

# Selective and Quickly Reversible Inactivation of Mammalian Neurons In Vivo Using the *Drosophila* Allatostatin Receptor Neurotechnique

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## Summary

Genetic strategies for perturbing activity of selected neurons hold great promise for understanding circuitry and behavior. Several such strategies exist, but there has been no direct demonstration of reversible inactivation of mammalian neurons in vivo. We previously reported quickly reversible inactivation of neurons in vitro using expression of the *Drosophila* allatostatin receptor (AlstR). Here, adeno-associated viral vectors are used to express AlstR in vivo in cortical and thalamic neurons of rats, ferrets, and monkeys. Application of the receptor's ligand, allatostatin (AL), leads to a dramatic reduction in neural activity, including responses of visual neurons to optimized visual stimuli. Additionally, AL eliminates activity in spinal cords of transgenic mice conditionally expressing AlstR. This reduction occurs selectively in AlstR-expressing neurons. Inactivation can be reversed within minutes upon washout of the ligand and is repeatable, demonstrating that the AlstR/AL system is effective for selective, quick, and reversible silencing of mammalian neurons in vivo.

## Introduction

A major goal of systems neuroscience is to elucidate the roles of individual cell types within complex neural circuits and to understand their contributions to perception and behavior. To this end, several laboratories have developed genetically encoded modulators of neural activity. The primary advantage of genetic methods is that expression can be targeted to the cell type(s) of interest (Callaway, 2005; Gong et al., 2003); modulation of activity in targeted cells can then allow investigation of the contributions of specific cell types.

Genetic strategies for manipulating neural activity differ in their degree and speed of reversibility as well as

other factors that influence their utility for in vivo manipulation. Because any given experimental paradigm will have unique goals and limitations, no single method will be ideal for all future applications. Pioneering genetic strategies selectively and irreversibly ablated targeted cells (Isles et al., 2001; Kobayashi et al., 1995). Methods developed subsequently possess the potential for reversibility via temporally regulated gene expression. For instance, neurons have successfully been silenced, sometimes reversibly, using K<sup>+</sup> channel overexpression (Ehrensgruber et al., 1997; Johns et al., 1999; Nitabach et al., 2002). Another highly effective strategy blocks receptors and channels via toxins tethered to the plasma membrane (Ibanez-Tallon et al., 2004). These approaches offer slow temporal regulation at best, and are thus best suited for studies requiring long-term inactivation. The inability to achieve more transient inactivation presents difficulties related to compensatory changes: K<sup>+</sup> channel overexpression, for instance, has yielded unwanted side effects such as cell death and hyperexcitability (Nadeau et al., 2000; Sutherland et al., 1999).

To minimize effects caused by long-term changes in excitability, more recent efforts have adopted strategies with faster timescales of reversibility. Light-based methods for eliciting or inhibiting spiking in neurons (Banghart et al., 2004; Boyden et al., 2005; Li et al., 2005; Lima and Miesenböck, 2005; Zemelman et al., 2002, 2003) offer extremely fast temporal control, as they are limited largely by the speed and efficiency of light delivery. Impressive effects have been demonstrated in the intact embryonic chicken spinal cord (Li et al., 2005). Difficulties related to delivery of light in deep neural tissues are likely, however, to prove problematic for many in vivo applications.

Pharmacologically based methods for inhibiting spiking or synaptic transmission (Coward et al., 1998; Karpova et al., 2005; Lechner et al., 2002; Slimko et al., 2002) allow access to deep tissues while retaining the potential for quick temporal control. Although several factors could reduce the temporal resolution of such methods, in vitro studies have demonstrated that methods based on G protein-coupled activation of K<sup>+</sup> channels can be very fast (milliseconds to minutes) and are therefore, in theory, limited only by the speed with which ligand can be effectively applied and removed from the system (Coward et al., 1998; Lechner et al., 2002).

Despite the great promise of these varied approaches, no reversible method has directly demonstrated in vivo inactivation of neurons in a mammalian system. The Molecules for Inactivation of Synaptic Transmission (MIST) approach (Karpova et al., 2005), based on reversible blocking of synaptic transmission, has been shown to elicit behavioral deficits following application to mouse cerebellar Purkinje cells; in this study, however, in vivo neural activity was not directly assayed. The MIST system is reversible in vivo on a timescale of hours to days and is likely to prove extremely useful for applications requiring long periods

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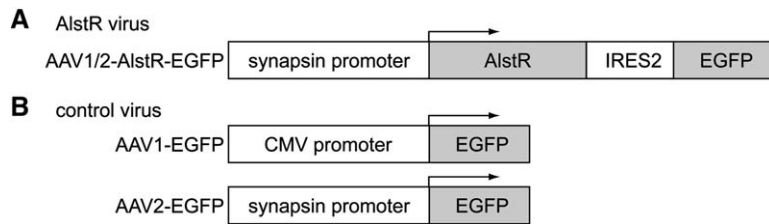


Figure 1. Diagrams of Plasmids Used to Make Viruses in This Study

These diagrams illustrate the genetic constructs placed in the AAV backbone and therefore carried into infected cells by virus. (A) AlstR viruses. A synapsin promoter was used to drive expression of AlstR; an IRES2 element was used to obtain additional expression of EGFP. The construct was identical for both AAV1 and AAV2 viruses. (B) Control viruses. EGFP expression was driven by a CMV promoter (AAV1 construct) or by a synapsin promoter (AAV2 construct). AAV1 was used in cortical experiments; AAV2 was used in thalamic experiments.

of inactivation without fast onset or recovery. For many physiological studies, however, the ideal system would elicit inactivation within minutes, would allow inactivation to persist for an hour or longer, and could allow temporally controlled recovery within minutes.

Here, we present such a genetic system for use in mammalian neurons *in vivo*. In a previous report, we demonstrated selective, quickly reversible inactivation of ferret cortical neurons *in vitro* using the allatostatin receptor/allatostatin (AlstR/AL) system (Birgul et al., 1999; Lechner et al., 2002). AL effectively reduced membrane potential and input resistance by opening G protein-coupled inward-rectifying K<sup>+</sup> (GIRK) channels in AlstR-expressing neurons *in vitro*, indicating that it may be capable of inactivating neurons *in vivo*. Here, we overcome many of the difficulties inherent in genetic manipulation and direct assay of neuronal activity in order to study the efficacy of the AlstR/AL system *in vivo*. Using adeno-associated viral (AAV) vectors to express AlstR in mammalian neurons, we demonstrate that the AlstR/AL system is effective for quick and reversible inactivation of rat and ferret cortical neurons, as well as ferret and monkey thalamic neurons, *in vivo*. Additionally, we demonstrate that AL can effectively silence neurons in spinal cords of transgenic mice conditionally expressing AlstR.

## Results

### Overview

AAV vectors were used to express AlstR in several different preparations, including the rat barrel cortex, ferret visual cortex, and lateral geniculate nucleus (LGN) of ferrets and monkeys. Experiments conducted in rat barrel cortex tested for inactivation of *in vivo* cortical activity generated by strong and synchronous activation of afferent input following electrical stimulation of the whisker pad. Experiments conducted in the LGN (ferrets and monkeys) and visual cortex (ferrets) tested the ability of AL to eliminate responses to visual stimuli *in vivo*. In additional experiments, AlstR was expressed conditionally in transgenic mice following Cre recombination. These experiments examined the effects of AL on the activity of motor neurons in an *in vitro*, isolated spinal cord preparation. In all preparations, the ability of AL to induce inactivation was tested both in neurons expressing AlstR and in control neurons not expressing AlstR.

### Viral Constructs

To achieve AlstR expression, AAVs were used to deliver genetic constructs (Figure 1). Due to differences in transduction efficiency, AAV serotype 1 (AAV1) was used in cortical experiments, while AAV serotype 2 (AAV2) was used in LGN experiments. AlstR expression was under the regulation of the neuron-specific synapsin promoter, which drives expression indiscriminately in all neuron types; an IRES2 element drove additional expression of EGFP as a marker for virus-infected, AlstR-expressing cells (Figure 1A). For control experiments, AAVs encoding only EGFP under the CMV or synapsin promoter were used (Figure 1B). For simplicity, we will refer to the AlstR-encoding viruses as “AAV1-AlstR-EGFP” and “AAV2-AlstR-EGFP;” the control viruses will be referred to as “AAV1-EGFP” and “AAV2-EGFP.”

### Inactivation of Neurons in Rat Barrel Cortex

To examine whether the AlstR/AL system could effectively silence cortical cells in rat, we injected AAV1-AlstR-EGFP into the barrel cortex of adult rats. Following at least 35 days to allow expression of the delivered genes, local field potentials (LFPs) evoked by electrical stimulation of the whisker pad were recorded from virus-injected sites of anesthetized rats (see [Experimental Procedures](#)). Subsequent histological staining was used to determine whether virus injection resulted in successful AlstR/EGFP expression and whether the recordings were made from the region of AlstR expression.

Figure 2 shows representative LFPs recorded from a region of AlstR expression in the barrel cortex of a rat. Recordings included a large stimulus artifact coinciding with the onset of whisker pad electrical stimulation. A large positive voltage deflection typically appeared about 7 ms after the stimulus artifact, followed by a sizeable negative deflection and sometimes by additional small fluctuations in voltage. An example response is illustrated in Figure 2A for a recording made prior to application of AL in a cortical region that was later confirmed to coincide with a region of AlstR expression (Figure 3A2). Following application of 0.1 μM AL directly onto the cortical surface, the stimulus-evoked response was essentially eliminated (Figure 2B) and then returned following washout of the AL with saline (Figure 2C). After several rounds of repeated inactivation with AL (see below), effects of the GABA<sub>A</sub> agonist muscimol were characterized for comparison, since muscimol should maximally inactivate cortical

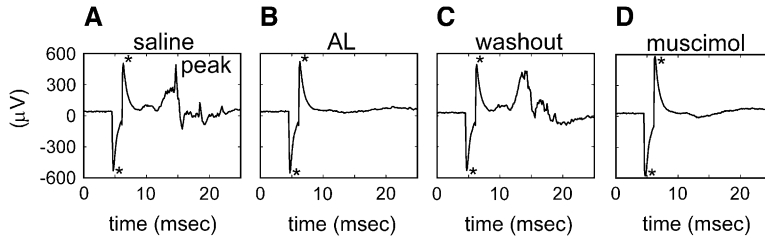


Figure 2. Representative Local Field Potentials Recorded from the Barrel Cortex of a Rat Expressing AlstR

Recordings are in response to electrical stimulation of the whisker pad. Example LFPs are illustrated in chronological order: (A) prior to application of AL to the cortical surface; (B) 11 min after application of AL; (C) 10 min after washout of AL with saline; and (D) 20 min after application of muscimol. AL application resulted in complete elimination of stimulus-evoked LFP, comparable to the effects of muscimol, and washout resulted in complete recovery. Asterisks indicate the electrical stimulus artifact, which was not affected by AL or muscimol.

responses. Figure 2D shows that the stimulus-evoked LFP following inactivation with muscimol is similar to that observed following AL application (Figure 2B).

To test the speed of inactivation and recovery following application and washout of AL, whisker stimulation-evoked LFPs were recorded at 10 s intervals. Peak LFPs measured over the entire course of the same experiment for which representative recordings are shown in Figure 2 are plotted in Figure 3A1. An example from a second rat is shown in Figure 3B1. In both examples, neurons inactivated within minutes of 0.1  $\mu$ M AL applica-

tion and recovered nearly completely within minutes of saline washout. This effect was repeatable, indicating that the AlstR/AL system can inactivate neurons repeatedly without apparent desensitization. Longer AL applications result in longer-lasting inactivation (Figure 3B1, second AL application), suggesting that the duration of AlstR/AL-mediated inactivation is dictated by the presence of AL, rather than being short-lived (see further results below). Moreover, a dose of 0.1  $\mu$ M AL appears to be sufficient to elicit maximal inactivation in this experimental paradigm, as increasing the dose 10-fold does

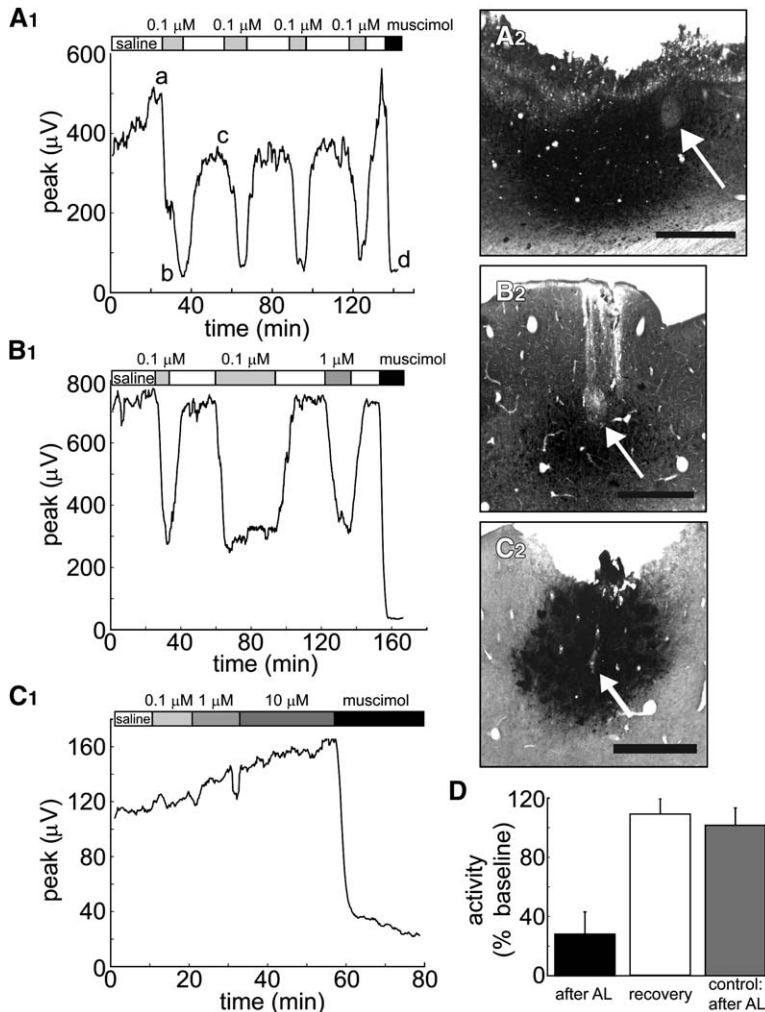


Figure 3. The AlstR/AL System Quickly and Reversibly Eliminates Stimulus-Evoked LFPs in Rat Barrel Cortex

(A1–C1) Peaks of LFPs evoked at 10 s intervals, plotted over time, from rats infected with AAV1-AlstR-EGFP (A1–B1) and a control rat infected with AAV1-EGFP (C1). Letters a–d in panel A1 correspond to the times of LFP recordings illustrated in Figure 2 (panels [A]–[D], respectively), which were from the same animal. Field potential responses disappear within minutes of AL application in AlstR-expressing neurons and recover within minutes of washout; the effect is repeatable. (A2–C2) Sagittal sections from rats shown in (A1)–(C1), respectively, demonstrating EGFP expression (black staining) and recording sites (lesions indicated by white arrows). Scale bar, 200  $\mu$ m; top, pia; left, anterior. In all cases, recordings were made from the area of EGFP expression.

(D) Average LFP responses  $\pm$  SEM from three AlstR-expressing and three control rats. Averages represent responses relative to baseline during the 3 min after application of 0.1  $\mu$ M AL or saline; data were pooled across AL applications for each animal (see Experimental Procedures for details). LFPs inactivated upon AL application and recovered to baseline levels in AlstR-expressing rats; no effect was observed in control rats.

not increase the degree of inactivation (Figure 3B1, third AL application). Comparison of the effect of AL application to the effect of muscimol suggests that inactivation was more complete in the example shown in Figure 3A than in Figure 3B. This may be related to less efficient infection in the latter case, as suggested by the weaker EGFP staining in superficial cortical layers (compare Figure 3A2 with Figure 3B2).

No effect was observed when AL was applied to the cortex of control rats injected with AAV1-EGFP, even when 100-fold higher AL concentrations (10  $\mu$ M) were applied (Figure 3C1). Summary data from three AlstR-expressing and three control rats are shown in Figure 3D. To quantify inactivation, responses to AL application were compared to responses to muscimol application. Muscimol was presumed to result in complete inactivation, so peak LFPs measured following muscimol were defined as a baseline below which LFPs could not fall. Inactivation following AL application was expressed as a percentage of the difference between the peak LFP before AL application and the baseline LFP following muscimol application (see Experimental Procedures). On average, neurons from the AlstR-expressing rats inactivated to  $28.2\% \pm 14.9\%$  of baseline levels upon AL application and recovered to  $108.9\% \pm 9.9\%$  of baseline levels after washout of AL. (0% corresponds to complete inactivation while 100% corresponds to no effect/complete recovery.) Neurons from control rats did not inactivate, their responses remaining at  $101.6\% \pm 11.7\%$  of baseline levels upon AL application. Similar values were obtained when field potential areas, rather than peaks, were analyzed (inactivation to  $25.9\% \pm 13.4\%$  of baseline and recovery to  $115.8\% \pm 8.0\%$  for AlstR-expressing rats;  $101.3\% \pm 12.9\%$  of baseline following AL application for controls; see Experimental Procedures for details of analysis). In all cases included in this analysis, we had histological confirmation that recordings were made from the virus-infected region of cortex (Figures 3A2, 3B2, and 3C2).

The incomplete inactivation observed in some AlstR-expressing rats is likely due to the fact that LFPs reflect responses from many neurons, including both AlstR-expressing and nonexpressing neurons: the non-AlstR-expressing neurons presumably retain their activity upon AL application, resulting in residual activity in the field potential response. An alternative possibility, however, is partial inactivation of AlstR-expressing neurons and/or variability in the levels of AlstR expression. This ambiguity prompted us to perform single-unit recordings in ferret visual cortex, as described below.

#### Inactivation of Neurons in Ferret Visual Cortex

The rat somatosensory cortex experiments described above indicate that the AlstR/AL system can effectively inactivate LFP activity in a reversible fashion. However, because field potential recordings reflect both presynaptic and postsynaptic activity from a population of neurons, the response of individual neurons remained unclear, particularly in cases where inactivation was incomplete. To clarify this issue, and to assess responses to AL using a sensory stimulus rather than an artificial electrical stimulus, we performed a series of single- and multi-unit recordings from visual cortical neurons of adult ferrets.

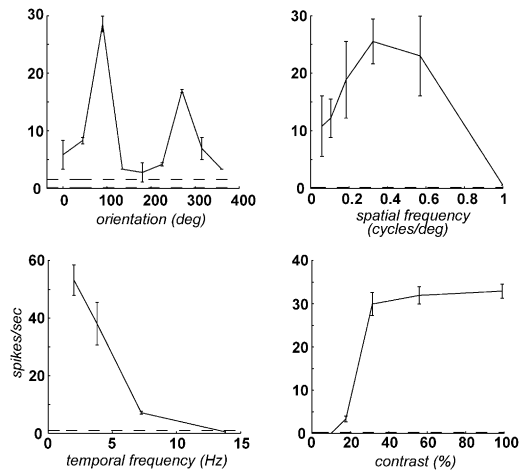


Figure 4. Tuning Properties of a Visual Cortical Neuron from a Ferret Expressing AlstR

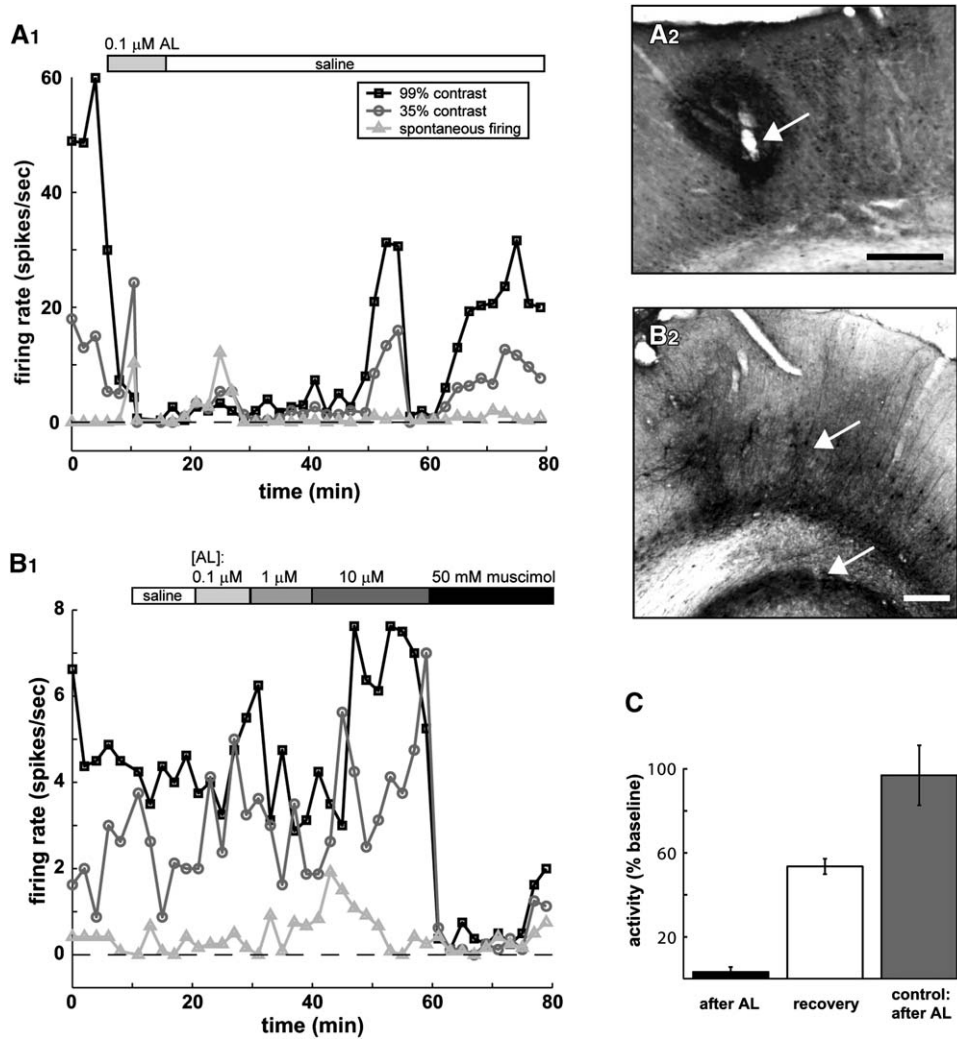
Separate plots indicate visual responses to drifting sinusoidal gratings with varying stimulus orientation, spatial frequency, temporal frequency, or contrast. Each point indicates the mean firing rate  $\pm$  SEM for two presentations of each stimulus; dotted lines indicate mean response  $\pm$  SEM to a blank stimulus. In the absence of AL, responses of this neuron are typical for a layer 5 ferret visual cortical neuron; this neuron's activity in the presence of AL is shown in Figure 5A.

In this set of experiments, AAV1-AlstR-EGFP was injected into Area 17 of adult ferrets. After establishment of transgene expression, extracellular recordings were made from the virus-infected area in anesthetized, paralyzed ferrets. Ferrets viewed drifting grating visual stimuli while AL or saline was applied to the brain surface. After recordings were completed, ferrets' brains were sectioned, stained with an anti-EGFP antibody, and examined to locate the recording site and its relationship to regions of viral infection.

After infection with the AlstR-encoding virus and prior to application of AL, visual cortical cells exhibited apparently normal visual response properties. Figure 4 shows the tuning properties of an example unit, recorded in cortical layer 5 at a depth of 880  $\mu$ m from the pial surface, from an AlstR-expressing region of ferret cortex before application of AL. This unit had strong orientation tuning, moderate direction selectivity, and bandpass spatial frequency tuning. All units studied ( $n = 5$ ) were tuned for values typical of ferret Area 17 neurons (Alitto and Usrey, 2004; Baker et al., 1998). Cells had preferred spatial frequencies of 0.02–0.32 cycles/deg and optimal temporal frequencies of 1–10 Hz. Nearly all cells examined were orientation-tuned and had bandpass spatial frequency tuning.

Application of 0.1  $\mu$ M AL to the cortical surface completely abolished both spontaneous and visually evoked activity of the unit characterized in Figure 4 (Figure 5A1). Activity dropped to 8% of baseline levels within 5 min of AL application, with complete inactivation occurring 30 s later. Upon saline washout, administered 11 min after AL application, activity did not recover immediately: recovery began to occur 30 min after washout, reaching a peak of 57% of baseline 40 min after washout (see Experimental Procedures for details of analysis). This result was typical of our ferret cortical recordings, and





**Figure 5. The AlstR/AL System Quickly and Reversibly Inactivates Ferret Visual Cortical Neurons**  
(A1 and B1) Mean firing rate (y axis) versus time (x axis) for AlstR-expressing (A1) and EGFP control (B1) ferrets. (A1) is a single-unit recording; (B1) is a multi-unit recording. In both plots, responses are to drifting grating stimuli of 99% and 35% contrast (black squares and dark gray circles, respectively); stimuli were optimized for spatial and temporal frequencies. Light gray triangles indicate responses to blank stimuli; the dashed line indicates a firing rate of 0 spikes/s. Timing of saline, AL, and muscimol applications to the cortical surface are indicated above each plot. The unit from the AlstR-expressing ferret (A1) inactivated quickly and completely in response to 0.1  $\mu$ M AL and began to recover 30 min after saline wash; units from the control ferret (B1) did not inactivate, even at 10- and 100-fold higher AL concentrations. Muscimol inactivated the control units, indicating that they did not correspond to recordings from afferent axons.  
(A2 and B2) Histology from ferrets shown in A1 and B1, respectively. Antibody staining for EGFP (black staining) reveals area of virus infection; lesion in (A2) and electrode track in (B2) (white arrows) mark recording sites. Scale bars, 250  $\mu$ m.  
(C) Summary data from three AlstR-expressing ferrets and four controls (two EGFP, two uninjected). Neurons from AlstR-expressing ferrets inactivated to  $3.3\% \pm 2.4\%$  of baseline firing rates in response to AL application and recovered to  $53.5\% \pm 3.7\%$  of baseline; neurons from control ferrets fired at  $97.0\% \pm 14.3\%$  of baseline levels after AL was applied.

contrasts with the results of our rat LFP experiments, in which complete recovery was achieved within minutes of saline wash. The lack of full recovery is likely to be due in part to loss of unit isolation over the course of the experiment: since it is not possible to monitor changes in spike shape or amplitude for units that are inactive, a fully recovered unit might sometimes fail to meet the spike shape criteria required for inclusion. The difference in recovery time likely reflects a difference in ability to remove AL from the cortical surface: in the ferret, unlike in the rat, a large physical gap exists between the skull and the brain, making it difficult to replace fluids once they are applied. In analogous

experiments performed in ferret LGN, where surrounding brain structures do not permit washout of AL, we observed a similar time course of recovery from AL-induced inactivation (see below).

Figure 5C shows summary data from the three AlstR-expressing ferrets examined in this study. Cells from AlstR-expressing ferrets fired at  $3.3\% \pm 2.4\%$  of baseline levels (in the 5–10 min time interval) following AL application and typically recovered to  $53.5\% \pm 3.7\%$  of baseline within 35–80 min of saline washout (see [Experimental Procedures](#) for details of analysis). Processed brain sections from the ferret represented in Figure 5A1 provided visual confirmation that recordings

were made from the region of virus infection (Figure 5A2). In the remaining ferrets, where histology was not recovered, correct placement of the electrode was inferred, as inactivation was never observed in non-AlstR-expressing ferrets.

AL had no effect on visual responsiveness or spontaneous firing of Area 17 neurons in control ferrets ( $n = 2$  ferrets, three recordings) infected with AAV1-EGFP (Figures 5B1 and 5C). This was true even when 100 times the effective dose of AL was applied. Application of the GABA<sub>A</sub> agonist muscimol resulted in complete inactivation of the neurons (Figure 5B1), confirming that recordings were from cortical neurons rather than from afferent fibers, which would have been unresponsive to AL regardless of the virus used. In all cases, examination of processed brain sections confirmed that recordings were made from the region of virus infection (see Figure 5B2 for an example). These results indicate that virus infection alone does not confer sensitivity to AL. Similar results were observed in recordings made in noninjected hemispheres of virus-injected ferrets ( $n = 2$  ferrets, two recordings). On average, neurons from EGFP-expressing and normal controls fired at  $97.0\% \pm 14.3\%$  of baseline levels following application of AL (Figure 5C). These results, combined with our observations in rat, support our conclusion that AL does not elicit any detectable effects in cortical neurons not expressing AlstR.

In addition to these normal and EGFP-only controls, three ferrets injected with AAV1-AlstR-EGFP in Area 17 showed extremely low AlstR expression (as inferred from anti-EGFP staining; see the Supplemental Data). In these cases, we did not observe any effect of AL application, providing further support to our conclusion that cells expressing little or no AlstR are not affected by the doses of AL administered.

A drop in activity in the experiments described here might be interpreted as a loss of unit isolation rather than inactivation of the cells whose activity was being recorded. This possibility is unlikely, as the observed reductions were always rapid, nearly complete, and well-correlated in time with AL application (e.g., Figure 5A1). Moreover, dramatic reductions in activity such as those observed here never occurred except in response to AL, indicating that AL was the likely mediator of those effects. We therefore conclude that the observed drops in activity reflect a response to AL rather than a loss of unit isolation.

The results described here indicate that in Area 17 of ferrets, complete inactivation of AlstR-expressing neurons occurs within minutes of AL application. Recovery of responses to 50% of baseline can occur within 40 min when AL cannot be quickly washed out. These effects are specific: the firing properties and gross morphology of neurons do not appear to be affected by AlstR expression, and AL has no detectable effect on non-AlstR-expressing neurons.

### Inactivation of Neurons in Ferret LGN

To investigate whether the AlstR/AL system can effectively inactivate neurons in thalamus, we injected AAV2-AlstR-EGFP into LGNs of adult ferrets. Extracellular recordings were made from the virus-infected regions of anesthetized, paralyzed ferrets after establish-

ment of AlstR expression. Ferrets viewed drifting grating stimuli on a computer monitor while AL or saline was pressure-injected into the region through separate barrels of a glass micropipette positioned in or near the LGN.

AlstR/AL-mediated inactivation in ferret LGN was rapid and complete. In the multi-unit recording shown in Figure 6A1, conducted in an LGN infected with AAV2-AlstR-EGFP, an initial injection of saline ( $2 \mu\text{l}$  at  $t = 5$  min) resulted in a small pressure artifact. A subsequent injection of AL ( $2 \mu\text{l}$  of  $0.1 \mu\text{M}$  at  $t = 11$  min) elicited a slightly larger firing rate reduction. Lack of complete inactivation here is probably due to reflux of cerebrospinal fluid into the pipette tip over the course of the experiment, as evidenced by the rising level of the meniscus observed in the pipette; this would cause the injected fluid to consist largely of cerebrospinal fluid with very little AL. A second AL injection ( $2 \mu\text{l}$  of  $0.1 \mu\text{M}$ ) from the same pipette, just 12 min later ( $t = 23$  min), elicited a long-lasting and nearly complete reduction in neuronal firing and responsiveness.

After a complete drop in activity at  $t = 28$  min (5 min after the second AL injection), cells remained inactive for about 20 min and then gradually recovered to above baseline firing rates at  $t = 88$  min, delineating an approximate 60 min time window for complete recovery. Because surrounding brain structures do not permit wash-out of AL after injection into the LGN, this time window represents the natural time course of recovery, which presumably reflects the time for dissipation and/or breakdown of AL. Two additional saline injections ( $2 \mu\text{l}$  each) applied after recovery ( $t = 99$  and  $110$  min) elicited small pressure artifacts similar to that observed for the initial saline injection. A final injection of  $2 \mu\text{l}$  of  $0.1 \mu\text{M}$  AL ( $t = 121$  min) caused a complete, albeit possibly briefer, reduction in firing. It was unclear whether the subsequent brief increase in measured spike rates (at  $t = 142$  min) reflected changes in unit isolation or true recovery, but the experiment was concluded before full recovery could be tested. Histological processing confirms that recordings from this ferret were made from the area of virus infection and that the AL-containing pipette was located in the LGN,  $300 \mu\text{m}$  lateral to the recording site (Figures 6A2 and 6A3).

The effects of AL were examined in three additional LGN recordings from three AlstR-expressing ferrets. Of these, one recording showed AL-induced inactivation and two failed to inactivate. In the former, the spike rate fell to 15% of baseline levels within 5 min of AL injection and remained near zero for over 4 hr. In the latter two recordings, cells fired at 84.4% and 139% of baseline levels following AL injection. In all three cases, examination of processed brain sections confirmed correct placement of both the recording electrode and the AL-containing pipette (see Experimental Procedures for inclusion criteria). Potential reasons for lack of inactivation in the latter two cases are discussed below.

AL injection had no effect on firing rates of LGN cells in control ferrets. Figure 6B1 shows firing rates, both spontaneous and visually evoked, from a multi-unit recording made in the LGN of a normal, non-virus-infected ferret. Two injections of AL at a 100-fold increased concentration ( $10 \mu\text{M}$ ,  $2 \mu\text{l}$  each) were administered during the 40 min recording session. In neither of these cases did

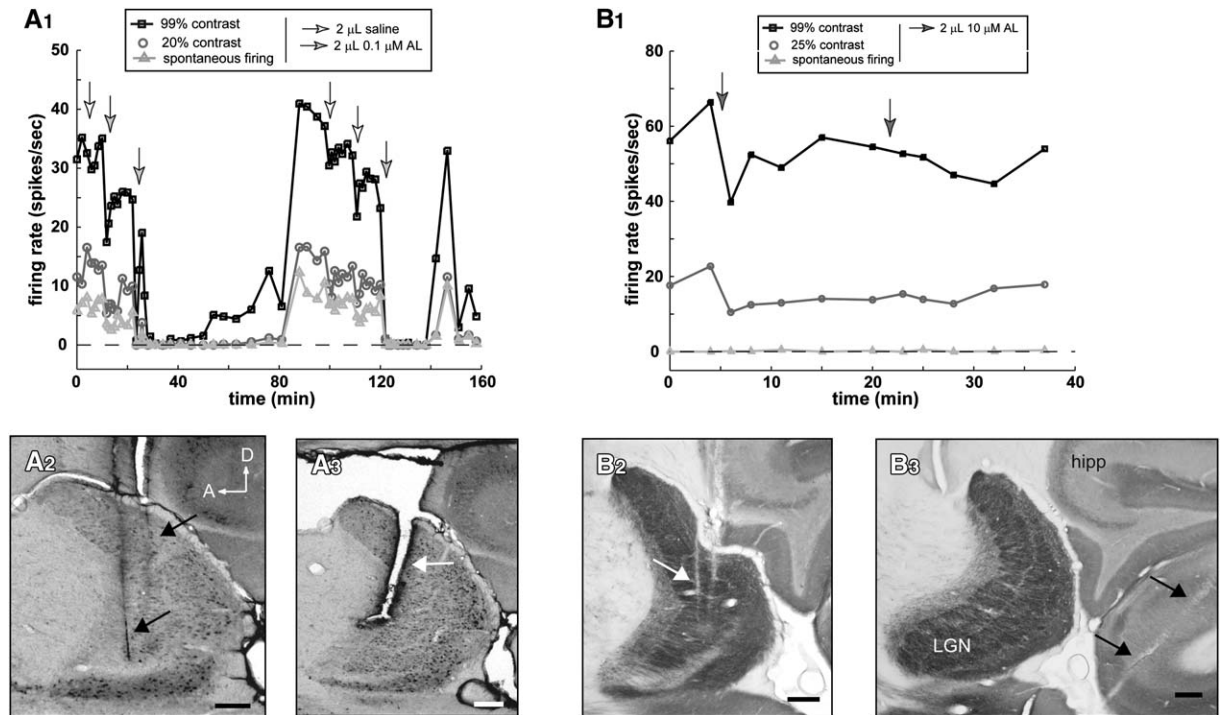


Figure 6. The AlstR/AL System Can Quickly and Reversibly Inactivate Ferret LGN Neurons

(A1 and B1) Multi-unit recordings from LGN of an AlstR-expressing (A1) and a non-virus-injected control (B1) ferret. Format of plots are same as in Figure 5; note different stimulus contrasts used. Arrows indicate times of injections of saline or AL into or near the LGN. AlstR-expressing neurons were inactivated in response to AL (gray arrows) but not to saline (white arrows); recovery occurs approximately 60 min later. Control neurons were not affected by AL.

(A2, A3, B2, and B3) Histology from ferrets shown in A1 and B1, respectively. LGN in (A) was stained for EGFP; LGN in (B) was stained for cytochrome oxidase. Arrows mark tracks from recording electrodes ([A2] and [B2]) and from the pipette used to inject AL ([A3] and [B3]); the pipette was located 300  $\mu$ m medial and 450  $\mu$ m lateral to the electrode for (A3) and (B3), respectively. Two electrode tracks are present in (A2) (arrows) and the recordings shown in (A1) are from the more anterior penetration. Scale bars, 250  $\mu$ m; top, dorsal; left, anterior; hipp, hippocampus.

injection of AL noticeably reduce the firing rate. Histological processing from this ferret confirms that the recording was made in the LGN and that the injection pipette was located near the LGN, 450  $\mu$ m medial to the recording site (Figures 6B2 and 6B3).

Similar results were observed in additional recordings from control ferrets ( $n = 12$  recordings, one normal ferret, one ferret infected with AAV2-EGFP), even at 1000 times the effective AL dose (100  $\mu$ M). On average, control neurons fired at  $87.4\% \pm 5.2\%$  of baseline firing rates following injection of AL. The slight decrease in firing rate most likely reflects a loss of unit isolation over the course of the experiment, rather than a reduction caused by AL. Processing of brain sections confirmed proper placement of the recording electrode and AL-containing pipette. Taken together, these observations indicate that AL does not have any apparent endogenous effects on thalamic neurons.

The experiments described here demonstrate that it is possible to inactivate ferret LGN cells using the AlstR/AL system. When inactivation occurs, it is quick, complete, and long-lasting. Effects are specific, as application of AL does not affect the activity of cells not expressing AlstR. There are cases, however, where inactivation was not achieved. In these cases, several explanations are possible. The most likely explanation is that AL did not reach cells at the recording site in some cases, likely

because the distance between recording electrode and AL-containing pipette was greater than estimated by our methods, as it is difficult to pinpoint the exact locations of the electrode and pipette tips. Even when the electrode and pipette were well-positioned, AL may not have successfully traversed the distance of dense subcortical tissue to reach its site of action. A second possibility is that recordings were from non-AlstR-expressing cells despite being in a virus-infected region, as we cannot correlate our recordings to virus infection on a cell-by-cell basis. Finally, it remains a possibility that AL does not reliably inactivate AlstR-expressing cells in ferret LGN, even when it reaches those cells in appropriate concentrations. Because it was impossible to control all variables in these experiments, and because we have few recordings from ferret LGN, it is difficult to determine unequivocally which of these potential explanations is correct.

#### Inactivation of Neurons in Monkey LGN

The AlstR/AL system is a tool that could prove very useful in organisms such as monkeys, where neural structure/function/behavior relationships are heavily studied but standard transgenic methods for manipulating gene expression are not practical. To test whether the AlstR/AL system can effectively inactivate monkey

neurons, we expressed AlstR in the LGN of a macaque monkey and tested responses of LGN cells to AL.

AAV2-AlstR-EGFP was injected into the LGN of a macaque monkey. Virus was injected into all layers of the LGN and in regions that spanned a range of parafoveal receptive fields (2–20° azimuth [az], –12–0° elevation [el]), as determined from electrical recordings made from the virus-containing pipette immediately prior to virus injection. Extracellular recordings were then made from the LGN of the monkey during four recording sessions conducted approximately 1, 1.5, 2, and 16 months after virus injection. The monkey was anesthetized and paralyzed for each recording session, during which optimized drifting grating stimuli were presented. Responses to visual stimuli, as well as spontaneous firing rates, were recorded before and after pressure injection of AL through a glass micropipette positioned in or near the LGN.

Figure 7 shows the results from a representative recording session. The first recordings of this series, shown in Figure 7A, were from multiple units that had a receptive field location outside of the range covered by our virus injections (0.7° az, –4.8° el). Firing rate and visual responsiveness did not change in response to AL injection, presumably due to lack of AlstR expression at the recording site. In two additional recordings from this penetration, neurons similarly did not inactivate following additional AL injections.

After repositioning the electrode to a location where recorded receptive field positions more closely matched those at the sites of virus injection, a second set of recordings was made. The recordings shown in Figure 7B, conducted 4 hr after those shown in Figure 7A and representative of the second set of recordings, responded to visual stimulation at 5.4° az and –11.1° el. Activity dropped briefly and recovered in response to the initial two injections of 0.2 μM AL. Lack of complete inactivation in these cases is likely due to reflux of cerebrospinal fluid into the tip of the pipette, as evidenced by changes in the position of the meniscus observed within the pipette; this is a strong possibility in light of the small volumes injected (0.3 μl and 0.5 μl, respectively). After a third AL injection (1.5 μl of 0.2 μM), cells inactivated completely: activity fell to 11.1% of baseline values within 5 min of AL injection, and cells inactivated completely within 10 min of AL injection.

After inactivation of the cells shown in Figure 7B, more than 60 min elapsed without recovery. At this time, the electrode was advanced ventrally in small increments, covering a distance of 600 μm. There was very little recorded activity along this stretch of LGN, likely reflecting continued inactivation of most neurons in the vicinity. We were, however, able to occasionally locate weakly visually responsive neurons along this stretch. One such neuron, recorded 90 min after the previous AL injection, completely inactivated in response to a new AL injection (1.8 μl of 0.2 μM). Five minutes later the electrode was moved again, and 130 min after the previous AL injection, another visually responsive neuron was isolated. Data from this recording are illustrated in Figure 7C. The neuron quickly inactivated and recovered several times in response to repeated injections of AL (0.4–0.8 μl of 0.2 μM). This recording is likely reflective of atypical neurons, perhaps expressing low levels of

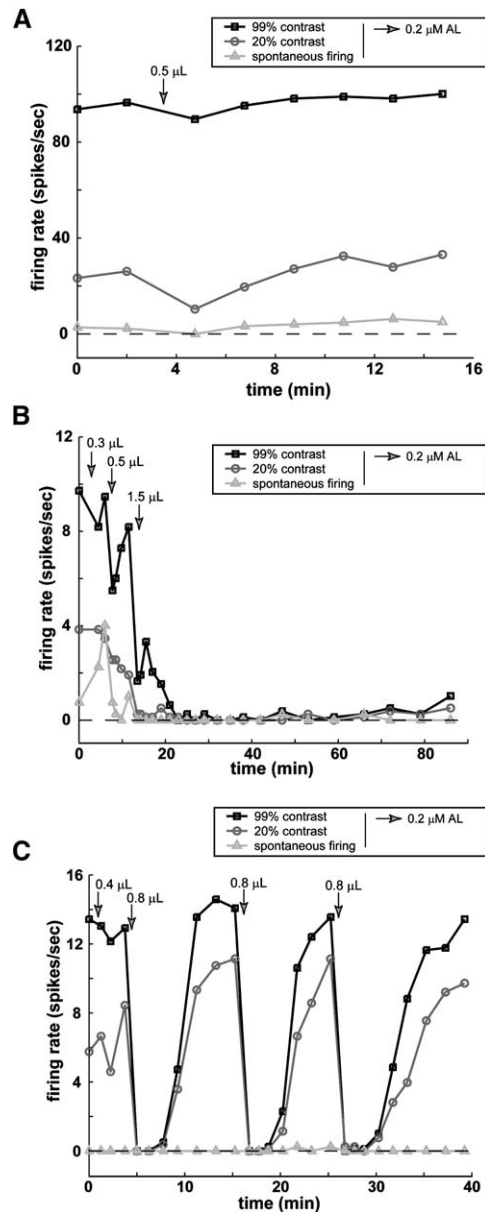


Figure 7. The AlstR/AL System Can Quickly and Reversibly Inactivate Monkey LGN Neurons

Recordings at three different sites ([A]–[C]), all from the same recording session in the LGN of a monkey expressing AlstR, are illustrated in chronological order. Format of plots is the same as in Figure 5 (note different stimulus contrasts used). (A) Recording from a single unit whose receptive field location was outside of the range covered by AlstR virus injections. This cell was not affected by addition of AL, presumably due to lack of AlstR expression. (B) Multi-unit recording, made 4 hr after the recording shown in (A); this recording was from a new penetration where receptive fields aligned with those at the site of AlstR virus injection. AL injections resulted in complete silencing so that even responses to optimal stimuli were eliminated. Inactivation remained nearly complete for approximately 70 min; very little activity could be detected, even at other sites within 600 μm of this site when the electrode was repositioned 90 min after AL injection. (C) Single-unit recording made from the same penetration as in (B), 130 min after the last AL injection indicated in (B). This cell inactivated and recovered quickly and repeatedly in response to repeated AL injections.



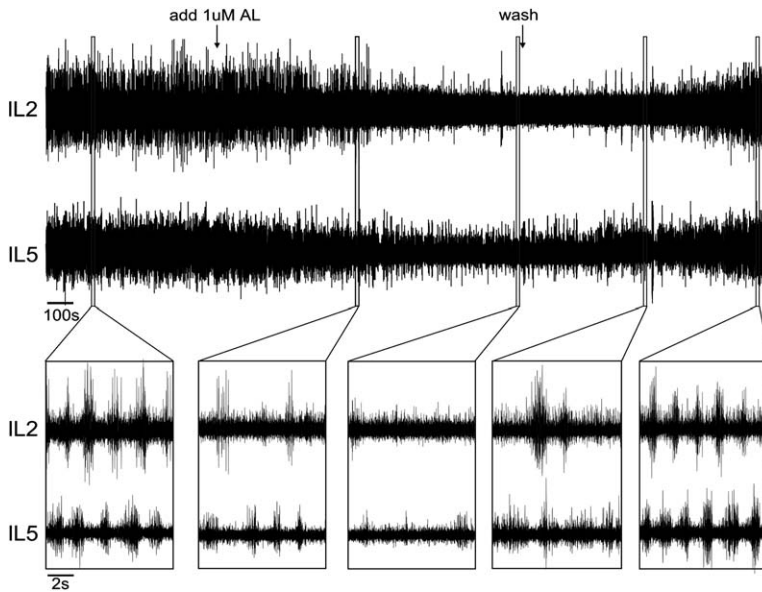


Figure 8. The AlstR/AL System Quickly and Reversibly Inactivates Mouse Spinal Motor Neurons

Ventral root electroneurogram recordings from left L2 and left L5 ventral roots in isolated spinal cords of P0 *nestin<sup>Cre</sup>; AlstR192* mice. Recordings shown at the top are at a slow timescale while insets show selected points at a faster timescale. Rhythmic motor activity was induced by drug application to the preparation (see [Experimental Procedures](#)). The rhythmic activity alternating between the L2 and L5 ventral roots can be seen in the first inset. After adding 1  $\mu$ M AL to the bath, activity gradually reduced over the next 5–10 min. Reduced activity and disrupted rhythmicity are apparent in the second inset, and nearly complete inactivation of motor activity is apparent in the third inset. After the AL is washed out of the bath, normal activity gradually recovers and appears similar to activity before AL.

AlstR: such neurons would be less sensitive to AL and able to recover from inactivation more quickly. In the recordings shown here, it is likely that the initial AL injection was insufficient to elicit long-lasting inactivation, whereas subsequent injections transiently increased the local AL concentration adequately to briefly abolish activity.

As illustrated above, we found cells that were both responsive and unresponsive to AL in each of our first three recording sessions in monkey. In cases where inactivation was not observed, there are several potential explanations: (1) recordings may have been made from non-AlstR-expressing cells; (2) the AL pipette may have been far from the recording site, making it impossible for critical concentrations of AL to reach the cells being recorded; and (3) cerebrospinal fluid, rather than AL, may have been injected from the pipette tip due to reflux. The first two are very likely possibilities, as we had no histological confirmation of electrode or pipette positions following our recording sessions. In view of the observation that nearly all neurons appeared to be inactivated in a particular region whenever inactivation was observed (see above), other explanations, such as variability in intrinsic properties of neurons or susceptibility to inactivation by these methods, seems unlikely. Because our number of recordings is small, it remains a formal possibility that, even under ideal conditions, AL-induced inactivation might not occur reliably in the monkey LGN.

A fourth recording session was conducted in the same monkey, 16 months after injection of virus into the LGN. In this session, we performed just one recording from a group of cells with a receptive field of 11.4° az, -6.7° el. Although this receptive field location corresponds to the area injected with virus, we did not observe a response to AL. This could be due to dropoff of AlstR expression during the 16 month period following virus injection. However, because AAV2 has been shown to confer stable gene expression lasting more than 18 months ([Xiao et al., 1997](#)), the lack of inactivation is more likely to be due to one of the reasons described

above. Because we did not process histological sections from this monkey, we have no physical indication of what the AlstR levels were.

#### Inactivation of Neurons from Mouse Spinal Cord

Mice are an excellent mammalian species for manipulating gene expression and have been widely examined in a variety of research areas, making them an ideal organism for future applications of the AlstR/AL system. To examine whether the AlstR/AL system can reversibly silence neurons in transgenic mice, we generated transgenic mice that conditionally express AlstR and EGFP following Cre recombination (*AlstR192* mice). We described these mice in a previously published study in which AL-induced, reversible inactivation of a specific class of spinal cord inhibitory neurons was shown to result in specific changes in locomotor activity ([Gosgnach et al., 2006](#)). We reiterate the utility of these mice here by illustrating effects of more widespread inactivation of spinal cord neurons. In these experiments, the *AlstR192* mice were crossed with mice expressing Cre from the nestin promoter (*nestin<sup>Cre</sup>; AlstR192* mice), which results in widespread expression of EGFP (and presumably AlstR) throughout the population of spinal cord neurons ([Gosgnach et al., 2006](#)). Locomotor-like oscillations, characterized by repetitive oscillatory bursting of motor neurons, were induced in the isolated spinal cord by applying the excitatory neurotransmitter agonists N-methyl-D-aspartate and 5-hydroxytryptamine ([Gosgnach et al., 2006](#)). Before addition of AL, agonists induced a pattern of alternating left flexor activity (IL2) and left extensor activity (IL5) in spinal cords isolated from *nestin<sup>Cre</sup>; AlstR192* mice ([Figure 8](#)). Application of 1  $\mu$ M AL strongly silenced rhythmic motor activity, and washout of AL resulted in recovery of rhythmic motor activity within 5 min. In contrast, allatostatin (100 nM–5  $\mu$ M) had no effect on rhythmic motor activity in isolated spinal cords of control mice (data not shown). These results indicate that the AlstR/AL system can be used to inactivate neurons from transgenic mice in a selective and quickly reversible manner.

## Discussion

Here we have shown that the AlstR/AL system is an effective genetic method for quick and reversible inactivation of mammalian neurons *in vivo*. Using AAV to express AlstR in thalamic and cortical neurons of rat, ferret, and monkey, we have demonstrated that the AlstR/AL method can effectively inactivate both field potential and single-unit activity. Application of AL also resulted in reversible inactivation of spinal cord neurons of transgenic mice conditionally expressing AlstR. Taken together, these results indicate that the AlstR/AL system can be used *in vivo* to effectively inactivate a wide range of neuron types in a variety of mammalian systems.

In addition to its versatility, the AlstR/AL system has many features that make it well-suited for detailed studies of neural circuitry, perception, and behavior. An important feature of this system is its high degree of specificity. Even at 100–1000 times the effective dose, AL does not have detectable effects on neurons not expressing AlstR. Moreover, AlstR is unresponsive to a variety of common mammalian peptides (Birgul et al., 1999), suggesting that expression of AlstR alone does not affect the excitability of AlstR-expressing cells. This is consistent with our observation that visual neurons had normal activity levels and tuning properties in the absence of AL. These characteristics make the AlstR/AL system ideal for targeting cells for inactivation without unwanted side effects.

Another attractive feature of the AlstR/AL system is its potency. Application of AL to neurons expressing AlstR could completely inhibit spiking, leaving no residual activity, even to optimized visual stimuli. AL also very reliably inactivated cortical LFP activity in rats expressing AlstR. Although some activity remained after AL application, in most cases this residual activity likely represents the activity of neurons with low levels or no expression of AlstR. This conclusion is supported by our single-unit recordings in ferret and monkey, where spiking could be completely abolished in individual neurons.

All of our results are consistent with the conclusion that when AL reaches AlstR-expressing neurons, inactivation is reliable and complete. When inactivation was not observed, it is likely that either AL did not reach the AlstR-expressing neurons or the recorded neurons did not express AlstR. In our LGN experiments, we encountered difficulties in assuring that AL was delivered to the AlstR-expressing cells. In some of these cases, we could not later verify whether recorded neurons expressed AlstR, thus confounding clear interpretation of the cause of the negative result. Because of these limitations, our method will prove easiest to use when applied to neurons that can be readily accessed by AL—on the cortical surface or in subcortical structures adjacent to a ventricle, for example. Although we cannot rule out the possibility that the AlstR/AL system is unable to inactivate some LGN cell types, our results remain consistent with the potential for inactivation of all cell types.

The time course of AL-mediated inactivation and recovery makes it ideal for many physiological and behavioral studies. The temporal resolution of AlstR/AL-mediated inactivation is limited only by the speed with which AL can be delivered and then removed. The primary factors influencing speed of inactivation there-

fore appear to be diffusion and proximity. Even when AL is applied up to 1 mm distally (e.g., cortical neurons far from the brain surface), AlstR-expressing cells inactivate within minutes, indicating that the time limitation posed by these factors is minimal. Inactivation persists without desensitizing in the continued presence of AL, and recovery is similarly rapid when AL is efficiently removed from the area, as in our rat LFP experiments. Recovery occurs more slowly when AL is not easily removed: in our ferret experiments, for example, recovery typically required an hour or more. The ability to achieve inactivation within minutes and maintain that inactivation for extended periods of time makes it a very useful method for physiological studies.

In these experiments, we studied the effects of direct application of AL to neurons. We did not test the possibility of inactivation by delivering AL to the ventricles or systemically. AL delivered to the ventricle is likely to inactivate AlstR-expressing neurons along or near the ventricular surface, as AL was able to inactivate neurons when applied to surfaces 1 mm distally in our cortical experiments. Systemic delivery may not be as successful, as a peptide such as AL is unlikely to cross the blood-brain barrier. For this reason, future development of small-molecule AlstR ligands capable of overcoming this limitation would be helpful. However, systemic administration would likely result in some loss of temporal control and would eliminate specificity obtainable by applying AL locally. The level of temporal control could, nevertheless, be satisfactory for conducting behavioral experiments.

Several genetic methods for perturbing neuronal activity exist, and the choice of method for a particular application will depend on the temporal resolution desired. Light-based methods (Banghart et al., 2004; Boyden et al., 2005; Li et al., 2005; Lima and Miesenböck, 2005; Zemelman et al., 2002, 2003) offer millisecond temporal control, and are ideal for studying issues such as the effects of spike timing on synaptic plasticity. The MIST system (Karpova et al., 2005) reversibly blocks synaptic transmission with temporal control on the order of hours: such a system is well-suited for behavioral studies, in which the desired period of inactivation is hours to days. A disadvantage of this method is that the extent of transmission block has not been directly measured *in vivo* and therefore remains unknown. Long-term inactivation of neurons via temporally regulated gene expression (e.g. Johns et al., 1999) are best suited for studies of development and plasticity, where the desired period of inactivation is days to weeks. Finally, the AlstR/AL system holds a unique position as a method with a temporal resolution ideal for electrophysiological studies of circuit function: the timescale of this method is rapid enough to allow direct comparisons of a normally functioning circuit with its selectively inactivated counterpart within the brief period during which stable recordings can be obtained.

In conclusion, the AlstR/AL system is a promising method for reversibly inactivating mammalian neurons *in vivo* on a timescale of minutes and extending up to hours. Here, we have shown that the AlstR/AL method can be used in a variety of mammalian systems, demonstrating its wide applicability. When combined with promoter-based, cell-type-specific expression, this

method will greatly advance our understanding of neural circuits and behavior (Gosgnach et al., 2006). In the future, in vivo development of other genetically based methods for altering neural excitability will likely complement this one to further enrich our understanding of brain function.

## Experimental Procedures

### Adeno-Associated Virus Preparation

AAV was prepared according to methods described previously (Rabinowitz et al., 2002; Xiao et al., 1998; Zolotukhin et al., 1999). AAV was generated by transfection of 293T cells in 150 mm dishes with 22.5  $\mu\text{g}$  of pXX6-80 (Ad5 genome), 7.5  $\mu\text{g}$  of either pXR1 (for serotype 1) or pXX2 (for serotype 2), and 7.5  $\mu\text{g}$  of a cloning vector containing an expression cassette flanked by the AAV ITRs by using lipofectamine 2000 (Invitrogen) according to the instructions. Cells were harvested at 48–72 hr after transfection, resuspended in 15 ml of Gradient Buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 10 mM  $\text{MgCl}_2$ ), subjected to four cycles of freeze/thaw in addition to passing through a syringe with a 21G–23G needle, and treated with 50 U/ml of Benzonase (Sigma) for 30 min at 37°C. Clarified supernatants containing AAV were obtained by centrifugation (3000  $\times$  g, 15 min, 4°C), and virus was purified using iodixanol gradients as described elsewhere (Zolotukhin et al., 1999). The titer of AAV genome-containing particles per milliliter was determined by real-time PCR using SYBR Green I double-stranded DNA binding dye and an ABI Prism 7700 sequence detection system (PE Biosystems).

Four viral constructs were used in this study. These viruses differed from each other only in the genetic sequence that was packaged into the viral genome and/or the capsid protein (serotype 1 or 2). The viruses were (1) an AAV1 containing an AlstR-IRES2-EGFP expression cassette under the control of a synapsin promoter (termed “AAV1-AlstR-EGFP,” initial titer  $9.2 \times 10^{11}$  particles/ml); (2) an AAV2 containing the same promoter and cassette (termed “AAV2-AlstR-EGFP,” initial titer  $1.35 \times 10^{10}$  particles/ml); (3) an AAV1 encoding EGFP under the control of a CMV promoter (termed “AAV1-EGFP,” initial titer  $1.5 \times 10^{11}$  particles/ml); and (4) an AAV2 encoding EGFP under the control of a synapsin promoter (termed “AAV2-EGFP,” initial titer  $3.83 \times 10^9$  particles/ml).

### Rat Experiments

All animal procedures described in the [Experimental Procedures](#) section were approved by the Salk Institute Animal Care and Use Committee.

#### Virus Injections

Six rats aged 33–37 days postnatal at the time of AAV injection were used in this study. Rats were initially anesthetized in a chamber containing 2.5% isoflurane. Animals then were intubated and placed in a stereotaxic apparatus, with anesthesia maintained using inhaled isoflurane (1.5%–2% in oxygen). End-tidal  $\text{CO}_2$  ( $\text{EtCO}_2$ ), Pulse oxygen ( $\text{SpO}_2$ ), and heart rate were monitored continuously to judge the animals' health and to maintain proper anesthesia levels. A small craniotomy was then made over the area of interest, and the underlying dura was slit in several locations to allow penetration by the virus-containing pipette. AAV was pulled into a glass micropipette (~30  $\mu\text{m}$  tip diameter) by suction, and injected by pressure using a Picospritzer II (General Valve Corp.) at a rate of 0.2–2  $\mu\text{l}/\text{min}$  in 10–20 ms bursts of pressure (10–40 psi). A total of 5–8  $\mu\text{l}$  of virus was typically injected at 3–5 sites at depths of 300  $\mu\text{m}$ , 600  $\mu\text{m}$ , and 1 mm from the cortical surface. After virus injections, Gelfoam (Pharmacia & Upjohn Company, MI, USA) was placed over the craniotomy and the scalp was sutured shut. We then waited 3–9 weeks for expression of AlstR and/or EGFP.

#### Field Potential Recording and Data Collection

Field potential recordings were made at least 35 days after AAV injection, and at this time rats were aged 71–122 days postnatal (“adult”). In preparation for recording, rats were initially anesthetized in a chamber containing 2.5% isoflurane in oxygen. Animals were then intubated and surgical anesthesia was maintained using 1.5%–3% isoflurane in oxygen. After placing the rat in a stereotaxic apparatus, the scalp was retracted to expose the pre-existing craniotomy; the craniotomy was expanded as necessary if healing had

occurred. The dura and scar tissue around the site of virus injection was removed. Following the initial surgical procedure, anesthesia was maintained with 1%–1.5% inhaled isoflurane: EKG,  $\text{SpO}_2$ ,  $\text{EtCO}_2$ , and heart rate were monitored continuously to judge the animal's health and to maintain proper anesthesia levels. Whiskers were then trimmed contralateral to the recorded barrel cortex and the cheek pierced with 27G needles used to stimulate the whisker pad with an electric pulse (10 V, 1 ms duration, 0.1 Hz) generated from a Grass S44 stimulator and SIU5 stimulus isolation unit (Grass Instrument). LFP recordings were made using epoxyite-coated tungsten electrodes (1 M $\Omega$  resistance, FHC, Bowdoinham, ME). Signals from 300 Hz to 10 kHz were passively filtered and amplified using a DAM-50 amplifier (WPI) and actively filtered at 60 Hz using a HumBug (Quest Scientific), and sent to a computer running custom LabVIEW 7.1 software (National Instruments) for data storage.

#### AL and Saline Application

The *Drosophila* allatostatin peptide Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH<sub>2</sub> (Birgul et al., 1999) was synthesized in-house and stored in 100  $\mu\text{M}$  aliquots in water at  $-80^\circ\text{C}$ . For experiments, AL was diluted to its final concentration in saline (0.9% NaCl) and stored on ice until use. To test effects on cortical neurons, a saturating volume of AL was dropped onto the cortical surface using a handheld manual pipette. For saline washes, fluid from the craniotomy was absorbed by applying a cotton swab to the portion of skull surrounding the craniotomy. After removal of most fluid from the craniotomy, additional saline was then applied. This procedure was repeated several times for a given wash.

#### Data Analysis

Field potentials recorded in rat barrel cortex were collected at a sampling rate of 10 kHz for a duration of 0.5 s, beginning 5 ms prior to each electrical stimulus. We used LabVIEW 7.1 software to control stimulus timing and NI DAQPad-6015 (National Instruments) for data acquisition. To correct for rapid fluctuations in the LFP, raw data were smoothed by replacing the value at each time point by the average of 10 values preceding and following that data point. All subsequent analyses were then based on these smoothed LFPs (e.g., [Figure 2](#)). Peak values, taken as the highest positive value in the signal after termination of the stimulus artifact, were then plotted for the entire length of the experiment as shown (e.g., [Figure 3](#)). An alternative analysis used the total area under the initial positive deflection of the LFP as a quantitative measure of the response to whisker stimulation and yielded essentially the same results (see [Results](#)).

To estimate the effects of AL on LFP activity, changes in LFP peaks and areas following AL application and recovery were compared to the effects of muscimol. Since muscimol presumably results in complete inactivation of cortical neurons, any activity remaining after muscimol is assumed to reflect contributions from other sources, such as afferent axons terminating in the recorded region. Therefore, the average LFP values during the final 3 min of recordings following muscimol application were defined as a baseline value and subtracted from all other LFP measures. Percent inactivation and recovery were calculated as the average LFP response during the 3 min following AL application and saline washout, respectively, divided by the average response 3 min prior to AL application, multiplied by 100.

#### Histology

After cortical recordings, electrolytic lesions ( $-3$  to  $-4 \mu\text{A}$ , 3–5 s) were made to mark the recording site. In some experiments, electrodes were coated with Dil (0.25% in 100% ethanol, then dried) to aid recording site identification. After lesions were made, the animal was deeply anesthetized with an overdose of sodium pentobarbital (>100 mg/kg, i.p.). The animal was then perfused transcardially with a phosphate buffered saline (PBS) rinse, followed by fixation with 4% paraformaldehyde in PBS. The brain was removed and cryoprotected overnight in a solution containing 30% sucrose in PBS. It was then frozen and cut sagittally into 40  $\mu\text{m}$  sections using a freezing microtome. Cytochrome oxidase (CO) staining was performed using methods described elsewhere (Wiser and Callaway, 1996). Sections were then antibody-stained for EGFP as follows: endogenous peroxidase activity was quenched using 10% hydrogen peroxide in PBS, and sections were incubated in a blocking buffer (10% normal goat serum, 2% bovine serum albumin, 0.25% Triton X-100, in PBS). Sections were then incubated in a rabbit polyclonal antibody against



EGFP (Molecular Probes, 1:1000 in blocking buffer), followed by an incubation in a biotinylated anti-rabbit secondary antibody (Vector Laboratories, 1:200 in blocking buffer). Sections were then incubated in an HRP-conjugated avidin-biotin complex (ABC Peroxidase Standard Kit, Vector Laboratories), and HRP localization was revealed by reacting the sections in a solution containing 0.05% DAB, 0.028% cobalt chloride, 0.02% nickel ammonium sulfate, and 0.0015% hydrogen peroxide in PBS. Finally, sections were mounted on gelatin subbed slides, dried, dehydrated, cleared, and coverslipped in Permount (Fisher Scientific).

### Ferret Experiments

Fifteen adult female ferrets (*Mustela putorius furo*, 0.7–1 kg) were used in this study. Ten ferrets were used in cortical studies, and for each of these, AAV was injected into Area 17 of a single hemisphere. Of these ten ferrets, eight were injected with AAV1-AlstR-EGFP and two with AAV1-EGFP. One animal injected with AAV1-AlstR-EGFP received recordings only in the noninjected hemisphere; one animal injected with AAV1-EGFP received recordings from both injected and noninjected hemispheres. All others received recordings only in the injected hemisphere. Five ferrets were used in the LGN studies, and four of these had AAV injected into the LGN in a single hemisphere; three were injected with AAV2-AlstR-EGFP and one with AAV2-EGFP. All four of these animals received recordings in the injected hemisphere. LGN recordings were also made from a fifth ferret that was not injected with AAV.

### Virus Injections

Virus injections in ferret visual cortex were performed as described above for rat experiments, with the following exceptions. Recordings were first made with a tungsten electrode to identify potential injection sites (see below for recording methods); a silver wire was then inserted into the virus-containing pipette for electrical confirmation of receptive fields at the sites of virus injection. A total of 4–8  $\mu\text{l}$  of virus was typically injected at several depths along two to four penetrations of the cortex per ferret. Additionally, red and green latex microspheres (LumaFluor, Inc) were injected near the site of virus injection to facilitate subsequent identification of the virus-infected area for recording purposes.

Virus injections in ferret LGN were performed as described above for cortical injections, with the following exceptions. Ferrets were initially anesthetized with an intramuscular injection of 40 mg/kg ketamine prior to intubation and subsequent maintenance of anesthesia with inhaled isoflurane. Virus was targeted to the ventral portions of the LGN, corresponding to central visual fields. A total of 8–12  $\mu\text{l}$  of virus was typically injected along two to four penetrations per ferret LGN.

### Electrophysiological Recordings

Surgical preparation for recording was conducted as described above for rats, except that ferrets with LGN virus injections were initially anesthetized with an intramuscular injection of 40 mg/kg ketamine rather than in a chamber containing isoflurane. Additionally, dexamethasone (0.5 mg/kg) and atropine (0.05 mg/kg) were administered to all ferrets intramuscularly in order to reduce brain swelling and salivation, respectively. For stabilization of cortical recordings in some ferrets, cerebrospinal fluid (CSF) was removed from the ventricle via a cisternal puncture, creating a large gap between the skull and brain surface. Even when CSF was not removed, a sizeable gap ( $\sim 1$  mm) existed between the skull and brain of the ferret.

Following the initial surgical procedure, anesthesia was maintained with 1%–2% inhaled isoflurane (LGN recordings) or 0.5%–1% isoflurane in a 2:1 mixture of oxygen and nitrous oxide (cortical recordings). Ferrets were paralyzed with pancuronium bromide (0.1–0.2 mg/kg/hr, i.v. or i.p.) and artificially ventilated. Pupils were dilated with 1% atropine, nictitating membranes were retracted with 1% phenylephrine hydrochloride, and corneas were protected with noncorneal, gas-permeable contact lenses. EEG, EKG,  $\text{SpO}_2$ ,  $\text{EtCO}_2$ , heart rate, and body temperature were continuously monitored to judge the animal's health and to maintain proper anesthesia levels.

Recordings were made using epoxy-coated tungsten electrodes (FHC, Bowdoinham, ME) of 2–5 M $\Omega$  resistance. Before collecting data, receptive fields were tested for correspondence with those recorded at the time of virus injection (in cortical experiments, latex microspheres aided in correct electrode placement). Signals

were passively filtered and amplified using a DAM-50 amplifier (WPI), and actively filtered at 60 Hz using a HumBug (Quest Scientific); spikes were sorted online and spike times were stored, along with stimulus parameters, using custom software (PEP, Dario Ringach).

In some cortical experiments, data were collected using quartz/platinum-tungsten electrodes and a multielectrode drive (Mini-05 microdrive, Thomas Recording Inc; electrode impedances 1–2 M $\Omega$ ). Neuronal signals were recorded extracellularly and waveforms were stored using the Multichannel Acquisition Processor system (Plexon, Inc). Single neurons were isolated on-line for analysis with Rasputin software (Plexon, Inc), and again off-line with Plexon Off-line Sorter (Plexon, Inc).

### Visual Stimulation

Stimuli were generated by a Silicon Graphics O2 computer, 24-bit color, using custom software (PEP, Dario Ringach) and were displayed on an SGI GDM-17E21 CRT display at 100 Hz refresh rate. Stimuli were typically circular patches of drifting sinusoidal grating, of radius  $1^\circ$ – $2^\circ$ , presented on a constant gray background (each linearized gun at half-maximal intensity), of mean luminance  $\sim 28$  cd/m $^2$ . Stimuli were shown for 4 s at a distance of 100 cm from the animal. For some minimally responsive neurons, a square-wave grating was shown on a black background for the entire recording session. Stimulus location, spatial and temporal frequencies, and orientation were optimized, and stimulus parameters were kept constant for each unit. Each stimulus was presented twice, and a blank trial appeared after each grating stimulus to acquire a measure of spontaneous firing rate.

### AL and Saline Application

For cortical recordings, AL application and saline washes were performed as described above for rat experiments. For LGN experiments, AL was drawn into a glass micropipette similar to those used for virus injections. The micropipette was inserted at an angle, and its tip was positioned at the same depth as, and 0.5–1 mm away from, the tip of the recording electrode. In some experiments, a silver wire inserted into the AL-containing pipette enabled electrical recordings, and the pipette's position in the LGN was confirmed by detection of visually responsive cells with peripheral receptive fields. AL was injected from the micropipette by pressure as described for virus injections using a Picospritzer II (General Valve Corp.).

In some LGN experiments, saline and AL were injected through separate barrels of a triple-barrel pipette. In these cases, pipettes were pulled on a List-Medical puller (L/M-3P-A) from standard-wall triple-barrel capillary glass (FHC), and tips were broken to about 40  $\mu\text{m}$ . Barrels were back-filled using MicroFil needles (WPI), polyethylene tubing was inserted into the ends of the barrels, and quick-setting epoxy was applied at the junction to create an airtight seal. Positioning of the pipette, electrophysiological confirmation of LGN placement, and AL injection were conducted as described above.

### Data Analysis

For both cortical and LGN experiments, mean spike rates in response to two presentations of each visual stimulus were averaged and plotted as shown in the Results. In Figure 4, spontaneous firing rates represent average responses to blank trials presented during a single stimulus set; in Figures 5 and 6 and Figure S1 in the Supplemental Data, spontaneous firing rates are calculated from blank trials presented throughout the entire length of the recording session.

For calculations of silencing and recovery in cortical experiments, responses to 99% contrast visual stimuli were used. Baseline was taken as the average response during the 10 min (or fraction thereof, if data was not collected for 10 min) preceding AL application, regardless of AL concentration. Responses were averaged over 5 min bins following any fluid application. Percent remaining activity after AL treatment was calculated from the 5–10 min bin following application of 0.1  $\mu\text{M}$  AL (two ferrets) or 0.05  $\mu\text{M}$  AL (one ferret); in one case, a 0.01  $\mu\text{M}$  concentration of AL had been previously applied, with no effect. Recovery was taken as the 5 min bin yielding the highest average response to a 99% contrast stimulus after application of the saline wash. Responses during these 5 min bins were divided by baseline responses to yield the silencing and recovery indices reported.

In control experiments conducted in ferret cortex, no reduction in firing was observed in response to AL applied in a range of doses from 0.1–10  $\mu\text{M}$ . To calculate percent remaining activity in these



cases, mean responses during the 5–10 min bin following each AL application were averaged to yield a single mean response for each ferret. This response was then divided by the baseline response to yield the silencing index shown in results. All calculations were made using responses to 99% contrast stimuli.

In LGN experiments, AL was injected multiple times during multiple recordings for a given animal. In order to eliminate possible effects of previous AL applications from our analysis, we included in our analysis only responses to the first AL injection from the first recording in each ferret in which AL was applied (with one exception; see below). For both *AlstR*-expressing and control ferrets, inactivation was calculated as described for cortical experiments. Because no response to AL was observed in control ferrets, recordings were included in our analysis even if AL had been previously applied.

The following are criteria for exclusion of recordings, both cortical and thalamic unless otherwise indicated, from our analysis: (1) AL had previously been applied to the area, (2) cells were not visually responsive, (3) the recording site could not be identified after the experiment, (4) the recording site was found to lie outside of the virus-infected area, (5) injury discharges contributed to the spike count during the experiment, (6) the recording did not silence upon muscimol application, or did not exhibit orientation tuning if muscimol was not applied (cortical experiments only), or (7) the medial-lateral distance between the recording electrode and AL-containing pipette was not 0.2–1 mm (LGN recordings only). The last criterion was intended to exclude potential pressure artifacts while ensuring that the AL pipette was positioned reasonably close to the electrode. There was one exception to these exclusion criteria: one LGN experiment was included in which AL had been applied 4.5 hr previously, but the recording was made in a new electrode penetration.

#### Histology

Electrolytic lesions were made at the recording sites as described above for rat experiments. Quartz/platinum-tungsten electrodes used in some ferret recordings (see above) were incapable of producing electrolytic lesions; in these cases, a Dil-coated tungsten electrode was inserted after recording electrodes were retracted and lesions were made to mark the approximate recording site. Animals were perfused and sections were processed for CO and EGFP staining as described above, except that sections were 50  $\mu$ m thick. Also, following perfusion with 4% paraformaldehyde in PBS (“fixative”), ferrets were perfused with solutions of 10% and then 20% sucrose in fixative prior to removal of the brain.

### Monkey Experiments

#### Chamber Implantation

A recording chamber was implanted on the single Rhesus macaque monkey (*Macaca mulatta*, 8 kg) used in this study; virus injections and electrical recordings were then conducted with this recording chamber in place. Surgery was performed under inhaled isoflurane anesthesia (1%–1.5% in oxygen) after the monkey was sedated with 0.01 mg/kg acepromazine and 10 mg/kg ketamine. A stainless steel recording chamber was affixed to the skull with dental acrylic and stainless steel screws. The chamber was centered at approximately AP +7.5 mm and ML +11.5 mm to allow microelectrode penetration into the LGN along the dorsoventral axis. The chamber was capped and the skin drawn up around the margin of the cranial implant. After healing, the chamber was cleaned weekly with sterile saline, 3% hydrogen peroxide, and dilute povidone-iodine.

#### Virus Injections

Virus was injected into the monkey LGN as described above for ferret LGN experiments, with the following exceptions. The monkey was initially anesthetized in 0.01 mg/kg acepromazine and 10 mg/kg ketamine (i.m.); lactated Ringer’s and pancuronium bromide (0.075–0.1 mg/kg/hr) were administered intravenously during the surgery to maintain hydration and to paralyze the monkey. The recording chamber was cleaned as described above, the dura was thinned with a scalpel, and an incision was made in the dura to allow penetration. Corrective, gas-permeable contact lenses were applied to protect the corneas. As in the ferret experiments, electrical recordings were made from the LGN both before and during virus injections to locate potential injection sites and identify visual receptive fields at the sites of injection. To aid in receptive field assignments, a reversing ophthalmoscope was used to map the fovea and blind spot in each eye. A total of 20  $\mu$ l of virus (AAV2-*AlstR*-

EGFP) was injected at several depths along three penetrations in the monkey’s left LGN. After virus was injected, the chamber was recapped, and the dura healed before the next recording session.

#### Electrophysiological Recordings

Surgical preparation for recording was conducted as described above for ferrets, with the following exceptions. The monkey was initially anesthetized with 0.01 mg/kg acepromazine and 10 mg/kg ketamine (i.m.), and paralyzed with vecuronium bromide (7.5–12.5  $\mu$ g/kg/hr, i.v.) during the recording session. Phenylephrine hydrochloride was not applied to the eyes. Additional corrective lenses were placed in front of the eyes to focus the visual stimulus. Appropriate magnification of lenses was determined based on responses of neurons to drifting gratings (Chatterjee and Callaway, 2002). Recordings were conducted using 2–5 M $\Omega$  resistance epoxy-lite-coated tungsten electrodes; mapping of the fovea and blind spot using a reversing ophthalmoscope aided in assignment of receptive fields.

#### Visual Stimulation

Visual stimuli were displayed as described above for ferret experiments. All stimuli shown in monkey experiments were circular patches of drifting sinusoidal grating. When necessary, the color composition of the stimulus was optimized for the neurons whose activity was being recorded.

#### AL Application and Data Analysis

AL application and data analysis were conducted as described above for ferret LGN experiments.

#### Mouse Electroneurogram Recordings

*nestin<sup>Cre</sup>*; *AlstR192* mice were generated as described previously (Gosgnach et al., 2006). In vitro electrophysiological experiments were performed on early postnatal (P0) mice in accordance with the ethical rules stipulated by the NIH. Animals were anesthetized and decapitated, and spinal cords were dissected out in ice-cold Ringer’s solution (see Lanuza et al., 2004). Recordings were made in Ringer’s solution at room temperature (20°C) by placing the second and fifth lumbar ventral roots (i.e., rL2, IL2, rL5, IL5) in bipolar suction electrodes. Electroneurogram (ENG) signals were amplified, band pass filtered (100 Hz to 1 kHz), digitized, and collected using the Axoscope software (Axon Instruments, Foster City, CA). Rhythmic locomotor activity was induced by adding N-methyl-D-aspartic acid (NMDA, 5  $\mu$ M, Sigma) and 5-hydroxytryptamine (5-HT, 10  $\mu$ M, Sigma) to the perfusing Ringer’s solution. Effects of allatostatin on the locomotor pattern were examined by adding the peptide to the perfusion solution.

#### Supplemental Data

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/51/2/157/DC1/>.

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