genus in the world, but despite absolute protection of the species and its island habitats, 25% of known populations have become extinct in the past century (Table 1). Management intervention to save any endangered population has been limited. This contrasts markedly with the highly successful manipulative techniques applied to some threatened New Zealand birds[22]. Disparities in management intervention, possibly to the detriment of tuatara, are greatest on four islands where tuatara are now on the brink of extinction (Table 2); threatened birds have been introduced and sometimes intensively managed on all four, without intervention on behalf of tuatara.

Taxonomies are not irrelevant abstractions, but the essential foundations of conservation practice[23]. The present study shows that neglect of described taxonomic diversity may unwittingly have consigned one subspecies of tuatara, *S. p. reischeki*, to extinction. A second species, *S. guntheri*, has survived on one small island only by good fortune[24]. □

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Requirement for subplate neurons in the formation of thalamocortical connections

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THE neurons of layer 4 in the adult cerebral cortex receive their major ascending inputs from the thalamus. In development, however, thalamic axons arrive at the appropriate cortical area long before their target layer 4 neurons have migrated into the cortical plate. The axons accumulate and wait in the zone below the cortical plate, the subplate, for several weeks before invading the cortical plate. The subplate is a transient zone that contains the first postmitotic neurons of the telencephalon. These neurons mature well before other cortical neurons, and disappear by cell death after the thalamic axons have grown into the overlying cortical plate. The close proximity of growing thalamocortical axons and subplate neurons suggests that they might be involved in interactions important for normal thalamocortical development. Here we show that early in development the deletion of subplate neurons located beneath visual cortex prevents axons from the lateral geniculate nucleus of the thalamus from recognizing and innervating visual cortex, their normal target. In the absence of subplate neurons, lateral geniculate nucleus axons continue to grow in the white matter past visual cortex despite the presence of their target layer 4 neurons. Thus the transient subplate neurons are necessary for appropriate cortical target selection by thalamocortical axons.

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The role of subplate neurons in the formation of geniculocortical connections was tested by selectively ablating subplate neurons during development. The neurotoxin kainic acid was injected into the occipital subplate of fetal cats on embryonic day 42 (E42; gestation is 65 days), a time when the first axons from the lateral geniculate nucleus (LGN) have arrived in the visual subplate but before they have entered the cortical plate[1] (see Fig. 1). At this age, many neurons of cortical layer 4 are being generated at the ventricular zone, while others are migrating through the subplate *en route* to the cortical plate. Subplate neurons, by contrast, are generated between E24 and E30 (ref. 2), and have already acquired many characteristics of mature neurons by E42 (refs 3, 4). For example, they (and not other cortical neurons) are sensitive to kainate excitotoxicity; thus a kainic acid injection at E42 selectively kills subplate neurons, leaving the neurons of the cortical plate intact[5] (see Fig. 2*c*, *d*).

During normal development in mammals, LGN axons wait in the subplate below visual cortex from days to months depending on the species[6–8]. In the cat this occurs between E38 and E50, after which LGN axons first grow into the deep layers of the cortical plate[1] (see Fig. 1; A.G. and C.J.S, manuscript in preparation). Geniculocortical axons can be visualized by placing crystals of DiI into the LGN of an aldehyde-fixed brain (Fig. 2). Figure 2*a* shows this projection in an E60 control brain injected with saline at E42. As in normal animals, LGN axons leave the optic radiations to branch extensively in the subplate and in the deep layers of the visual cortex. (Deep-layer neurons which project subcortically are also labelled retrogradely, as expected[9].) To determine how the absence of subplate neurons affects LGN axons, we examined the morphology of these axons at E60, after kainic acid had been injected to delete subplate neurons at E42. In these animals, the entire zone that normally contains subplate neurons and waiting axons has collapsed, and LGN axons no longer arborize below the cortical plate (Fig. 2*b*). Instead of fanning out and branching in the subplate, the axons grow past the visual cortex in a tight fascicle. These

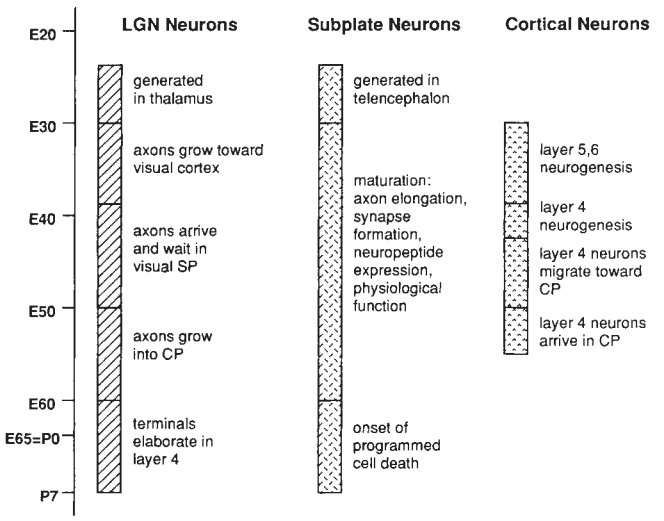

FIG. 1 Timing of events associated with the development of the thalamocortical pathway, subplate neurons, and layer 4 cortical neurons, in the visual system of the fetal cat. Abbreviations: SP, subplate; CP, cortical plate (for review, see ref. 8).

observations suggest that subplate neurons are necessary for LGN axons to stop and arborize below their normal cortical target before they grow into the cortical plate.

Several control experiments were performed to determine whether these effects are specifically due to the absence of subplate neurons. First, MAP2 immunocytochemistry was used to examine the histological organization of the cortical plate and subplate after injections of kainic acid or saline at E42. Both subplate neurons and cortical plate neurons stain heavily for MAP2 at E60 in saline-injected controls (Fig. 2*c*). By contrast, in brains lesioned by kainic acid, immunoreactivity is completely absent from the subplate (Fig. 2*d*), as has been shown previously[5]. The intensity of staining and the thickness of the cortical plate are unaffected by the lesion, consistent with the suggestion that cortical plate neurons are too immature at E42 to be susceptible to kainate toxicity.

The effect of kainic acid lesions at E42 was also examined on two other cell types that might be required for the normal development of LGN axons: radial glial cells, the predominant glial subtype in the developing cortex[10,11], and layer 4 neurons, the ultimate targets of thalamic axons. The morphology and patterning of radial glial cells revealed by DiI labelling were indistinguishable in the saline- and kainic acid-injected hemispheres at E60 (Fig. 2*g*, *h*), indicating that this cell type is not destroyed by injections of kainic acid at E42. Second, to confirm that the genesis, migration and survival of layer 4 neurons were not affected, we labelled these cells with [^{3}H]thymidine

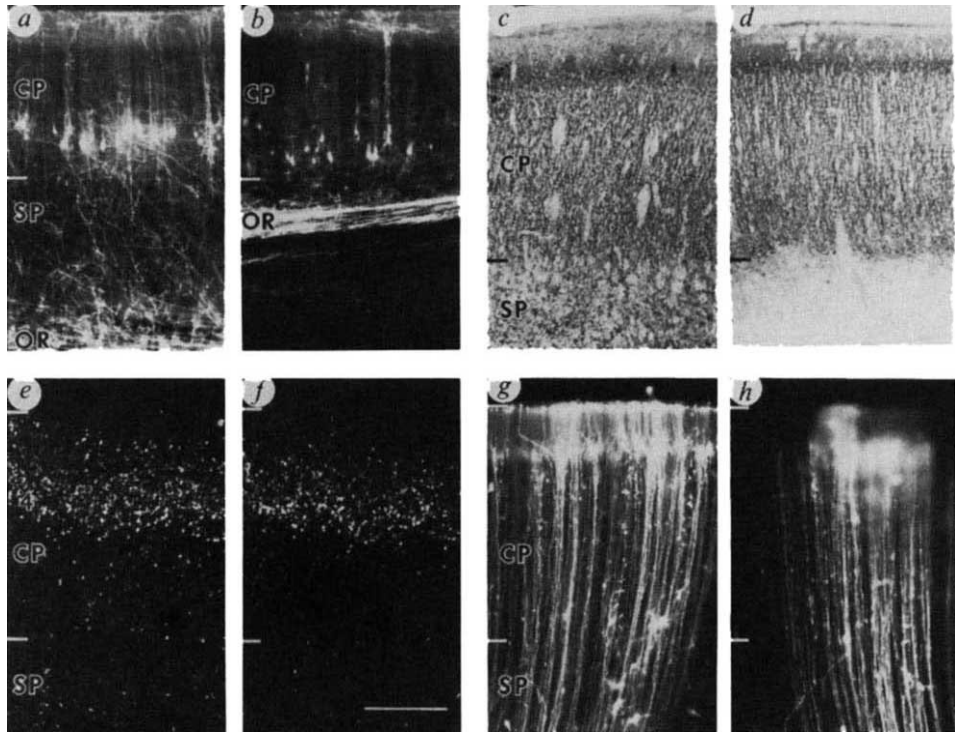

FIG. 2 Sections through the visual cortex at E60 after intracortical injections of kainic acid (*b, d, f, h*) or saline control (*a, c, e, g*) at E42, showing that kainic acid lesions result in the specific ablation of subplate neurons and marked changes in LGN axon morphology at E60. *a, b*, In the control hemisphere (*a*), anterogradely labelled LGN axons leave the optic radiations (OR) to branch below the visual cortex in the subplate (SP). Retrogradely labelled neurons in the deep cortical layers (CP) are also present as expected, as they project subcortically. In the kainate-lesioned hemisphere (*b*), LGN axons fail to branch and remain fasciculated in the OR, running past the visual cortex in a tight bundle. *c, d*, Extent of ablation verified by MAP2 immunocytochemistry[5]. *c*, Control hemisphere shows dense MAP2 immunoreactivity associated with the somata and the dendrites of neurons in both the cortical plate and subplate. *d*, In the kainate-lesioned hemisphere, cortical plate neurons remain and exhibit MAP2 immunoreactivity, but the subplate neurons are missing. The thickness of the cortical plate is indistinguishable from that of controls. *e, f*, Dark-field autoradiographs of the visual cortex on E60, after injection of [^{3}H]thymidine on E42 to label layer 4 neurons[12]. Layer 4 neurons have migrated into their normal positions in both the control (*e*) and kainic acid-lesioned (*f*) hemispheres. *g, h*, DiI-labelled radial glial cells (see methods) are indistinguishable in control (*g*) and kainate-treated (*h*) hemispheres. Scale bar (shown in *f*): 450 μm (*a, b*); 300 μm (*c–h*).

METHODS. Subplate neurons were ablated by injecting 0.6–0.8 μl of 10 mg ml^{-1} kainic acid (Sigma) in 0.9% NaCl into the visual subplate in one hemisphere in fetal cats at E42 as previously described[5]. The other hemisphere was injected with an equal volume of 0.9% NaCl (saline) as a control. Fetal surgeries were performed under halothane anaesthesia using sterile surgical procedures[2]. In four fetuses, 500 μCi [^{3}H]thymidine in 0.1 ml sterile saline was injected intraamniotically after the kainate injections to label layer 4 neurons. A total of 11 fetuses were injected with kainic acid at E42 and perfused on E50(2), E59(1), E60(2), E63(4), P5(1) or P8(1) with 4% paraformaldehyde. To visualize the geniculocortical pathway, small crystals of DiI (Molecular Probes, D282) were placed into the LGN of fixed brains. The brains were stored in the same fixative for 2–4 months to allow for the diffusion of the dye[14,17]. Coronal sections (200 μm) were cut on a vibratome and viewed under rhodamine epifluorescence optics. Radial glial cells were visualized by preparing 1-mm thick coronal slices of aldehyde-fixed brains, into which crystals of DiI were placed into the ventricular zone to label radial glial processes by means of their ventricular endfeet. MAP2 immunocytochemistry and [^{3}H]thymidine autoradiography were performed as described previously[2,5].

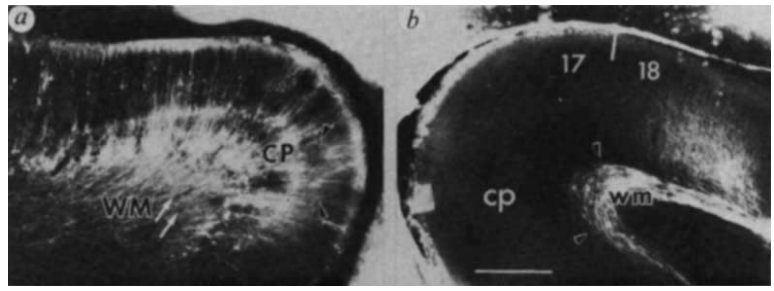

FIG. 3 LGN axons examined in postnatal life have failed to recognize and innervate visual cortex after subplate ablation at E42. *a*, Coronal section through the visual cortex of a normal animal at P2 shows a wealth of labelled LGN axons in the cortical plate, as well as retrogradely labelled pyramidal neurons in the deep cortical layers, resulting from a DiI injection into the LGN. LGN axons fan out radially as they enter the cortex (arrows) and branch extensively in layer 4 (arrowheads). *b*, Section through the visual cortex at P5 after ablation of subplate neurons underlying primary visual cortex (area 17) at E42 shows that LGN axons fail to recognize and innervate primary visual cortex and instead grow past the area, confined to the white matter (wm, arrowheads). These axons can be followed underneath the splenial sulcus and into the cingulate gyrus (not shown). A subset of LGN axons have entered area 18, a normal target of LGN axons where subplate neurons are spared. Scale bar, 750 μm.

METHODS. Subplate neurons were ablated with kainic acid, and geniculocortical axons were labelled with DiI as described in Fig. 2.

immediately after injection of kainic acid and followed their subsequent development. Previous [^{3}H]thymidine-labelling studies showed that neurons comprising the upper half of layer 4 are born at this time[12]. Autoradiographs of control and kainic acid-treated brains at E60 (Fig. 2*e*, *f*) indicate that layer 4 neurons generated on E42 can indeed migrate through the kainic-treated zone and take up their appropriate position in the cortical plate.

Finally, to address the possibility that LGN axons might be directly affected by the kainate treatment, we examined whether axons exposed early on to kainic acid would still innervate the cortical plate later on. Kainic acid was injected into the subplate below temporal cortex (future auditory cortex) at E38, just when LGN axons *en route* to visual cortex are growing past. Subsequent DiI injections into visual cortex at E50 retrogradely labelled LGN cells, implying that exposure to kainate had not compromised the ability of these LGN axons to innervate cortex (A.G. and C.J.S., manuscript in preparation). Thus it seems unlikely that LGN axons are affected directly by the kainic acid injection. Taken together, these controls strongly suggest that the effects of the kainic acid lesion on LGN axons are specifically due to the absence of subplate neurons.

To determine whether LGN axons can eventually recognize and innervate the visual cortex despite the absence of the subplate, we deleted subplate neurons at E42 and labelled LGN axons with DiI 30 days layer, at postnatal day (P) 5. In normal animals, LGN axons grow into layer 4 during the first postnatal week[1,13]. Figure 3*a* shows that at P2 both anterogradely labelled axons and retrogradely labelled neurons in the deep cortical layers were present. As expected at this age, LGN axons fan out radially as they enter the cortex and branch extensively in layer 4.

A section through visual cortex at P5 following an E42 subplate deletion is shown in Fig. 3*b*. In contrast to that of normal animals, the trajectory of LGN axons is radically modified after a deletion of subplate neurons. LGN axons fail to innervate the primary visual cortex, their appropriate target. Instead, these DiI-labelled axons have grown past their normal cortical target in a dense fascicle restricted to the white matter, seemingly unaware of their correct cortical destination. Serial sections spanning the extent of visual cortex show that where subplate neurons are missing, the entire geniculocortical projection has been rerouted. The axons grow past visual cortex and into the white matter underlying the adjacent cingulate gyrus (not shown); we do not so far know whether these axons invade and innervate this inappropriate cortical area.

A subset of LGN axons does, however, enter area 18, a normal target of LGN axons (Fig. 3*b*). MAP2 immunocytochemistry and cresyl violet staining show that although subplate neurons are absent underneath area 17, they are present below area 18 in the region where axons have entered the cortical plate. Thus, where subplate neurons are present, LGN axons can innervate directly overlying cortical areas. Subplate neurons may also have a role in the formation of the feedback connection from the cortex. We have previously shown that subplate neurons pioneer the first axon pathway from cortex to thalamus very early in development, a pathway that is followed later by corticofugal axons[14]. Consistent with this observation, few cortical layer 6 neurons could be retrogradely labelled from the LGN after subplate ablation (Fig. 3*b*), suggesting that these cortical neurons also fail to innervate their appropriate thalamic target in the absence of subplate neurons.

The results of this study imply that interactions between growing geniculocortical axons and subplate neurons are required for axons to select and grow into their appropriate target, the visual cortex. Thus, one function of subplate neurons may be to mediate the formation of orderly sets of connections between different thalamic nuclei and their appropriate cortical target areas.

Much discussion has addressed the issue of how cortical areas are specified during development[15,16]. One view is that cortical areas have intrinsic differences that are mapped out very early in development, perhaps even in the ventricular zone. Alternatively, cortical areas may emerge gradually from an undifferentiated 'protocortex' through epigenetic influences such as afferent inputs. Our results indicate that subplate neurons stand as a crucial link in cortical target recognition, whichever strategy is used. The observation that LGN axons grow past the visual cortex in the absence of subplate neurons indicates that the cortical plate alone has insufficient information to allow the ingrowth of appropriate axons. It remains to be seen whether in these animals, thalamic axons invade and innervate layer 4 of inappropriate cortical regions as a result of subplate ablation. Such findings could shed light on the issue of whether intrinsic differences among subplate neurons specify the identity of overlying cortical areas. □

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