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Genetic Strategies for Elucidating Neural Circuit Function in the Brain

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in

Neurosciences

by

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2006

DEDICATION

I would like to dedicate this work to my family:

…to my mother Sharon, for supporting me through some of the toughest years of my life. A mother's love is endless.

…to my father Chor-Kheng, for encouraging me in Math and Science from a young age.

…to my sister Stella and my daughter Corinne for always cheering me up.

…and finally, to my loving husband Jared, without whom the long journey through life would be unbearable. You have enriched my life in countless ways, and I hope that you continue to do so for many years to come.

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Tan E, Ding XQ, Saadi A, Agarwal N, Naash MI, Al-Ubaidi MR (2004). Expression of cone-photoreceptor-specific antigens in a cell line derived from retinal tumors in transgenic mice. *Invest Ophthalmol Vis Sci* 45:764-8.

ABSTRACT OF THE DISSERTATION

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The brain is a complex organ, responsible for our intelligence, awareness, and ability to sense and interact with the world around us. In order to understand how the brain performs such advanced computations, it is important to elucidate the circuitry of the brain and to determine how that circuitry underlies its function. In practical terms, solving this problem requires targeted perturbations of specific neuron types. Such a strategy will allow for comparisons of brain function in intact versus partially handicapped brains. Chapter I of this dissertation outlines this problem, and explains how genetic strategies for targeting individual cell types can advance our understanding of brain function. Examples of such strategies, along with a discussion of the advantages and disadvantages of each, are outlined in Chapter II. Chapter III

presents novel data describing a particular genetically-based strategy, termed the allatostatin receptor/ allatostatin (AlstR/AL) system, in detail. Using adeno-associated viral vectors to express the AlstR/AL system *in vivo*, we demonstrate that it is an effective method for quickly and reversibly inactivating neurons in a variety of mammalian species. Such a method can be applied to investigate the role of individual cell types in neural circuits and overall brain function. The applications of the AL/AlstR method, along with a discussion of technical hurdles that were overcome to develop the method, are discussed in Chapter IV.

I. Introduction

The brain is an amazing organ, capable of performing complex computations. An incredible diversity of neuron types populates the brain, and in order to understand brain function, we must decipher the contributions of each individual type of neuron to overall brain function.

Historically, this has been an elusive goal. Different types of neurons, defined by differences in properties such as morphology, immunoreactivity, and firing properties, are generally intermingled in a given brain area. This makes individual subgroups of neurons difficult to target using conventional techniques. Inactivation of a group of cells by ablation or drug application is useful for discerning function when all neurons in a given brain nucleus subserve the same function. However, application of this strategy to a structure such as the cerebral cortex, where excitatory and inhibitory neurons, all with different connections and firing properties, are interlaced is of limited use, as several different neuron types are simultaneously inactivated (Figure 1-1).

A more useful strategy would allow an individual cell type to be specifically targeted for a manipulation such as ablation or inactivation. This strategy would allow comparisons of normal brain circuit function to function in the absence of contribution of the targeted cell type. With the availability of modern molecular biological methods, this type of targeting is possible: a

Figure 1-1. Diagram of a conventional lesion in the brain. Conventional lesions are achieved by permanently ablating or applying a drug to temporarily silence a region of the brain. Such methods provide limited insight into fine-scale circuitry-function relationships, as they simultaneously affect several subtypes of neurons (depicted here as neurons with different coloration and soma shapes).

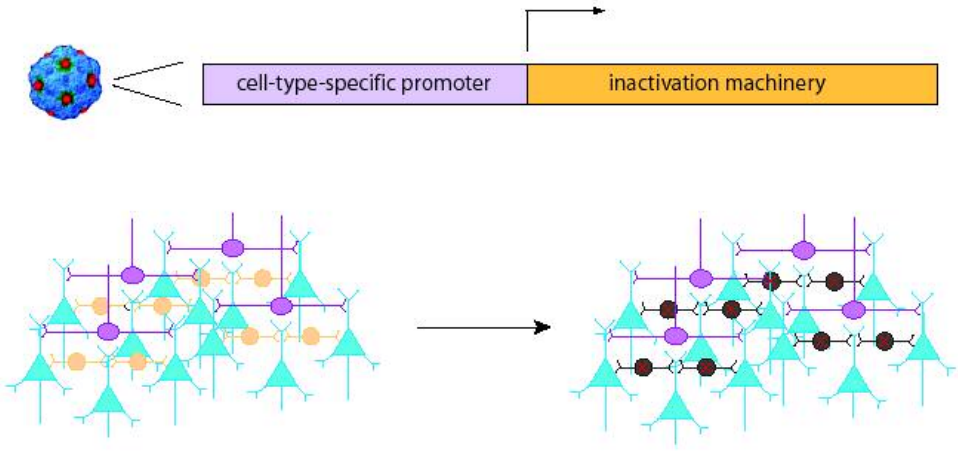

Figure 1-2. Genetically targeted lesions using cell-type-specific promoters. Subpopulations of neurons can be selectively inactivated by using a promoter specific for the targeted neuron type ("cell-type-specific promoter") to drive expression of a factor that will shut down activity in the neuron ("inactivation machinery"), as depicted in the upper portion of this figure. Because the inactivation machinery will only be synthesized in neurons that recognize the cell-type-specific promoter, this approach will result in selective lesioning of the targeted cell type (bottom portion of figure; targeted neurons are depicted with circular somas). In this schematic, the DNA carrying the promoter and encoding the inactivation machinery is delivered using a virus (upper left); however, DNA can be delivered to neurons using a variety of other methods.

promoter with specificity for the targeted cell type can be used to drive expression of a factor, such as a potassium channel, that interferes with normal cell function. This factor, by virtue of the fact that its expression is controlled by a cell-type-specific promoter, would only be present in the targeted cell type; the targeted cell type would thereby be selectively inactivated, and its contribution to circuit function could be discerned. This principle is illustrated in Figure 1-2.

A main drawback of the strategy just described is the permanent nature of the alteration. Permanent modifications can cause compensatory responses that would mask the consequences of knocking out a particular cell type. To avoid this potential problem, reversible methods have been developed that allow short-lived perturbations of circuit function. This can be achieved by placing a genetically-encodable switch under the control of a cell-type specific promoter, as illustrated in Figure 1-3. This switch, which again would only be expressed in the targeted cell type, could allow the experimenter to turn the targeted cells on or off by performing the appropriate manipulation. In addition to circumventing the problem of compensatory changes, this strategy, if reversible on a fast time scale, could allow direct comparisons of circuit function within the course of a single experiment.

This dissertation describes genetic methods available for investigations of the function of brain circuits. In Chapter II, I describe several methods, both permanent and reversible, currently in existence. Chapter III describes my graduate research, which has focused on development of a single method for reversible inactivation of mammalian neurons *in vivo*.

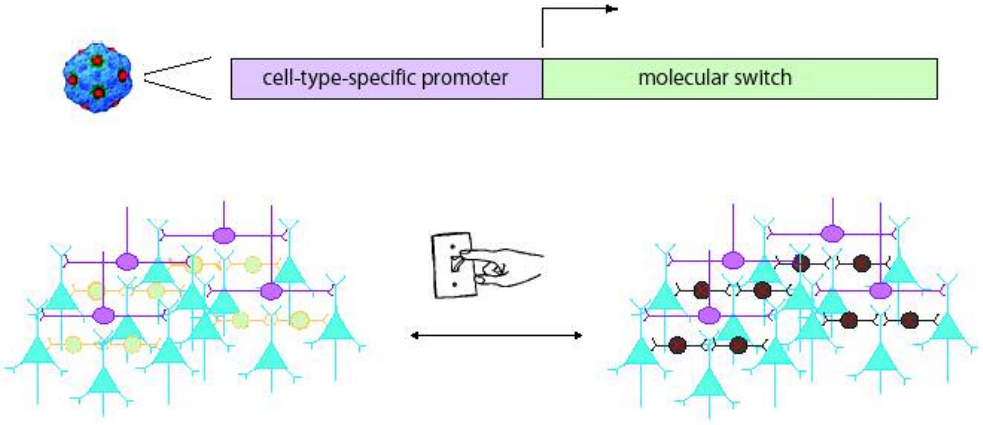

Figure 1-3. Reversible inactivation of selected neuron subpopulations. As in Figure 1-2, specific subpopulations of neurons can be genetically targeted for silencing. This effect can be made reversible through the introduction of an inducible silencer ("molecular switch") in place of permanent inactivation machinery. The molecular switch will be synthesized selectively in neurons recognizing the cell-type-specific promoter, and will silence those neurons only when the appropriate inducing agent is applied. Neurons can be rescued from silencing by removing the inducing agent, much like flipping an electrical switch. As discussed in Chapter II, inducing agents include drugs, peptides, and light (UV and visible).

II. Genetic methods for altering neural excitability

Genetic methods for altering neural excitability are useful because they allow specific neuron types to be targeted for alteration. Early methods were crude, while methods developed more recently have increased in sophistication, offering greater temporal control. This chapter provides an overview of the currently available methods, with special mention of the allatostatin receptor/allatostatin system, which is highlighted in Chapter III of this dissertation.

A. Ablation of targeted cells

Early genetic strategies for dissecting nervous function simply ablated the cells of interest. One strategy (Kobayashi et al., 1995) utilizes expression of the immune receptor interleukin receptor 2α (IL-2α) in cells of interest. Application of the immunotoxin anti-Tac(Fv)-PE40 then leads to cell death in cells expressing IL-2α. In an initial study by this group (Kobayashi et al., 1995), the strategy was used to ablate catecholaminergic cells in the central nervous system of transgenic mice using the dopamine β-hydroxylase (DBH) promoter: behavioral defects were observed, including ataxia, frequent falling, and hindlimb stiffness. In a later study by the same group (Sawada et al., 1998), the anti-Tac(Fv)-PE40 immunotoxin was applied systemically to ablate peripheral catecholaminergic cells in transgenic mice. This ablation led to a deficit in locomotor activity, along with a slowing of the heart rate and abnormal EKG.

A similar method, developed by Isles and colleagues (Isles et al., 2001), expresses the *E. coli* nitroreductase (NTR) gene in neurons of interest. The prodrug CB1954 is metabolized into a cytotoxic compound by NTR-expressing neurons, leading to their selective ablation. In an initial test of this method, the group expressed NTR in olfactory neurons of transgenic mice using the olfactory marker protein (OMP) promoter. Application of CB1954 led to a reduction in cell number in the olfactory epithelium as well as a disorganization of olfactory bulb structure. Behavioral tests indicated that NTR-expressing mice that had received CB1954 treatment were unable to detect novel odors.

B. Potassium channel overexpression in *Drosophila*

More recent strategies have utilized potassium (K^+) channel overexpression to reduce excitability in targeted cells. Attempts made in the fruitfly *Drosophila melanogaster* have been largely successful, and their application has led to advancements in our understanding of some neural phenomena. In one study (White et al., 2001), the Shaker K^+ channel was modified such that its fast inactivation was eliminated and its voltage-dependent activation occurred at a more hyperpolarized membrane potential than the wild-type channel. Expression of this modified channel, termed the electrical knockout (EKO) channel, in photoreceptors of *Drosophila* led to a defect in the electroretinogram; this effect was partially reversed when the channel blocker 4-AP was applied. Expression of the EKO channel in the developing fly helped to clarify some principles of neuromuscular connectivity: expression of EKO in neurons led to aberrant

connections, while expression of EKO in muscles led to normal connections, implicating the role of presynaptic activity in the establishment of normal neuromuscular connections.

Overexpression of a different K^+ channel, the Kir2.1 channel, in *Drosophila* muscle cells led to a reduction in muscle excitability (Paradis et al., 2001). The investigators in this study observed a compensatory increase in presynaptic quantal content, as reflected by increases in mEPSC frequency and probability of release.

A third study conducted in *Drosophila* used K^+ channel overexpression to investigate the role of circadian pacemaker neurons in the establishment of the circadian rhythm (Nitabach et al., 2002). Expression of the *Drosophila* open rectifier K^+ channel (dORK) and Kir2.1 in pacemaker neurons led to greatly reduced levels of the PERIOD and TIMELESS proteins, essential for establishing circadian rhythms. In behavioral tests, flies overexpressing the dORK or Kir2.1 channel in their pacemaker neurons displayed highly irregular circadian rhythmicity in the dark, but near-normal rhythmicity in the presence of light/dark cues, outlining a role for the pacemaker neurons in establishing circadian rhythms in the absence of light/dark cues.

C. Potassium channel overexpression in mammalian neurons

In contrast to the results of efforts made in *Drosophila*, overexpression of K^+ channels in mammalian neurons has met with variable success. Very few *in vivo* efforts have been reported, perhaps due to difficulties in achieving *in vivo* K^+

channel expression without lethality. One successful effort (Ehrengruber et al., 1997) used adenoviral vectors to overexpress G-protein-coupled inward rectifier K^+ (GIRK) channels in cultured rat hippocampal neurons. Application of the $GABA_B$ agonist baclofen to neurons expressing the exogenous GIRKs should reduce their excitability, as $GABA_B$ receptors couple to GIRK channels. This is precisely what the investigators observed: membrane potential was significantly lowered, spontaneous firing decreased dramatically, and a larger positive current injection was required to elicit spiking in the presence of baclofen.

Similar studies have been conducted in cultured muscle cells, and because they could also be applied to neurons, I mention them here. In one study (Nuss et al., 1999), adenovirus was used to express the HERG (human ether-a-gɵ go-related gene) K^+ channel in cultured rabbit ventricular myocytes. Myocytes expressing HERG exhibited a large, sustained outward K^+ current, along with a shortening of the muscle action potential. The severity of the phenotype correlated with the level of HERG expression.

Another study (Falk et al., 2001) used a herpes simplex virus (HSV-1) to express a modified Kv1.4 in primary cultured rat muscle cells. In voltage clamp mode, these muscle cells exhibited a much larger K^+ current than their noninfected counterparts; in current clamp mode, a larger positive current injection was required to elicit an action potential in infected cells. Additionally, the hyperexcitability typically achieved by applying the Na^+ channel toxin anemone toxin II (ATX-II) was not present in infected muscle cells, implying that the

presence of exogenous K^+ channels counteracted the Na^+ component of the membrane excitability.

Despite these successes, there have been a comparable number of failed attempts to overexpress K^+ channels in excitable tissues of mammals. Nadeau and colleagues (Nadeau et al., 2000) expressed Kir1.1 in cultured rat hippocampal neurons using a lentiviral vector. Kir1.1-expressing cells died 2 days after infection, while cells infected with a control virus encoding green fluorescent protein (GFP) displayed normal morphology, indicating that virus infection itself was not harmful to the cells. The cell death was likely due to excessive K^+ efflux resulting from the increased K^+ conductance; this hypothesis is supported by the observation that minimizing K^+ efflux by increasing the extracellular K^+ concentration rescued Kir1.1-expressing cells.

The only *in vivo* attempt to overexpress K^+ channels in a mammal (Sutherland et al., 1999) yielded similarly unfavorable results. In this study, the investigators expressed a Shaker-type channel from *Aplysia* (AKv1.1a) in the central nervous system of transgenic mice using the human HPRT promoter. *In situ* hybridization revealed abnormal expression levels of various K^+ channels: some channels were drastically upregulated, while others were severely downregulated. These changes likely reflect compensatory responses to the presence of AKv1.1a. Additionally, transgenic mice expressing AKv1.1a exhibited hyperexcitability and epileptiform discharges in their EEG. These changes are surprising considering the prediction that K^+ channel overexpression

should lower excitability; however, they are likely again to reflect an overcompensation for the presence of AKv1.1a.

D. Other "irreversible" methods for altering neural excitability

Attempts to silence excitable cells by overexpressing K^+ channels have yielded variable results. This variability could be due to a number of factors – variability in organisms, tissues, and gene delivery methods used, as well as in expression levels achieved are just a few possibilities. An important factor is the incredible diversity of K^+ channels: each has unique properties, making it difficult to predict from a given study whether expression of a related channel will yield similar results. For this reason, and to expand the repertoire of available methods, several investigators have taken alternative approaches to altering neural excitability.

One alternative approach has targeted Na^+, rather than K^+ channels, to reduce excitability in neurons (Nadeau and Lester, 2002). In this study, the investigators expressed NRSF (neuron restrictive silencer factor), a transcriptional repressor of Na^+ channel expression, in cultured rat hippocampal neurons. Na^+-mediated currents in hippocampal cells declined gradually over several days following infection with a lentivirus encoding NRSF, and expression of Na^+ channels not affected by NRSF appeared normal, suggesting an absence of compensatory mechanisms.

Another approach blocks synaptic transmission, rather than altering neurons' intrinsic excitability. Tetanus toxin light chain (TeTxLC) blocks synaptic

transmission by cleaving the synaptic vesicle-associated protein, synaptobrevin (VAMP2). Expression of TeTxLC throughout the nervous system of *Drosophila* (Sweeney et al., 1995) yielded flies with morphologically normal synaptic contacts, but defective synaptic transmission: glutamate-evoked postsynaptic currents were intact, while postsynaptic currents in response to electrical stimulation of motor neurons were abolished. Screens of flies expressing TeTxLC under various promoters using the GAL4-UAS system (Brand and Perrimon, 1993) yielded a mutant with a defective olfactory escape response, demonstrating that expression of TeTxLC can be used to investigate the roles of selected populations of neurons in behavior. The same group later used TeTxLC to investigate the role of synaptic input on the development of motor neurons in *Drosophila* (Baines et al., 2001): block of synaptic activity led to morphologically normal development but altered intrinsic excitability in motor neurons, indicating a compensatory response to lack of input.

A third strategy for altering neural excitability directly blocks receptors and ion channels (Ibanez-Tallon et al., 2004). In this strategy, genetically-encodable bungarotoxins and conotoxins are tethered to the plasma membrane via an ectopic glycosyl phosphatidyl inositol (GPI) linkage. The proximity afforded by membrane-tethering then allows the toxins to bind constitutively to their targets: nicotinic acetylcholine receptors (nAChRs), Na^+ channels, and Ca^{2+} channels. When toxins were co-expressed with their appropriate targets in *Xenopus* oocytes, target-mediated currents were not detectable. Expression of tethered α-bungarotoxin in embryonic Zebrafish muscle cells yielded similar results:

acetylcholine-induced currents were not detectable, presumably due to the blockage of nAChRs by the tethered α-bungarotoxin.

E. Reversibility via temporally-regulated gene transcription

The methods presented thus far alter neural excitability in a seemingly irreversible manner. However, with the exception of the cell ablation methods, all of the strategies mentioned above can be made reversible through the use of inducible promoters. An inducible promoter allows expression of the target gene to be controlled by the experimenter: generally, application of an inducing agent allows the gene to be transcribed and expressed, while withdrawal of the inducing agent blocks transcription (Saez et al., 1997). In some cases, the methods mentioned above have been combined with the use of inducible promoters to achieve reversible changes in the excitability of neurons.

In a study conducted by Johns and colleagues (Johns et al., 1999), an adenoviral vector was used to express the K^+ channel Kir2.1 in cultured rat superior cervical ganglion neurons. Expression of Kir2.1 was under the control of an ecdysone-inducible promoter: application of the ecdysone analog muristerone A led to expression of Kir2.1, as evidenced by the appearance of an inward-rectifying K^+ current. Cells expressing Kir2.1 had a lowered resting membrane potential and required a larger positive current injection to elicit spiking when compared to controls. This effect was not observed in the absence of muristerone A.

Another study (Yamamoto et al., 2003) has combined expression of TeTxLC with the tetracycline-controlled reverse trans-activator system (Gossen et al., 1995). Administration of the drug doxycycline in transgenic mice with the Tet transgene under the control of the GABA-α6 promoter and the TeTxLC gene under the control of the TRE/CMV promoter leads to restricted expression of TeTxLC in cerebellar granule cells. Synaptobrevin was effectively cleaved and glutamate release was drastically reduced in cerebella of these mice, while several other synaptic proteins remained unaffected. The mice exhibited motor impairments in behavioral tests including the fixed-bar and rota-rod tests; these impairments disappeared after withdrawal of doxycycline. Although effective, the reversibility of this method, like that of other inducible promoters, is quite slow: expression reaches its peak at 5 days after doxycycline administration begins, and low levels of transgene are still detectable 7 days after withdrawal of doxycycline.

The temperature-sensitive allele of the *shibire* gene (shi^{ts1}) in *Drosophila* operates on a similar principle (Kitamoto, 2001): *shibire* encodes the protein dynamin, which is essential for synaptic vesicle recycling. At restrictive temperatures (>29ºC), synaptic transmission is inhibited. In a series of experiments, Kitamoto applied this principle to investigate the functions of various groups of neurons. Flies expressing shi^{ts1} in cholinergic neurons displayed an extreme loss of motor activity at the restrictive temperature. When shi^{ts1} was expressed in photoreceptor cells, flies had impaired electroretinograms and lost their behavioral preference for light. These effects were easily reversed by returning flies to their permissive temperature. This method is quite effective and

is certain to prove extremely useful in studies of circuitry and behavior in flies; unfortunately, however, it cannot be applied to mammals.

F. Pharmacological methods for reversibly altering neural excitability

The reversibility achieved by regulating gene transcription occurs on a timescale of hours to days, and is ideal for developmental studies as well us studies of plasticity. Other types of studies, however, could benefit from more quickly reversible methods for altering neural excitability. Pharmacologically based methods offer this advantage.

One pharmacologically-based approach is termed the RASSL (receptors activated solely by synthetic ligands) system (Coward et al., 1998). In this approach, naturally-occurring receptors are engineered such that they no longer bind their endogenous ligands; instead, they retain a sensitivity only to synthetic agonists. In the first demonstration of this principle, the human κ-opioid receptor was engineered such that its ligand-binding domain was replaced by that of the δ-opioid receptor. Due to differences in binding sites, this rendered the receptor insensitive to its natural ligand, dynorphin, but still sensitive to small-molecule agonists such as sporadoline. This receptor, termed the Ro1 receptor, was expressed *in vivo* in hearts of transgenic mice under regulation of the tetracycline transactivator system (Redfern et al., 1999). Expression of Ro1, combined with activation by the agonist sporadoline, led to a roughly 55% decrease in heart rate within 30 seconds of activation. This effect reversed within 2 hours, presumably due to diffusion and/or breakdown of spiradoline. Long-term expression of the

receptor alone, however, led to lethality, resulting perhaps from a low level of receptor activation by its endogenous ligand (Redfern et al., 2000) . Although the Ro1 receptor activates on a fairly short time course and has been useful for studying the role of opioid receptors in regulating heart rate, its utility for studying nervous system function is limited: the nervous system contains high levels of endogenous κ-opioid receptors, which would be activated by the synthetic ligand sporadoline.

A second pharmacologically-based method (Slimko et al., 2002) takes advantage of a chloride channel from *C. elegans* that is directly gated by the neurotransmitter glutamate; this channel is also extremely sensitive to the drug ivermectin (IVM). Expression of the IVM channel in cultured rat hippocampal neurons led to increases in membrane conductance, as well as decreases in spontaneous and evoked firing rates, in response to IVM application. These effects occurred within seconds of IVM application, and reversed roughly 8 hours after washout of IVM. Later efforts by this group lowered the channel's sensitivity to glutamate (Li et al., 2002) and improved expression of the IVM channel through codon optimization (Slimko and Lester, 2003). Application of this method for future studies may be problematic, as the 8-hour time frame for recovery from inactivation is less than ideal for some types of experiments. More importantly, IVM can be toxic to the brain at levels slightly higher than those used in the studies described here, making it unfavorable for use in studies of nervous system function.

The allatostatin receptor/allatostatin (AlstR/AL) system, featured in this dissertation and discussed in further detail below, is based on similar principles but provides the advantage of a high level of specificity. The method relies on ectopic expression of the *Drosophila* allatostatin receptor (AlstR), which triggers opening of GIRK channels upon binding its ligand, allatostatin (AL). The system has a time resolution similar to that of the methods just described, yet has much more selective effects: AL does not have any effect on neurons not expressing AlstR, and AlstR does not bind endogenous peptides in the mammalian brain. These points are discussed in greater detail below in the section entitled "The Allatotstatin Receptor/Allatostatin System".

A fourth pharmacological method takes the approach of blocking synaptic transmission rather than altering the intrinsic excitability of neurons (Karpova et al., 2005). This method, termed MISTs (Molecules for Inactivation of Synaptic Transmission), uses a chemical inducer to trigger dimerization of modified presynaptic proteins. This dimerization inhibits synaptic transmission by sequestering synaptic vesicles and synaptic vesicle proteins from their site of action. Application of dimerizer to brain slices expressing the MIST proteins led to a roughly 50% reduction in EPSC size in 15 minutes. Network activity recorded in dissociated cultures expressing MISTs fell to about 30% of baseline levels 1 hour after dimerizer application, and recovered to baseline 1 hour after wash-out of dimerizer. The MIST method is the only reversible method to be demonstrated in a mammalian system *in vivo*: transgenic mice expressing MISTs in cerebellar Purkinje cells experienced learning impairments on the rotarod test in

the presence of dimerizer, an effect which was reversible on a timescale of about 2 days. This cleverly designed method is likely to prove useful for many behavioral studies in mammals, and has the clear advantage of having been demonstrated to work *in vivo*. However, its temporal resolution is slower than would be desired for some electrophysiological studies: for such studies, the experimenter will have to turn to other available methods.

G. Light-based methods for reversibly altering neural excitability

Because the delivery of light is extremely fast, the temporal control offered by light-based methods is even greater than that offered by the pharmacological methods just mentioned. Some methods offer millisecond control over neural activity, allowing for studies of precise events such as spike timing.

An early light-based strategy used phototransduction proteins from *Drosophila* to elicit spiking in cultured neurons (Zemelman et al., 2002). By expressing various combinations of *Drosophila* phototransduction proteins in *Xenopus* oocytes, the investigators found that the proteins ninaE, $G_{q\alpha}$, and arrestin2 were necessary and sufficient to elicit the positive inward currents that normally arise during *Drosophila* phototransduction. Expression of these proteins, along with the chromophore 11-*cis* retinal, in cultured rat hippocampal neurons, led to dramatic increases in spontaneous firing rates in the presence of light. These increases could occur within hundreds of milliseconds, and reversed within a minute of light withdrawal, demonstrating that the method is quick and effective for eliciting spiking in neurons. Despite being an impressive achievement, the

system is cumbersome, as it requires co-expression of three proteins, along with conjugation to 11-*cis* retinal, to achieve its effects.

Methods developed more recently have taken more elegant approaches to achieving light-mediated control of neural activity. A method developed by Banghart and colleagues (Banghart et al., 2004) uses a modified Shaker K^+ channel to control excitability. The modifications to the Shaker channel included elimination of the domains responsible for fast and slow inactivation, as well as a shift of the channel's voltage activation to more hyperpolarized potentials. Conjugation of a specifically-designed tetraethyl ammonium (TEA)-like molecule to the modified Shaker channel allows the channel to be blocked in the presence of visible light, as the TEA-like moiety physically blocks the channel's pore. In ultraviolet (UV) light, the TEA-like component changes conformation, allowing current to flow through the pore of the modified channel. Expression of this channel in cultured rat hippocampal neurons demonstrated that it can successfully be used to control excitability: spontaneous spiking disappeared within seconds of UV light application, and returned within seconds after the light stimulus was switched back to visible wavelengths. Although effective, a drawback of this method is that the expressed K^+ channels are open at rest until the TEA like molecule is applied, making plasticity resulting from long-term increases in K^+ conductance a concern.

More recent methods have used ectopic expression of individual, non-modified proteins to alter neural excitability. In one study (Li et al., 2005), expression of rat rhodopsin 4 (RO4) was used to inhibit spiking in cultured

hippocampal neurons. Upon exposure to light, retinal-conjugated RO4 opens GIRK channels via the $G_{i/o}$ pathway. Application of light to cultured hippocampal neurons expressing RO4 elicited a hyperpolarization comparable to that induced by the $GABA_B$ agonist baclofen; additionally, the number of spikes evoked by a positive current injection decreased in the presence of light. Importantly, a side effect was also observed: exposure to light also decreased quantal content in RO4-expressing neurons, presumably due to a $G_{i/o}$–mediated decrease in Ca^{2+} influx. Expression of RO4 in an isolated embryonic chick spinal cord preparation led to mixed results: continuous light application slightly lowered spontaneous bursting, but short (3 sec) light applications caused premature bursting.

The same study also investigated the ability of the green algae protein channelrhodopsin 2 (ChR2) to elicit spiking in neurons. ChR2 is a nonselective cation channel directly gated by light. When exposed to light, cultured hippocampal neurons expressing retinal-conjugated ChR2 increase their basal firing rates. Applying light pulses at rates up to 5 Hz faithfully elicited spike trains. Expression of ChR2 in an isolated embryonic chick spinal cord preparation led to increased spontaneous bursting in the presence of light.

A second group has also used ChR2 to elicit spiking in neurons (Boyden et al., 2005). In their study, ChR2 was expressed in cultured rat hippocampal neurons using lentiviral vectors. Application of long (1 sec) light pulses led to spike trains with variable timing. When brief (10-15 ms), randomly spaced light pulses were applied, the spike trains produced followed the stimulus with remarkable fidelity: spike trains with an average frequency of 10 Hz could be

evoked with a trial-to-trial jitter of less than 3 ms. Neurons were also able to reliably follow periodic light stimuli to 10 Hz, with some neurons able to follow at frequencies up to 30 Hz. Additionally, subthreshold potentials were achievable through light applications of lower intensity or shorter durations. Oddly, application of retinal was not necessary to achieve the effects observed in this study.

A final set of studies cleverly combines a pharmacologically based method with light activation. This strategy uses capsaicin, menthol, and ATP receptors, all of which conduct cationic current when activated by their respective ligands, to elicit spiking in neurons (Zemelman et al., 2003). Expression of these channels in cultured rat hippocampal neurons led to a dose-dependent increase in spiking within seconds of application of the appropriate ligand. Introduction of photocaged ligands offered an additional level of control: application and removal of light offered greater temporal control than wash-in and wash-out of the ligands, and although not demonstrated in this study, focal application of light could also enable activation of specific groups of neurons. This principle was applied *in vivo* in a later study (Lima and Miesenbock, 2005), where the $P2X_2$ ATP receptor was expressed in various populations of neurons in *Drosophila*. Expression of $P2X_2$ in the giant fiber system led to expected escape behaviors when flies were treated with photocaged ATP and exposed to light. The same method was used to investigate the role of dopaminergic neurons in the fly: stimulation of dopaminergic neurons led to increased locomotion in flies exhibiting low basal locomotor activity.

Light-based methods for altering neural excitability offer extremely fast temporal control. Due to the ease of delivering light, they are likely to prove very useful for many studies of nervous system function, including, but not limited to, studies requiring high temporal precision. However, light delivery to some brain structures is certain to be problematic, and it is therefore wise to develop methods that could be applied to subcortical and other light-inaccessible structures. Here, we have developed a pharmacological method, the allatostatin receptor/allatostatin system, for quickly and reversibly inactivating mammalian neurons. A brief description of the method, including relevant background information, follows.

H. The Allatostatin Receptor/Allatostatin System

Drosophila allatostatin is a member of the allatostatin family of insect hormones. Originally discovered in the cockroach *Diploptera punctata* (Woodhead et al., 1989), it is produced in the brain and inhibits synthesis of juvenile hormone by the corpora allata endocrine gland in *Diploptera* and other insects. However, its role in *Drosophila* is unclear: although head extracts from *Drosophila* are able to inhibit juvenile hormone synthesis in an *in vitro* preparation of *Diploptera* corpora allata, allatostatin localizes to various interneurons and motor neurons in *Drosophila*, rather than to the corpora allata as it does in other insects (Yoon and Stay, 1995). All known allatostatins contain the C-terminal consensus sequence –Tyr-X-Phe-Gly Leu-NH_2.

Drosophila allatostatin, along with its receptor, were first identified in a reverse physiological screen conducted by Birgül and colleagues (Birgul et al.,

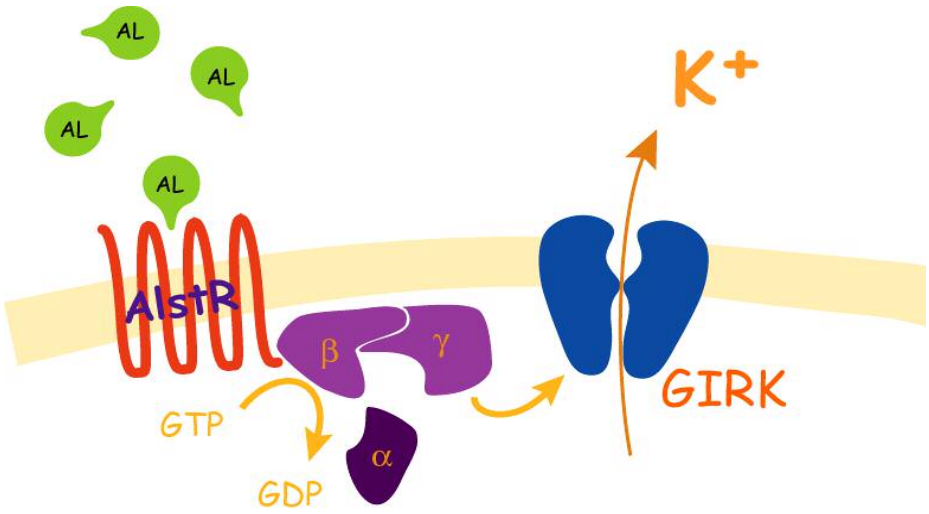

Figure 2-1. Mechanism of action of *Drosophila* allatostatin. Allatostatin (AL; sequence: Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH_2) binds to the allatostatin receptor (AlstR), activating the $G_{i/o}$ pathway. Pathway activation leads to opening of G-protein-coupled inward rectifier K^+ (GIRK) channels, leading to an efflux of K^+ ions and causing the AlstR-expressing cell to hyperpolarize.

1999). Using degenerate oligonucleotide primers based on the mammalian somatostatin receptors, the *Drosophila* allatostatin receptor (AlstR) was cloned from *Drosophila* mRNA. Sequence alignment of the cloned receptor with homologous receptors suggested a seven-transmembrane structure and a coupling to GIRK channels; sequence comparisons indicated that it was most closely related to the mammalian galanin receptors. Co-expression of*Drosophila* AlstR with GIRK channels in Xenopus oocytes revealed GIRK-mediated currents in response to *Drosophila* head extracts. Importantly, no currents were observed in response to a number of mammalian peptides, including somatostatin 14, somatostatin 28, leu-enkephalin, met-enkephalin, galanin, and proctolin. Purification of the active compound revealed that it was an allatostatin-like peptide with the sequence Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH_2.

Drosophila allatostatin (AL) acts by binding AlstR, activating $G_{i/o}$, and opening GIRK channels, as illustrated in Figure 2-1. This characteristic can be exploited for controlling the excitability of mammalian neurons, as our laboratory has previously demonstrated *in vitro* (Lechner et al., 2002). AlstR-expressing neurons in cultured ferret visual cortical slices showed decreases in input resistance and resting membrane potential after application of 1 nM AL (Figure 2-2). Additionally, a much larger positive current injection was required to elicit spiking after AL was applied as compared with pre-AL conditions. This effect reversed within minutes of AL wash-out, reflecting the amount of time necessary to completely replace the bath fluid.

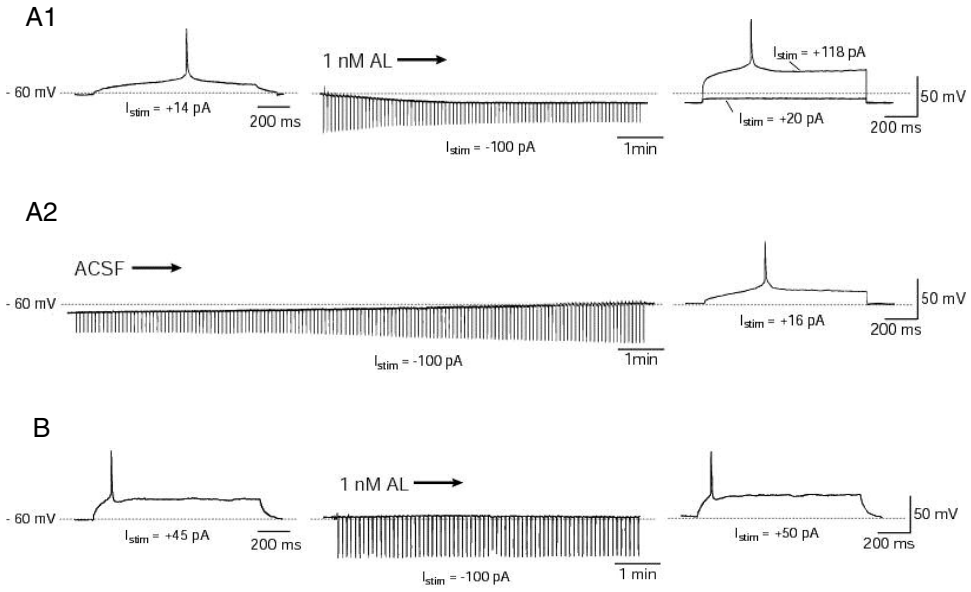

Figure 2-2. Reversible silencing of neurons *in vitro* using the AlstR/AL system. Brain slices from P13-P30 ferret visual cortex were transfected with plasmids encoding AlstR, GFP, GIRK1, and GIRK2 (experimental) or GFP, GIRK1, and GIRK2 (control). Whole-cell patch recordings were performed in cultured slices 1 day after transfection. **A1)** Prior to application of AL, a current injection of +14 pA is required to elicit spiking in an AlstR-expressing neuron *(left panel).* Application of 1 nM AL leads to decreases in resting membrane potential and input resistance, as reflected by smaller voltage deflections in response to 5 ms pulses of- 100 pA current *(middle panel).* After complete wash-in of AL, a current injection of +118 pA is now required to elicit spiking *(right panel)*; a current injection of +20 pA is no longer sufficient to trigger spiking. **A2)** Wash-out with artificial cerebrospinal fluid (ACSF) reverses the effects elicited by AL; resting membrane potential, input resistance, and spike threshold return approximately to initial values. **B)** No effect is observed in control neurons not expressing AlstR.

Adapted from Lechner et al., 2002.

Here, we demonstrate the feasibility of the AlstR/AL method *in vivo*. By using adeno-associated viral vectors to deliver AlstR to cortical and subcortical neurons in rat, ferret, and monkey, we demonstrate that the AlstR/AL system can be used to reversibly inactivate neurons on a timescale of minutes. This method has a high level of specificity compared to other pharmacologically-based methods for altering neural excitability: as described above, AlstR is not activated by a number of mammalian peptides, and as observed in our experiments, AL has no detectable effects on neurons not expressing AlstR. These characteristics make the AlstR/AL system an excellent tool for studying the contributions of specific neuron populations to overall circuit function and behavior.

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III. Selective and Quickly Reversible Silencing of Mammalian Neurons *in Vivo* Using Genetic Expression of the Drosophila Allatostatin Receptor

A. Abstract

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*.

B. 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 could 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^+ channel overexpression (Ehrengruber 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. However, for studies investigating effects of short-term inactivation, they are susceptible to compensatory changes resulting from long-term expression: K^+ 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 time scales 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 Miesenbock, 2005; Zemelman et al., 2002; Zemelman et al., 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^+ 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 time scale of hours to days and is likely to prove extremely useful for applications requiring long periods 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 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 to 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.

C. Results

Overview

We tested the ability of AL to induce inactivation, both in neurons expressing the AlstR and in control neurons not expressing AlstR. Experiments were conducted in several different preparations, including the rat barrel cortex, ferret visual cortex, and lateral geniculate nucleus (LGN) of ferrets and monkeys. In all of the experiments just mentioned, genetic material encoding AlstR was delivered using AAV vectors (see further details below). In additional experiments, AlstR was expressed conditionally in transgenic mice following Cre recombination. 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*. Finally, experiments in mice examined the effects of AL on the activity of motor neurons in an *in vitro*, isolated spinal cord preparation.

Viral constructs

To achieve AlstR expression, AAVs were used to deliver genetic constructs (Figure 3-1). 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

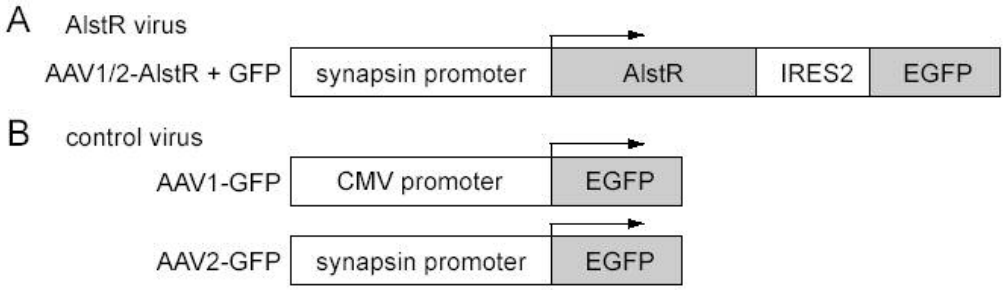

Figure 3-1. Diagrams of plasmids used to make viruses in this study. These diagrams illustrate the genetic constructs placed in the AAV backbone and therefore carried into infected cells by virus. **A)** AlstR viruses. A synapsin promoter was used to drive expression of AlstR; an IRES2 element was used 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.

indiscriminately in all neuron types; an IRES2 element drove additional expression of EGFP as a marker for virus-infected, AlstR-expressing cells (Figure 3-1A). For control experiments, AAVs encoding only EGFP under the CMV or synapsin promoter were used (Figure 3-1B). 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 3-2 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 msec 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 3-2A for a recording made prior to application of AL in a cortical region that was later

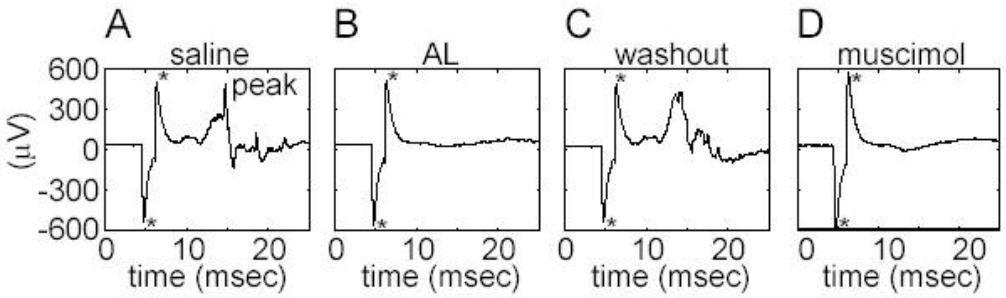

Figure 3-2. Representative local field potentials (LFPs) recorded from the barrel cortex of a rat expressing AlstR. Recordings are in response to electrical stimulation of the whisker pad. Example LFPs are illustrated in chronological order: **A)** prior to application of AL to the cortical surface; **B)** 11 minutes after application of AL; **C)** 10 minutes after washout of AL with saline; and **D)** 20 minutes 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.

confirmed to coincide with a region of AlstR expression (Figure 3-3A2). Following application of 0.1μM AL directly onto the cortical surface, the stimulus-evoked response was essentially eliminated (Figure 3-2B) and then returned following washout of the AL with saline (Figure 3-2C). After several rounds of repeated inactivation with AL (see below), effects of the $GABA_A$ agonist muscimol were characterized for comparison, since muscimol should maximally inactivate cortical responses. Figure 3-2D shows that the stimulus-evoked LFP following inactivation with muscimol is similar to that observed following AL application (Figure 3-2B).

To test the speed of inactivation and recovery following application and washout of AL, whisker stimulation-evoked LFPs were recorded at 10 second intervals. Peak LFPs measured over the entire course of the same experiment for which representative recordings are shown in Figure 3-2 are plotted in Figure 3-3A1. An example from a second rat is shown in Figure 3-3B1. In both examples, neurons inactivated within minutes of 0.1 μM AL application and recovered nearly completely within minutes of saline wash-out. 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 3-3B1, 2^{nd} 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 μM AL appears to be sufficient to elicit maximal inactivation in this experimental paradigm, as increasing the dose ten-fold does not increase the degree of

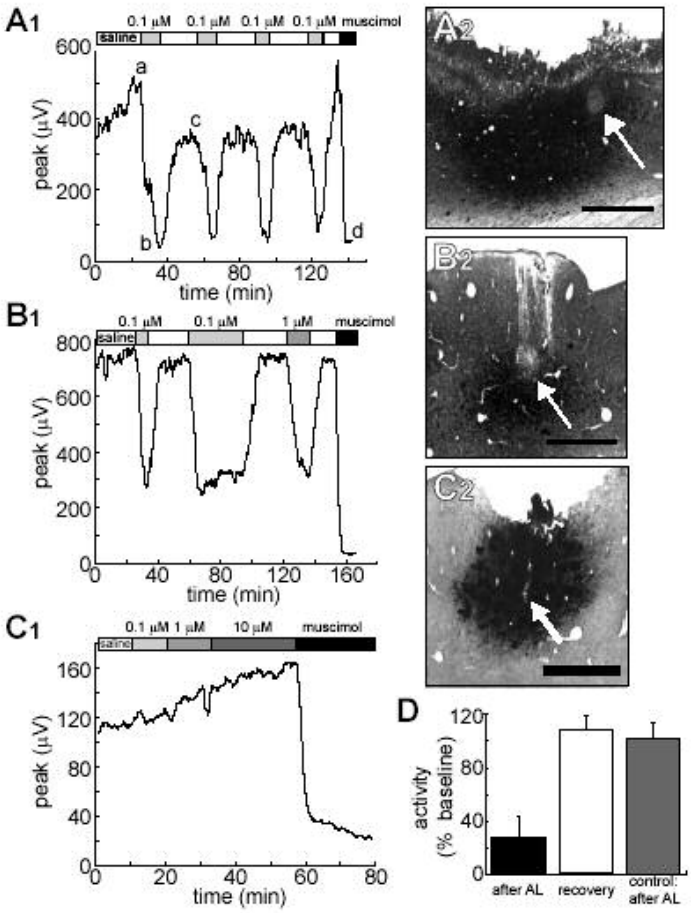

Figure 3-3. The AL/AlstR system quickly and reversibly eliminates stimulus-evoked LFPs in rat barrel cortex. A1-C1) Peaks of LFPs evoked at 10 second intervals, plotted over time, from rats infected with AAV1-AlstR-EGFP (A1 B1) and a control rat infected with AAV1-EGFP (C1). Letters a-d in panel A1 correspond to the times of LFP recordings illustrated in Figure 3-2 (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 µm; pia top, anterior left. In all cases, recordings were made from the area of EGFP expression. **D)** Average LFP responses ± SEM from 3 AlstR-expressing and 3 control rats. Averages represent responses relative to baseline during the 3 minutes after application of 0.1 µM 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.

inactivation (Figure 3-3B1, 3rd 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 3-3A than in Figure 3-3B. 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 3-3A2 to Figure 3-3B2)

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 μM) were applied (Figure 3-3C1). Summary data from 3 AlstR-expressing and 3 control rats are shown in Figure 3-3D. 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 ± 14.9% of baseline levels upon AL application and recovered to 108.9 ± 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 ± 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 ± 13.4% of baseline and recovery to 115.8 ± 8.0% for AlstR-expressing rats; 101.3 ± 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 3-3A2, 3-3B2, 3-3C2).

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 non-expressing 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 remains 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.

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 3-4 shows the tuning properties of an example unit, recorded in cortical layer 5 at a depth of 880 µm 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 µM AL to the cortical surface completely abolished both spontaneous and visually-evoked activity of the unit characterized in Figure 3-4 (Figure 3-5A1). Activity dropped to 8% of baseline levels within 5 minutes of AL application, with complete inactivation occurring 30 seconds later. Upon saline wash-out, administered 11 minutes after AL application, activity did not recover immediately: recovery began to occur 30 minutes after wash-out, reaching a peak at 57% of baseline 40 minutes after wash-out (see Experimental Procedures for details of analysis). This result was typical of our ferret cortical recordings, and

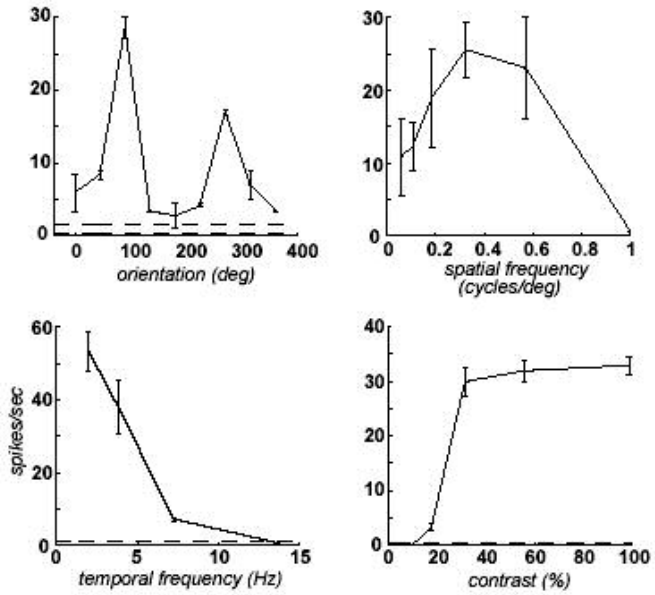

Figure 3-4. Tuning properties of a visual cortical neuron from a ferret expressing AlstR. Separate plots indicate visual responses to drifting sinusoidal gratings with varying stimulus orientation, spatial frequency, temporal frequency or contrast. Each point indicates the mean firing rate ± SEM for 2 presentations of each stimulus; dotted lines indicate mean response ± 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 3-5A.

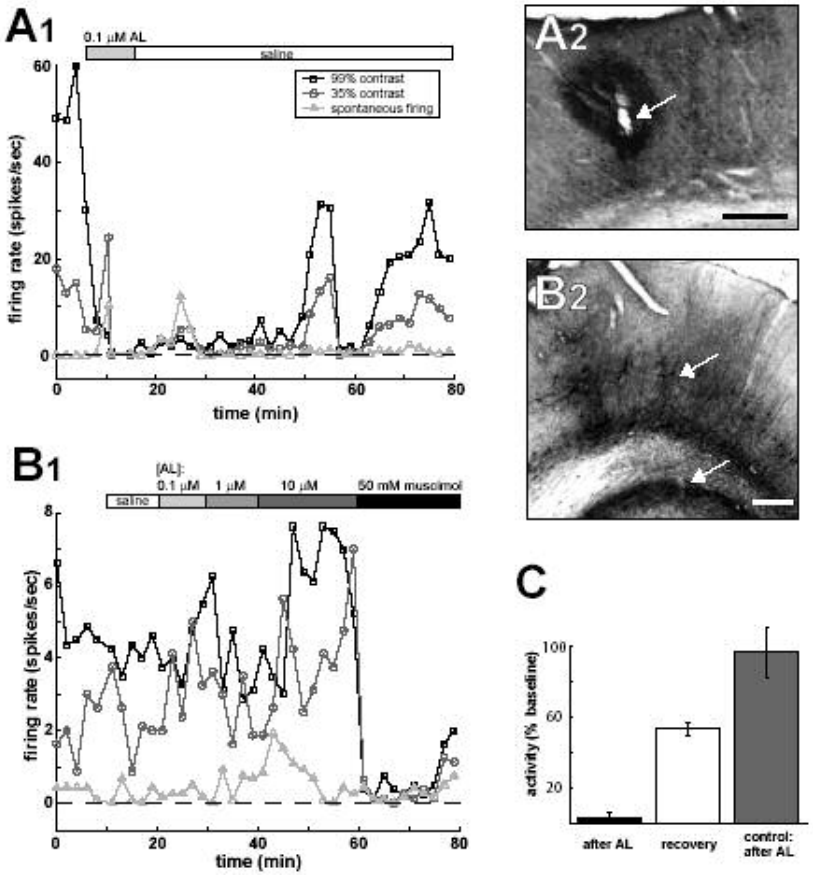

Figure 3-5. The AL/AlstR system quickly and reversibly inactivates ferret visual cortical neurons. A1, B1) Mean firing rate (y-axis) vs. time (x-axis) for AlstR-expressing (A1) and EGFP control (B1) ferrets. A1 is a single-unit recording; B1 is a multi- unit recording. In both plots, responses are to 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/sec. 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 µM AL and began to recover 30 minutes 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, 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 µm. **C)** Summary data from 3 AlstR-expressing ferrets and 4 controls (2 EGFP, 2 uninjected). Neurons from AlstR-expressing ferrets inactivated to 3.3 ± 2.4% of baseline firing rates in response to AL application and recovered to 53.5 ± 3.7% of baseline; neurons from control ferrets fired at 97.0 ±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 wash-out of AL, we observed a similar time course of recovery from AL-induced inactivation (see below).

Figure 3-5C shows summary data from the 3 AlstR-expressing ferrets examined in this study. Cells from AlstR-expressing ferrets fired at 3.3 ± 2.4% of baseline levels (in the 5-10 minute time interval) following AL application, and typically recovered to 53.5 ± 3.7% of baseline within 40-70 minutes of AL application (see Experimental Procedures for details of analysis). Processed brain sections from the ferret represented in Figure 3-5A1 provide visual confirmation that recordings were made from the region of virus infection (Figure 3-5A2). 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, 3 recordings) infected with AAV1-EGFP (Figure 3-5B1; Figure 3-5C). This was true even when 100 times the effective dose of AL was applied. Application of the $GABA_A$ agonist muscimol resulted in complete inactivation of the neurons, confirming that recordings were from cortical neurons, where virus-infected cells were located, 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 3-5B2 for example); these results indicate that virus infection alone does not confer sensitivity to AL. Similar results were observed in recordings made in non-injected hemispheres of virus-injected ferrets (n = 2 ferrets, 2 recordings). On average, neurons from EGFP-expressing and normal controls fired at 97.0 ± 14.3% of baseline levels following application of AL (Figure 3-5C). 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, 3 ferrets injected with AAV1-AlstR-EGFP in Area 17 showed extremely low AlstR expression (as inferred from anti-EGFP staining; see Figure 3-6). 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 tested.

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

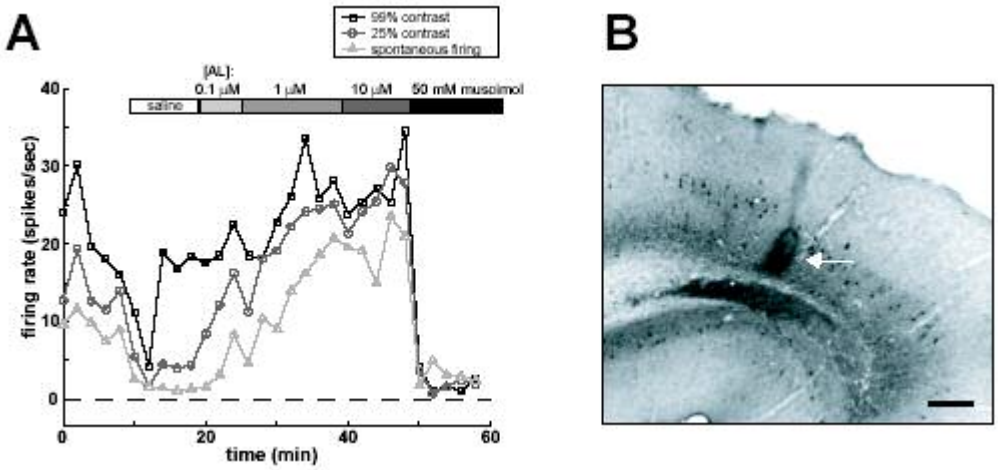

Figure 3-6. AL (0.1-10 µM) had no effect on activity recorded in Area 17 neurons of ferrets with extremely low AlstR expression. A) Example multi-unit recording from of a ferret with extremely low EGFP expression. Plot follows same format as shown in Figure 5. Firing rates are unaffected by AL application. Similar results were observed in 6 additional recordings from 3 ferrets, all with low expression. **B)** Sagittal section of Area 17 from ferret represented in A (pial surface, upper right). EGFP staining (black) is extremely weak, with the majority of virus-infected cells appearing in layer 5, distant from the location of recordings as marked by electrolytic lesion (white arrow). Scale bar, 250 µm.

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 3-5A1). 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 minutes 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. These characteristics make the AlstR/AL system an excellent tool for experiments requiring selective and reversible inactivation of mammalian neurons with temporal control on the order of minutes.

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 establishment 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 3-7A1, conducted in an LGN infected with AAV2-AlstR-EGFP, an initial injection of saline (2 µl at t = 5 minutes) resulted in a small pressure artifact. A subsequent injection of AL (2 µl of 0.1 µM at t = 11 minutes) 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 µl of 0.1 µM) from the same pipette, just 12 minutes later (t = 23 minutes), elicited a long-lasting and nearly complete reduction in neuronal firing and responsiveness.

After a complete drop in activity at t = 28 minutes (5 minutes after the second AL injection), cells remained inactive for about 20 minutes and then gradually recovered to above baseline firing rates at t = 88 minutes, delineating an approximate 60-minute 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 break-down of AL. Two additional saline injections (2 µl each) applied after recovery (t = 99 and 110 minutes) elicited small pressure artifacts similar to that observed for the initial saline injection. A final injection of 2 µl of 0.1 µM AL (t = 121 minutes) caused a complete, albeit

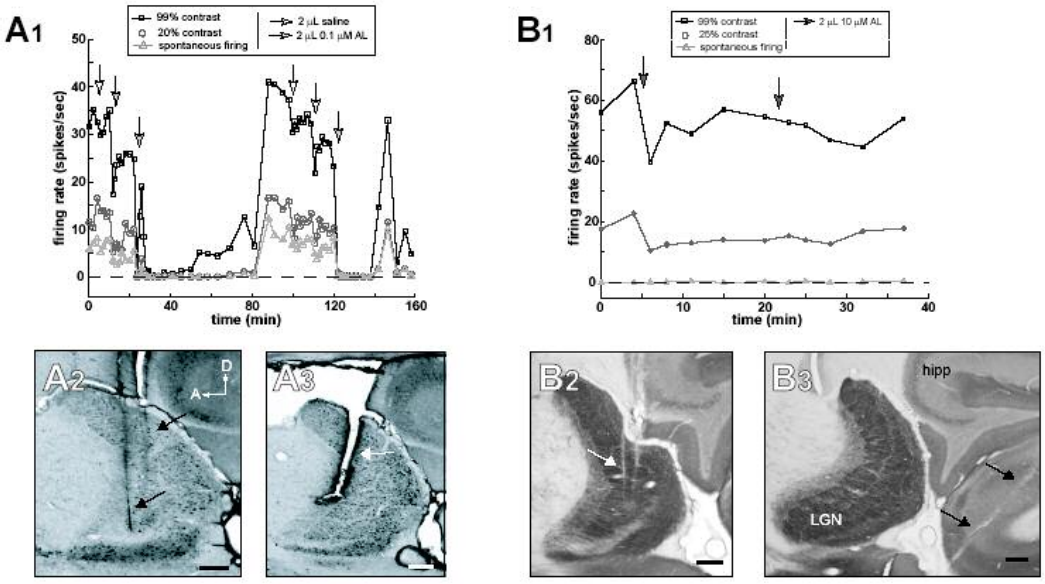

Figure 3-7. The AL/AlstR system can quickly and reversibly inactivate ferret LGN neurons. A1, B1) Multi-unit recordings from LGN of an AlstR-expressing (A1) and a non-virus-injected control (B1) ferret. Format of plots are same as in figure 5; note different stimulus contrasts used. Arrows indicate times of injections of saline or AL into or near the LGN. AlstR-expressing neurons inactivated in response to AL (grey arrows) but not to saline (white arrows); recovery occurs approximately 60 minutes later. Control neurons were not affected by AL. **A2, A3; B2, 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, B2) and from the pipette used to inject AL (A3, B3); the pipette was located 300 µm medial and 450 µm lateral to the electrode for A3 and B3 respectively. Dorsal top, anterior left; scale bars = 250 µm; *hipp*, hippocampus.

possibly briefer, reduction in firing. It was unclear whether the subsequent brief increase in measured spike rates (at t = 142 minutes) 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 µm lateral to the recording site (Figures 3-7A2, 3-7A3).

The effects of AL were examined in 3 additional LGN recordings from 3 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 minutes of AL injection and remained near zero for over 4 hours. 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 3-7B1 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 µM, 2 µl each) were administered during the 40-minute recording session. In neither of these cases did 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 µm medial to the recording site (Figures 3-7B2 and 3-7B3).

Similar results were observed in additional recordings from control ferrets (n = 12 recordings, 1 normal ferret, 1 ferret infected with AAV2-EGFP), even at 1000 times the effective AL dose (100 µM). On average, control neurons fired at 87.4±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 endogenous effects on thalamic neurons, making the AlstR/AL system an excellent tool for selective inactivation of targeted cell types without affecting surrounding cells.

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 since it is likely that in some cases, 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 can effectively inactivate neurons in such a system, 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 (2-20º azimuth, –12-0º elevation), 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 4 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 3-8 shows the results from a representative recording session. The first cell of this series, shown in Figure 3-8A, had a receptive field outside of the range covered by our virus injections (0.7º az, –4.8º el). This cell's 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 3-8B, conducted 4 hours after those shown in 3-8A and representative of the second set of recordings, responded to visual stimulation at 5.4º az and -11.1º el. Activity dropped briefly and recovered in response to the initial two injections of 0.2µM 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 0.2µM), cells inactivated completely: activity fell to 11.1% of baseline values

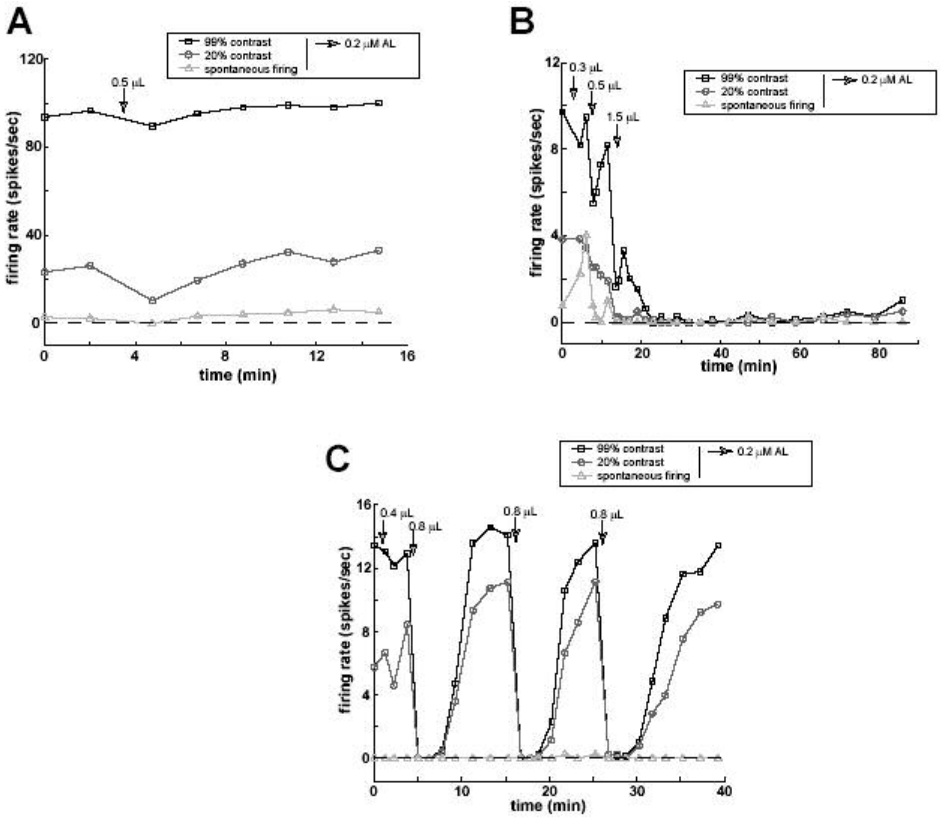

Figure 3-8. The AL/AlstR system can quickly and reversibly inactivate monkey LGN neurons. Recordings at 3 different sites (A-C), all from the same recording session in the LGN of a monkey expressing AlstR, are illustrated in chronological order. Format of plots is the same as in figure 5 (note different 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 hours 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 minutes; very little activity could be detected even at other sites within 600 μm of this site when the electrode was repositioned 90 minutes after AL injection. **C)** Single-unit recording made from the same penetration as in B, 130 minutes after the last AL injection indicated in B. This cell inactivated and recovered quickly and repeatedly in response to repeated AL injections.

within 5 minutes of AL injection, and cells inactivated completely within 10 minutes of AL injection.

After inactivation of the cells shown in Figure 3-8B, more than 60 minutes elapsed without recovery. At this time, the electrode was advanced ventrally in small increments, covering a distance of 600µm. 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 minutes after the previous AL injection, completely inactivated in response to a new AL injection (1.8 µl of 0.2 µM) which resulted in complete inactivation of this neuron. Five minutes later the electrode was moved again, and 130 minutes after the previous AL injection, another visually responsive neuron was isolated. Data from this recording are illustrated in Figure 3-8C. 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 expressing low levels of 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 3 recording sessions in monkey. In cases where inactivation was not observed, there are several potential explanations: a)

recordings may have been made from non-AlstR-expressing cells; b) 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; c) 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 11.4º az, -6.7º 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 drop-off 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 GFP 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*^{Cre}; *AlstR192* mice) which results in widespread expression of GFP (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 (lL2) and left extensor activity (lL5) in spinal cords isolated from*nestin* ^{Cre}; *AlstR192* mice (Figure 3-9). Application of 1 μM AL strongly silenced rhythmic motor activity, and wash-out of AL resulted in recovery of rhythmic motor activity within 5 minutes. In contrast, allatostatin (100 nM–5 μM) had no effect on

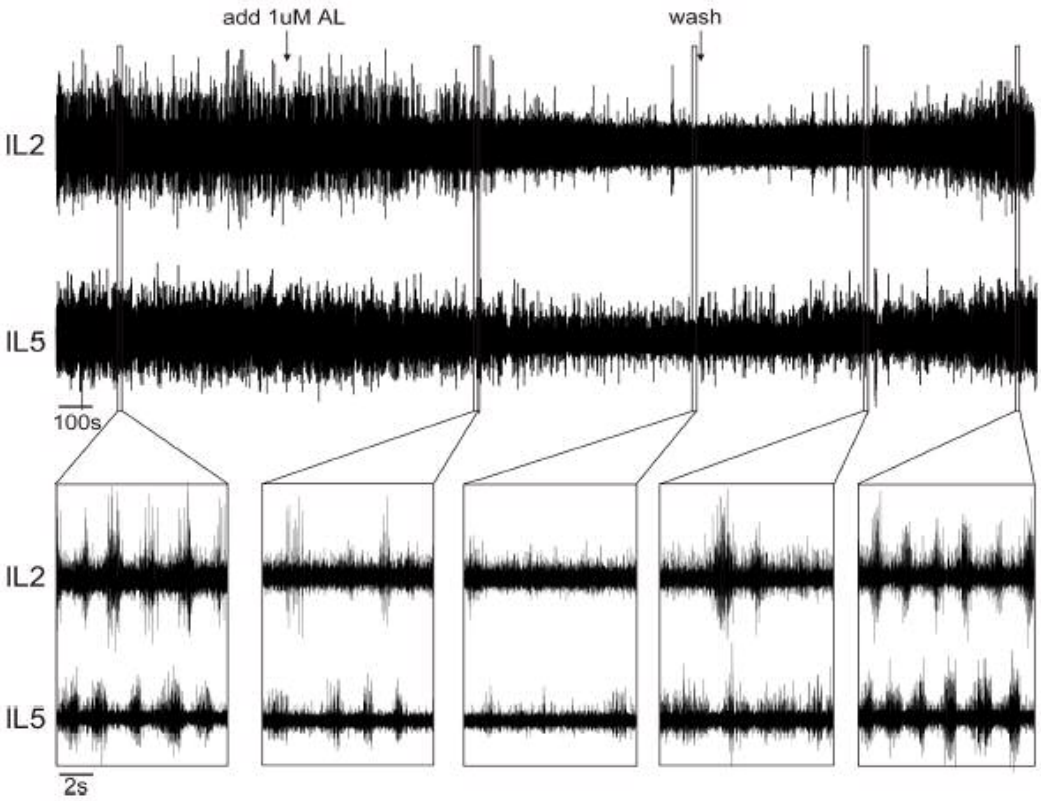

Figure 3-9. The AlstR/AL system quickly and reversibly inactivates mouse spinal motor neurons. Ventral root electroneurogram recordings from left L2 and left L5 ventral roots in isolated spinal cords of P0 *nestin*^{Cre}*;AlstR192* mice. Recordings shown at the top are at a slow time scale while insets show selected points at a faster time scale. 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 1μm AL to the bath, activity gradually reduced over the next 5-10 minutes. 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.

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.

D. 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-expresing 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 therefore 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 make 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 1mm 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 Miesenbock, 2005; Zemelman et al., 2002; Zemelman et al., 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 (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.

E. 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 ug of pXX6-80 (Ad5 genome), 7.5 ug of either pXR1 (for serotype 1) or pXX 2 (for serotype 2), and 7.5 ug 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 hours after transfection, resuspended in 15 ml of Gradient Buffer (10 mM Tris (pH 7.6), 150 mM NaCl, 10 mM MgCl2), subjected to four cycles of freeze/thaw in addition to passing through a syringe with a 21-23G 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 x 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 $9.2x10^{11}$ particles/ml); 2) an AAV2 containing the same promoter and cassette (termed "AAV2-AlstR-EGFP," initial titer $1.35x10^{10}$ particles/ml); 3) an AAV1 encoding EGFP under the control of a CMV promoter (termed "AAV1-EGFP," initial titer $1.5x10^{11}$ particles/ml); and 4) an AAV2 encoding EGFP under the control of a synapsin promoter (termed "AAV2-EGFP," initial titer $3.83x10^{9}$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

6 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 CO_2 ($EtCO_2$), Pulse oxygen (SpO_2) and heart rate were monitored continuously to judge the animal's 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 µm tip diameter) by suction, and injected by pressure using a Picospritzer II (General Valve Corp.) at a rate of 0.2-2 µl /min in 10-20 msec bursts of pressure (10-40 psi). A total of 5-8 µl of virus was typically injected at 3-5 sites at depths of 300 µm, 600 µM , 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, SpO_2, $EtCO_2$, 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 msec duration, 0.1 Hz) generated from a Grass S44 stimulator and SIU5 stimulus isolation unit (Grass Instrument). Local field potential (LFP) recordings were made using epoxylite-coated tungsten electrodes (1 MΩ 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_2 (Birgul et al., 1999), was synthesized in-house and stored in 100 µM aliquots in water at –80ºC. 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 sec beginning 5 msec 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 3-2). 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 3-3). 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 acitivity, 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 minutes 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 minutes following AL application and saline washout, respectively, divided by the average response 3 minutes prior to AL application, times 100.

Histology

After cortical recordings, electrolytic lesions (-3 to -4 μA, 3-5 s) were made to mark the recording site. In some experiments, electrodes were coated with DiI (0.25% in 100% ethanol) to aid recording site identification. After lesions were made, the animal was deeply anesthetized with an overdose of sodium pentobarbital (>100mg/kg, IP). 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 μm 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

15 adult female ferrets (*Mustela putorius furo*, 0.7-1 kg) were used in this study. Nine ferrets were used in cortical studies and for each of these, AAV was injected into area 17 of a single hemisphere. Of these 10 ferrets, 9 were injected with AAV1-AlstR-EGFP and 2 with AAV1-EGFP. One animal injected with AAV1-AlstR-EGFP received recordings only in the non-injected hemisphere; one animal injected with AAV1-EGFP received recordings from both injected and non-injected 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; 3 were injected with AAV2-AlstR-EGFP and 1 with AAV2-EGFP. All 4 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 µl of virus was typically injected at several depths along 2-4 penetrations in the cortex per ferret; in all cases, only 1 hemisphere was injected. 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 8-12µl of virus was typically injected along 2-4 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 (~1mm) 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 non-corrective, gas-permeable contact lenses. EEG, EKG, SpO_2, $EtCO_2$, heart rate, and body temperature were continuously monitored (to judge the animal's health and to maintain proper anesthesia levels).

Recordings were made using epoxylite-coated tungsten electrodes (FHC, Bowdoinham, ME) of 2-5 MΩ 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), 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 multi-electrode drive (Mini-05 microdrive, Thomas Recording Inc; electrode impedances 1-2 MΩ). 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 Offline 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°–2°, presented on a constant gray background (each linearized gun at half-maximal intensity), of mean luminance ~28 cd/m^2. Stimuli were shown for 4 seconds 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 inserted at an angle, and its tip was positioned at the same depth as, and 0.5-1mm 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 micropipettes 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 20-30 μm. 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 2 presentations of each visual stimulus were averaged and plotted as shown in results. In Figure 3-4, spontaneous firing rates represent average responses to blank trials presented during a single stimulus set; in Figures 3-5, 3-6 and 3-7, 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 minutes (or fraction thereof, if data was not collected for 10 minutes) preceding AL application, regardless of AL concentration. Responses were averaged over 5-minute bins following any fluid application. Percent remaining activity after AL treatment was calculated from the 5-10 minute bin following application of 0.1μM AL (2 ferrets) or 0.05 μM AL (1 ferret); in one case, a 0.01 μM concentration of AL had been previously applied, with no effect. Recovery was taken as the 5-minute bin yielding the highest average response to a 99% contrast stimulus after application of the saline wash. Responses during these 5-minute bins were divided by baseline responses to yield the silencing and recovery indices reported.

In control ferrets, no reduction in firing was observed in response to AL applied in a range of doses from 0.1-10 μM. To calculate percent remaining activity in these cases, mean responses during the 5-10 minute 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 evn if AL had been previously applied.

The following are criteria for exclusion of recordings from our analysis: a) AL had previously been applied to the area, b) cells were not visually responsive, c) the recording site could not be identified after the experiment, d) the recording site was found to lie outside of the virus-infected area, e) injury discharges contributed to the spike count during the experiment, f) the recording did not silence upon muscimol application, or did not exhibit orientation tuning if muscimol was not applied (cortical experiments only), or g) the medial-lateral distance between the recording electrode and AL-containing pipette was not 0.2-1mm (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 hours 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 DiI-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 μm 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 Ringers 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 µl of virus (AAV2-AlstR-EGFP) was injected at several depths along 3 penetrations in the monkey's left LGN. After virus was injected, the chamber was re-capped, 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 μg/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 MΩ resistance epoxylite 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 (ENG) Recordings

nestin^{Cre}; *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 NIH. Animals were anesthetized, decapitated and spinal cords were dissected out in ice-cold Ringers solution (see Lanuza et al., 2004). Recordings were made in Ringer's solution at room temperature (20^{o}C) by placing the second and fifth lumbar ventral roots (i.e. rL2, lL2, rL5, lL5) in bipolar suction electrodes. ENG signals were amplified, bandpass filtered (100 Hz-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 μM, Sigma) and 5-hydroxytryptamine (5-HT, 10 μM, Sigma) to the perfusing Ringer's solution. Effects of allatostatin on the locomotor pattern were examined by adding the peptide to the perfusion solution.

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IV. Conclusion

A. Conclusion

A major goal of systems neuroscience is to understand how individual cell types contribute to the overall function of neural circuits and behavior. As described in Chapter I of this dissertation, genetically-based methods for investigating circuitry allow the experimenter to target and perturb the activity of selected cell types, allowing investigation of the contribution of those cell types to neural circuit function and behavior. Genetic strategies for manipulating neural activity have taken many forms, outlined in Chapter II of this dissertation. Some of these strategies allow reversible changes in neural activity, differing in their timescales of reversibility and the agent used to elicit the change.

Although genetic approaches have led to numerous successful manipulations of neural activity *in vitro*, few *in vivo* successes have been reported, particularly in mammals. This is because translation of these methods for *in vivo* application is far from straightforward. DNA and pharmacological agents cannot be delivered to cells *in vivo* using the same strategies employed *in vitro*. Additionally, lower protein expression levels resulting from the available *in vivo* DNA delivery options may be insufficient to mediate the desired changes in excitability. Endogenously expressed proteins pose yet another challenge: some methods require sufficient levels of endogenous proteins for successful perturbation of activity, while other methods may experience interference from

endogenous proteins, either through non-specific responses to light or pharmacological stimuli or through interaction with the proteins responsible for mediating the desired changes in excitability. Indeed, very few of the methods described above have been successfully applied *in vivo* (Karpova et al., 2005; Lima and Miesenbock, 2005; Nitabach et al., 2002; Redfern et al., 1999; White et al., 2001). Of these, most are irreversible and/or employ invertebrate models, making them less than ideal for investigating neuronal circuitry and behavior in mammals.

Due to the large number of circuitry and behavior studies conducted in mammals, we sought to develop a genetically-based method that could be applied to mammalian neurons *in vivo*. We were particularly interested in using non-transgenic methods for expressing this system, as many organisms used for circuitry and behavior studies are not amenable to transgenics. In a previous report (Lechner et al., 2002), we described the allatostatin receptor/allatostatin (AlstR/AL) system for reversible inactivation of neurons *in vitro*. In Chapter III of this dissertation, this method was applied *in vivo*.

As mentioned above, considerable obstacles must be overcome for translating genetically-based *in vitro* methods to *in vivo* methods for perturbing neural activity. The AL/AlstR method is no exception, and several variables must be perfectly tuned for successful inactivation of neurons. The first of these is DNA delivery: to achieve long-term expression of AlstR in a large number of neurons without compromising tissue health (Xiao et al., 1997), we delivered DNA to neurons using AAVs. This strategy, however, presents a second problem:

it was unclear whether the level of AlstR expression obtained using AAVs would be sufficient to achieve complete inactivation of neurons. A third issue is the expression of endogenous proteins: proper functioning of the AL/AlstR system depends on endogenously-expressed G proteins and GIRK channels (Birgul et al., 1999), and sufficient levels of these proteins are necessary to successfully mediate inactivation. A final obstacle is the AL delivery method: AL can simply be washed into bath solutions in *in vitro* studies, but *in vivo* application requires more sophisticated delivery of AL.

The study presented in Chapter III of this dissertation successfully tackled these issues. Using AAV to express AlstR in cortical and thalamic neurons of rat, ferret, and monkey, we have demonstrated that the AL/AlstR method can be used to reversibly inactivate neurons in a wide variety of systems. Additionally, in isolated spinal cords from transgenic mice conditionally expressing AlstR, application of AL resulted in reversible inactivation of neurons on a timescale of minutes.

In conclusion, we have overcome many obstacles to transform the AlstR/AL system from an *in vitro* method to an effective *in vivo* method for quickly reversible inactivation of mammalian neurons. The method can be applied to many mammalian systems, making it very versatile. Application of this and other existing *in vivo* methods (Karpova et al., 2005) to various systems will greatly improve our understanding of neural circuits and behavior in mammals. With persistence, the future should bring us additional *in vivo* genetic methods that

will further elucidate the roles of individual populations of neurons in circuit function and behavior.

B. References

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