

Transgenic Silencing of Neurons in the Mammalian Brain by Expression of the Allatostatin Receptor (AlstR)

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¹Departments of Psychology and ³Biology, ²Institute of Neuroscience, and ⁴Transgenic Mouse Facility, University of Oregon, Eugene, Oregon; ⁵Oregon Health and Sciences University, Portland, Oregon; and ⁶The Salk Institute for Biological Studies, La Jolla, California

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Wehr M, Hostick U, Kyweriga M, Tan A, Weible AP, Wu H, Wu W, Callaway EM, Kentros C. Transgenic silencing of neurons in the mammalian brain by expression of the allatostatin receptor. *J Neurophysiol* 102: 2554–2562, 2009. First published August 19, 2009; doi:10.1152/jn.00480.2009. The mammalian brain is an enormously complex set of circuits composed of interconnected neuronal cell types. The analysis of central neural circuits will be greatly served by the ability to turn off specific neuronal cell types while recording from others in intact brains. Because drug delivery cannot be restricted to specific cell types, this can only be achieved by putting “silencer” transgenes under the control of neuron-specific promoters. Towards this end we have created a line of transgenic mice putting the *Drosophila* allatostatin (AL) neuropeptide receptor (AlstR) under the control of the tetO element, thus enabling its inducible expression when crossed to tet-transactivator lines. Mammals have no endogenous AL or AlstR, but activation of exogenously expressed AlstR in mammalian neurons leads to membrane hyperpolarization via endogenous G-protein-coupled inward rectifier K⁺ channels, making the neurons much less likely to fire action potentials. Here we show that this tetO/AlstR line is capable of broadly expressing AlstR mRNA in principal neurons throughout the forebrain when crossed to a commercially-available transactivator line. We electrophysiologically characterize this cross in hippocampal slices, demonstrating that bath application of AL leads to hyperpolarization of CA1 pyramidal neurons, making them refractory to the induction of action potentials by injected current. Finally, we demonstrate the ability of AL application to silence the sound-evoked spiking responses of auditory cortical neurons in intact brains of AlstR/tetO transgenic mice. When crossed to other transactivator lines expressing in defined neuronal cell types, this AlstR/tetO line should prove a very useful tool for the analysis of intact central neural circuits.

INTRODUCTION

The mammalian nervous system is composed of innumerable different neurons, each connected to thousands of other neurons. Communication between neurons is critically dependent on neurotransmitter release triggered by action potentials, which in turn depend on the membrane potential. Thus one can “silence” neurons by preventing the membrane potential from rising, effectively removing them from the circuit. The neuron-specific expression of silencer transgenes should enable investigators to perform circuit analysis of the intact mammalian brain: recording from one circuit element (i.e., neuronal cell type) before and after “shorting out” another one.

Toward this end, we have created and characterized a line of transgenic mice [called allatostatin (AL) neuropeptide receptor

(AlstR)/tetO] capable of expressing one such neuronal silencer, the allatostatin receptor (AlstR) under the control of the tetO element. The AL receptor is an invertebrate neuropeptide receptor (Birgul et al. 1999) developed by Callaway and colleagues for use as a silencer of mammalian neurons (Lechner et al. 2002). When expressed in mammalian neurons, AlstR is capable of activating G-protein-coupled inward rectifier K⁺ (GIRK) channels, nearly ubiquitous K⁺ channels that provide strong outward currents at subthreshold voltages (Gosgnach et al. 2006). GIRK activation thus serves to keep the membrane from depolarizing above threshold, thereby inhibiting action potentials. Because mammals do not have anything similar to the AL neuropeptide or its receptor, application of AL to the brains of intact animals should specifically turn off only those neurons that express the AlstR transgene.

The tetO element is part of the two-part system developed by Bujard and colleagues (Mansuy and Bujard 2000) to provide temporal control of transgene expression by combining elements of the bacterial tetracycline resistance operon with a viral transactivator. This two-part system involves crossing a transactivator line expressing the tTA fusion protein (also called “tet-off,” or its converse, rtTA or “tet-on”) from a cell-specific promoter to a “payload” line expressing a transgene under the control of the bacterial tetO promoter element. Wherever the tTA transgene is expressed, the expression of the transgenic payload can be controlled by the presence or absence of doxycycline in the animals’ diet. Because the AlstR silencer transgene is activated by a ligand, the temporal control afforded by the tetO/tTA system is of secondary importance. The key feature of this system for our purposes is its modularity: a tetO silencer line can be crossed to any tTA (or rtTA) line to take advantage of whatever anatomical specificity it may afford.

Here we describe the creation of a tetO/AlstR line via pronuclear injection of mouse oocytes and demonstrate its ability to specifically express mRNA for the AlstR silencer transgene in many regions of the forebrain when crossed to a commercially available tTA line. We then use in vitro patch-clamp recordings in the hippocampal slice preparation to show functional silencing of CA1 pyramidal cells. Finally, we demonstrate the utility of the line for in vivo electrophysiology in intact brains by reversibly silencing the responses of neurons in the primary auditory cortex to auditory stimuli. We expect these mice will lead to an enormous amount of information about the functional roles of the various elements comprising the circuitry of the mammalian brain when distributed to the

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scientific community and crossed to other transactivator lines that express only in defined sets of CNS neurons.

METHODS

Construct generation

The plasmid AlstR_pBMN was cut with *HindIII* and *XbaI* to generate a fragment containing the entire AL receptor coding sequence. This fragment was directionally ligated into the polylinker of the pTRE-tight plasmid (Clontech, Mountain View, CA). The resulting pTRE-AlstR plasmid was cut with *XhoI* to yield our injection construct (Fig. 1) consisting of the P_{tight} promoter, the full-length coding sequence of AlstR mRNA, and an SV40 polyadenylation site. P_{tight} is a modified tetracycline response element (TREmod) consisting of seven direct repeats of a 36-bp sequence that contains the 19-bp tet operator sequence (*tetO*) and the minimal CMV promoter (P_{minCMV}), which lacks the enhancer part of the complete CMV promoter. Consequently, P_{tight} is thought to be transcriptionally silent in the absence of binding of tTA or rtTA to the *tetO* sequences. The same *HindIII/XbaI* fragment was ligated into pBluescript to make antisense riboprobes for nonradioactive in situ hybridization.

Animals and genotyping

The preceding construct was injected into B6D2F1 oocytes (due to their relative ease of transgenesis) by the Transgenic Mouse Facility at the University of Oregon. Pups were then evaluated for transgene incorporation via PCR of genomic DNA isolated from tail biopsies (see Fig. 1 legend for genotyping primer sets). Because B6D2F1 is the F1 generation of a cross between C57BL/6J and DBA/2J, founders should ideally be backcrossed to C57BL/6J for at least five successive generations to obtain a more homogeneous genetic background. The animals described here have not yet been backcrossed enough generations to be considered congenic C57BL/6J, although these matings are ongoing. The reason for this is because not every genotypically positive founder line necessarily expresses transgene; we first tested for expression by crossing mice from each line to a commercially available transactivator line that expresses tTA in the forebrain from the CamKII α promoter [B6; CBA-Tg(Camk2a-tTA)1Mmay/J mouse; Jackson Laboratories, strain 003010, Bar Harbor, ME]. We then used in situ hybridization (ISH) to test whether genotypically double-positive (for both tetO-AlstR and tTA) individuals actually expressed AlstR mRNA in the forebrain. Finally, we used in vitro hippocampal and in vivo cortical electrophysiology to test whether expression led to functional silencing. Only then did we know whether a given line was worth backcrossing, and indeed, only one of the seven founders turned out to pass all of these tests. Only after all this did we know that the line was worth back-crossing to C57BL/6J. All procedures were in strict accordance with the National Institutes of Health guidelines as approved by the University of Oregon Animal Care and Use Committee.

In situ hybridization and anatomical analysis

Animals were anesthetized with a lethal dose of pentobarbital (50 mg/kg) and perfused transcardially with freshly made 4% paraformaldehyde in phosphate-buffered saline (PBS). Following craniotomy, brains were postfixed in the perfusion solution overnight at 4°C and then kept in 30% sucrose at 4°C until the brains no longer floated (typically 16–24 h). Brains used for slice electrophysiology were hemisected, and hemispheres not used for recordings were placed directly in the postfix solution and then otherwise treated identically. Cryostat sections (30 μ m) were affixed to microscope slides, air-dried, and kept at –80°C until needed. Room temperature sections were covered with 0.3 ml of hot (62°C) hybridization solution (50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 1 mg/ml rRNA, 1 $\times$ SSC in DEPC-treated H₂O), fitted with a coverslip, and hybridized overnight in a humidity chamber at 62°C.

Nonradioactive in situ hybridization was performed using a digoxigenin-labeled riboprobe at an estimated concentration of 0.0125 μ g/ μ l visualized by anti-digoxigenin sheep Fab fragments conjugated to horseradish peroxidase (Roche No. 11207733907). The riboprobe was a 746-bp transcript using T7 RNA polymerase in the presence of dig-labeled nucleotides using the pBlu-AlstR construct linearized with *PstI* as template. Sections were washed 3 $\times$ 30' at 62°C in wash buffer (50% Formamide, 0.5 $\times$ SSC, 0.1% Tween-20), then 3 $\times$ 30' at room temperature in MABT (1 $\times$ maleic acid, 20% Tween 20). Slides were then incubated in blocking solution (MABT + 20% sheep serum + 2% blocking reagent, Roche No. 11096176001) for 3 h. The alkaline phosphatase-linked anti-digoxigenin antibody was then added, and the slices incubated at RT overnight. The slices were then washed at RT with MABT buffer 5 $\times$ 5' and then AP staining buffer (0.1 M NaCl, 50 mM MgCl₂, 10% polyvinyl alcohol 100,000–150,000 MW, 0.1 M Tris-HCl, pH 9.5), 2 $\times$ 10', after which 3.5 μ l/ml NBT and 2.6 μ l/ml BCIP, and 80 μ l/ml levamisole were added, and the colorimetric reaction was allowed to develop for 3–7 h at 37°C under agitation, then stopped by washing twice with PBS (1% Tween-20), then twice in DI H₂O. Slides were then dehydrated in graded ethanols and mounted with Permount.

Hippocampal slice electrophysiology

Mice were anesthetized with isoflurane and rapidly decapitated. The brain was rapidly removed, and slices from the left middle third of the hippocampus were cut using a vibrating microtome (VT 1000s; Leica Instrument, Leitz, Nussloch, Germany) into 300 μ m thickness in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 119 NaCl, 26 NaHCO₃, 2.5 KCl, 1 NaH₂PO₄·H₂O, 1.3 MgCl₂·6H₂O, 2 CaCl₂·2H₂O, and 25 dextrose (saturated with carbogen). Slices recovered in the oxygenated ACSF at 35°C for 30 min, then maintained at room temperature for $\geq$ 1 h prior to recording. Experiments were performed at room temperature. Slices were transferred to a small volume (<0.5 ml) recording cham-

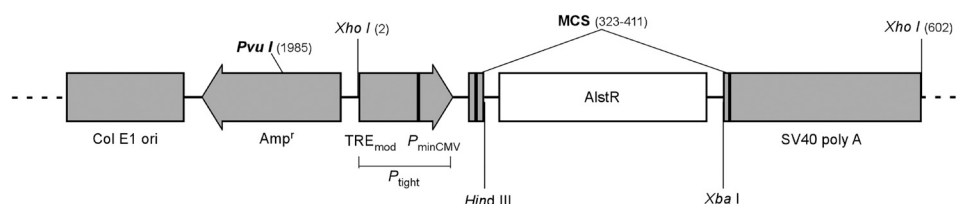


FIG. 1. Injection construct for the generation of allatostatin (AL) neuropeptide receptor (AlstR)/tetO mice. The cDNA encoding the AlstR transgene was excised with *BamHI* and *XbaI* and ligated into the multiple cloning site of the pTRE-tight vector (Clontech). The resulting plasmid was cut with *XhoI* to yield a fragment containing the pTRE element driving the entire coding sequence of the allatostatin receptor followed by the SV40 polyadenylation site. The 2 pairs (inner and outer) of genotyping primers used were OF: 5'-CACTGGAAACGGTAGTATC-3'; OR: 5'-CGTGACTCTGCGGAAGG-3'; IF: 5'-GGATCACAAT-GCCAACGAC-3'; IR: 5'-CAGATCTCCTCCTCCGTG-3'.

ber mounted on a fixed-stage, upright microscope (Axioskop FS2; Carl Zeiss, Thornwood, NY) equipped with infrared differential interference contrast (IR-DIC) optics. The recording chamber was continuously superfused with carbogen-saturated ACSF flowing at a rate of ~ 2 ml/min. Whole cell patch-clamp recordings were performed on visually identified CA1 pyramidal neurons. Patch electrodes were pulled from filamented, thick-walled borosilicate glass pipettes (BF150-86-10, Sutter Instruments, Novato, CA) and heat-polished to a resistance of $2\text{--}3\text{ M}\Omega$ when filled with the following internal solutions (in mM): 140 KMeSO₄, 10 KCl, 10 HEPES, 2 Mg₂ATP, 0.4 Na₃GTP, and 10 Tris-phosphocreatine; pH adjusted to 7.25 with KOH; final osmolarity ~ 290 mosM. Whole cell patch-clamp recordings were obtained from CA1 pyramidal cells using a Multiclamp 700A amplifier (Molecular Devices, Sunnyvale, CA), digitized using an Digidata 1440A analog-to-digital converter (Molecular Devices), and transferred to a computer using pClamp10 software (Molecular Devices). Experiments were performed in the

presence of SR95531 ($5\text{ }\mu\text{M}$), CGP55845 ($2.5\text{ }\mu\text{M}$), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, $25\text{ }\mu\text{M}$), and d-2-amino-5-phosphonopentanoic acid (D-AP5, $50\text{ }\mu\text{M}$) to block GABA_A, GABA_B, AMPA, and N-methyl-D-aspartate (NMDA) receptors, respectively. Current-clamp recordings were performed to assess the membrane properties of CA1 pyramidal neurons in control ACSF followed by bath application of AL (5 nM). Input resistance was measured with a series of hyperpolarizing current steps in 20-pA decrements. IE of the neurons was characterized with a series of depolarizing current steps in 25-pA increments. Solutions of the amygdalar complex (CxA, BMP, AHi, PMCo), and D-AP5 were purchased from Tocris Cookson (Ballwin, MO); AL peptide (Ser-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH₂) synthesized by Biomartik LLC, Wilmington, DE. All other drugs were purchased from Sigma (St. Louis, MO). CGP55845 was dissolved as a stock solution in DMSO. When used, the final concentration of DMSO was always $<0.1\%$.

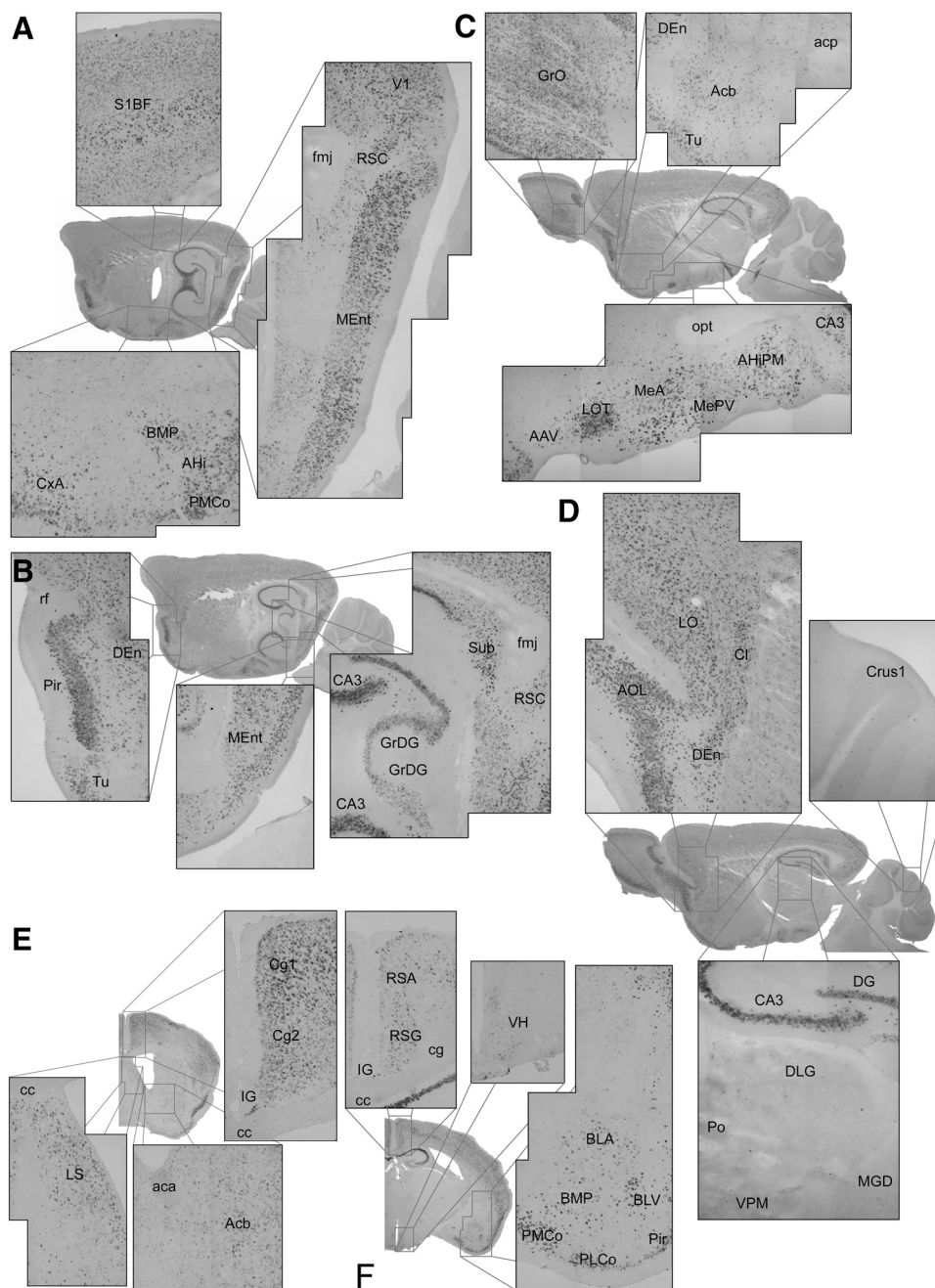


FIG. 2. AL receptor-associated mRNA expression was observed in principal neurons throughout the forebrain. The most striking exception to this expression pattern was the complete lack of expression in neurons of the thalamus. **A:** AlstR mRNA-expressing neurons were observed in visual (V1) and somatosensory (S1BF) cortices, the entorhinal cortex (MEnt), and components of the amygdalar complex (CxA, BMP, AHi, PMCo). **B:** AlstR mRNA-expressing neurons were observed, in addition to the aforementioned regions, in the piriform cortex (Pir), olfactory tubercle (Tu), dorsal endopiriform cortex (DEn), subiculum (Sub), and retrosplenial cortex (RSC). **C:** AlstR mRNA-expressing neurons were observed, in addition to the aforementioned regions, in the granule cell layer of the olfactory bulb (GrO), accumbens (Acb), nucleus of the lateral olfactory tract (LOT), and components of the amygdalar complex (AAV, MeA, MePV). **D:** AlstR mRNA-expressing neurons were observed, in addition to the aforementioned regions, in components of the olfactory system (AOL, LO) and the claustrum (Cl). mRNA expression was completely absent in thalamic nuclei (e.g., DLG, MGD, Po, VPM) as well as in the cerebellum (e.g., Crus1). **E:** AlstR mRNA-expressing neurons were observed, in addition to the aforementioned regions, in the anterior cingulate cortex (CG1, CG2), indusium griseum (IG), and the lateral septum (LS). **F:** AlstR mRNA-expressing neurons were observed, in addition to the aforementioned regions, in the retrosplenial cortex (RSA, RSG), components of the ventral hypothalamus (VH), and components of the amygdalar complex (BLA, BMP, BLV, PMCo, PLCo). aca: anterior commissure, anterior; acp: anterior commissure, posterior; cc: corpus callosum; fmj: forceps major; opt: optic tract; rf: rhinal fissure. $1\times$ photomicrographs illustrate whole coronal and sagittal sections. Outlines (gray) indicate locations of $5\times$ photomicrograph composites.

In vivo electrophysiology

We recorded from the left primary auditory cortex of anesthetized [(in mg/kg)120 ketamine, 0.24 medetomidine, 3 acepromazine] mice aged 3–3.5 mo. Recordings were made from well-tuned regions of auditory cortex (as determined by the frequency-amplitude tuning properties of multi-unit spiking responses and local field potentials), which probably correspond to cortical areas A1, AAF, or A2 (Linden et al. 2003). Subpial depth of recordings ranged from 330 to 435 μm , as determined from micromanipulator travel. For multi-unit recordings we used 1–2 M Ω tungsten electrodes amplified with an A-M Systems 1800 extracellular amplifier and band-pass filtered from 300–5000 Hz). 10 μM AL (Biomatik) in saline, or saline (0.9% NaCl) was continually superfused at a rate of 0.1–0.3 ml/min to the cortical surface. We used a pseudo-randomly interleaved stimulus array consisting of nine intensities of white noise bursts, linearly spaced from 0 to 80 dB SPL, with 25-ms duration, 3-ms 10–90% cosine-squared ramps, and a 500-ms interstimulus interval. We thresholded multiunit activity at a fixed voltage threshold for each recording site, which was set to 5 SDs of the extracellular voltage for the first measurement protocol for each site. We quantified spiking responses by counting spikes in a 100 ms window following sound onset. Recording sites for which spiking responses permanently increased or decreased during the recording, and did not recover, were excluded from analysis ($n = 3$ mice). In addition, at the end of each recording, we verified that the recording depth matched the distance traveled to return to the cortical surface; sites for which these two distances differed by $>50 \mu\text{m}$ were excluded from analysis ($n = 2$ mice).

RESULTS

Figure 1 shows our injection construct consisting of the AL receptor coding sequence under the control of a modified tetO element (pTRE-Tight, Clontech). Our injections yielded seven distinct founder lines, six of which transmitted genotypically. All lines were then mated to a commercially available line expressing tTA from the CamKII α promoter to check whether they were capable of driving AlstR expression in the forebrain. Only one of the lines (AlstR/tetO) was found to express significant levels of AlstR mRNA. Luckily, as shown in Fig. 2, this line expressed AlstR mRNA in all major brain areas that this tTA line has been shown to drive transgene expression (Mayford et al. 1996). As expected, the pattern of AlstR expression (Table 1) is quite similar to the distribution of the alpha subunit of calmodulin-dependent kinase II (αCamKII) (Liu and Jones 1996), the promoter used to drive tTA expression in this cross.

To determine the degree of penetrance of transgene expression (i.e., what percentage of cells of a certain type express the transgene), we compared cell counts of in situ results with the AlstR probe with Nissl stains (see Fig. 3). We concentrated on the hippocampus proper because the cell types are relatively well characterized there, sidestepping the quite involved task of defining cell types throughout the forebrain. We superimposed the Nissl stain with the in situ to count how many nuclei in the various layers of the hippocampus were positive for the AlstR mRNA. The results are shown in Fig. 3D, and although the quantitation may slightly exaggerate positive neurons in stratum pyramidale because the strong colorimetric signal spreads somewhat there, essentially all of the primary neurons (CA1 and CA3 pyramidal neurons and, to a lesser extent, dentate granule neurons) express AlstR mRNA. Consistent with both the native expression patterns of CamKII α (Liu and

TABLE 1. Regional distribution of AlstR mRNA

Category	Region	Cell Density	Expression
Sensory Cortices	L2,3	Dense	Mod
	L4	Dense	Light
	L5	Dense	Dark
	L6	Dense	Mod
Motor Cortex	L2,3	Dense	Light
	L5	Dense	Mod
	L6	Sparse	Light
Amygdalar	CxA	Dense	Dark
	ACo	Dense	Dark
	BLA	Mixed	Light
	BLP	Mixed	Light
	BMA	Mixed	Light
	BMP	Dense	Mod
	PLCo	Dense	Mod
	AHi	Dense	Dark
	PMCo	Dense	Mod
	AAV	Sparse	Light
	MePD	Dense	Light
	LOT	Dense	Dark
Striatum	CPu	Dense	Light
	LAcSh	Mixed	Light
	AcbSh	Mixed	Light
	AcbC	Mixed	Light
Hippocampal Formation	CA1,3	Dense	Dark
	DG	Dense	Light
	FC	Dense	Dark
	Sub	Dense	Dark
	PrS	Dense	Light
	PaS	Dense	Dark
	MEu	Dense	Dark
Agranular Insular Ctx	L2,3	Dense	Light
	L4	Dense	Mod
	L5	Dense	Dark
	L6	Dense	Mod
Retrosplenial Ctx	L2,3	Dense	Light
	L5	Dense	Mod
Anterior Cingulate Ctx	L6	Sparse	Light
	L2,3	Dense	Mod
	L5	Dense	Mod
Lateral Orbital Ctx	L6	Dense	Light
		Mixed	Light
Piriform Ctx		Dense	Dark
Olfactory Structures	Tu	Dense	Dark
	AOL	Mixed	Dark
	AOV	Dense	Dark
	EPI	Dense	Dark
	GI	Dense	Dark
	AOB	Dense	Dark
	AOD	Dense	Mod
	AOE	Dense	Mod
Cholinergic System	VDB	Sparse	Mod
	SI	Sparse	Light
	LS	Dense	Light
Other Structures	CI	Dense	Dark
	DEu	Dense	Dark
	SNR	Sparse	Dark
	DpMe	Sparse	Mod
	IG	Dense	Dark
	VHN	Sparse	Light

CxA, cortex-amygdala transition zone; ACo, anterior cortical amygdaloid nucleus; BLA, basolateral anterior amygdala; BLP, basolateral posterior amygdala.

Jones 1996) and prior work with this tTA line (Mayford et al. 1996), few if any interneurons express the transgene (as judged by the low ratio of positive neurons outside of stratum pyramidale). Thus the AlstR/tetO line appears to be able to strongly express AlstR mRNA in primary neurons throughout the forebrain.

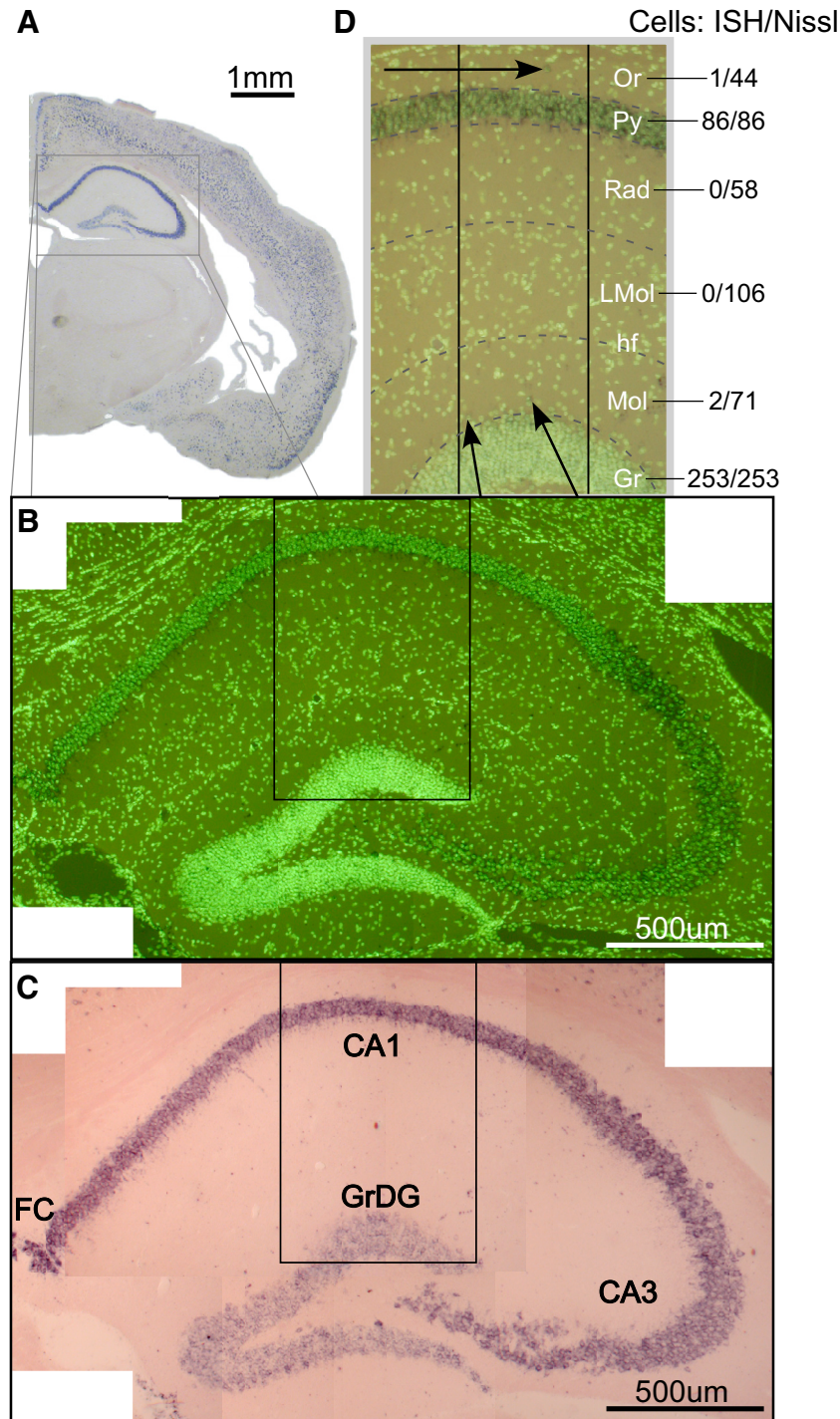


FIG. 3. Robust penetrance of allatostatin receptor-associated mRNA was observed in principal neurons of the hippocampus in contrast to an almost complete lack of receptor-associated mRNA in hippocampal interneurons. *A*: 1X bright-field photomicrograph of a coronal section from the hemisphere opposite that used for in vitro characterization of silencing resulting from bath-application of AL. *B*: a composite of 10X dark-field photomicrographs illustrating fluorescent Nissl labeling in the hippocampus. The composite corresponds to the rectangular outline illustrated in *A*. *C*: a composite of 10X bright-field photomicrographs illustrating mRNA labeling of principal neurons in cornu ammonis 1 and 3 (CA1 and CA3, respectively), granule cells of the dentate gyrus (GrDG), and fasciola cinereum (FC). *D*: a magnified view of the rectangular region identified in *B* and *C* illustrating a semi-transparent view of fluorescent Nissl labeling overlain on the corresponding view of mRNA expression. Numbers in *D* indicate the number of soma co-expressing AlstR-associated mRNA and fluorescence (numerator) compared with the total count of fluorescing cells (denominator). hf: hippocampal fissure; LMol: lacunosum moleculare; Mol: molecular layer; Or: stratum oriens; Rad: stratum radiatum.

However, the most important aspect of the AlstR/tetO line is its ability to functionally silence the neurons it is expressed in. Because of the strong AlstR mRNA expression in hippocampal pyramidal neurons (see Fig. 3), we used in vitro whole cell patch-clamp methods to record from CA1 neurons in hippocampal slices (Fig. 4). The current-clamp configuration was used to minimize complications of space clamp. Figure 4A shows that bath application of 5 nM AL to wild-type slices has no significant effect on the ability of known amounts of somatically injected current to drive spiking of the neurons (if anything, the trend is toward a requirement for less current

during AL application). However, as seen in Fig. 4B, slices made from double (tTA and tetO)-positive animals require more than twice as much current to get similar amounts of action potentials in the presence of AL. Consistent with the induction of GIRK channel activity, AL application also hyperpolarized the membrane (Fig. 4C) and decreased its input resistance.

To test whether the transgenic expression of AlstR can silence neurons in the intact brain, we obtained multi-unit responses to auditory stimuli from the middle layers of auditory cortex in ketamine-anesthetized mice. We measured the

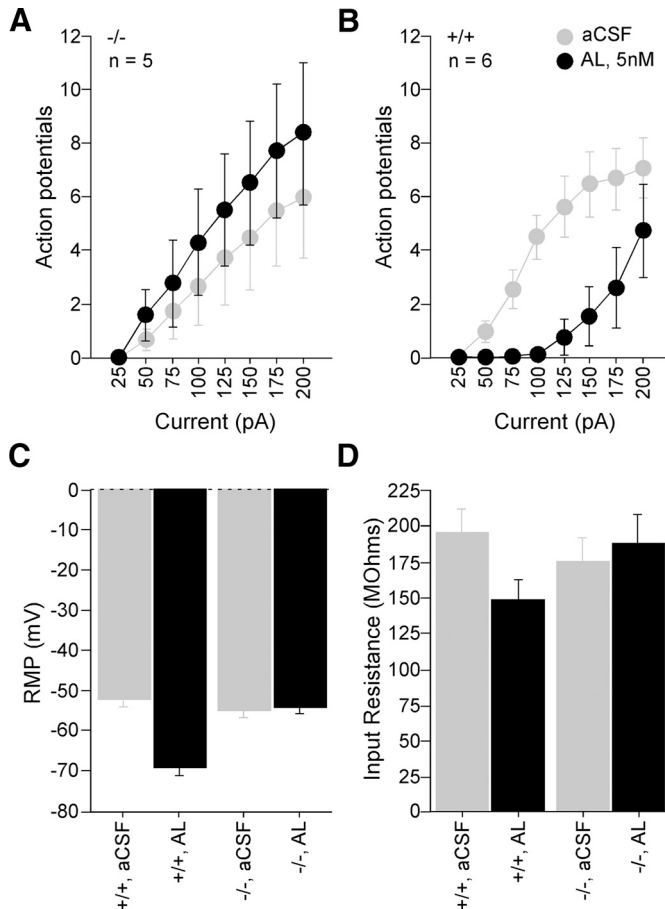


FIG. 4. *A* and *B*: AL significantly decreased intrinsic membrane excitability in neurons from +/+ (*B*) but not -/- (*A*) mice as evidenced by the increase in the number of action potentials elicited across a range of depolarizing current injections. Black represents recordings acquired in control artificial cerebrospinal fluid (aCSF); red represents recordings acquired following bath application of AL. *C* and *D*: consistent with G-protein-coupled inward rectifier K⁺ (GIRK) activation, bath application of AL significantly hyperpolarized the membrane (*C*) and reduced the resting input resistance (*D*) of CA1 pyramidal neurons from +/+ mice only.

spiking responses to brief bursts of white noise during continuous superfusion of the cortical surface with saline, then switched to superfusion with 10 μ M AL solution and then back to saline. Within 1–2 min of AL application, spiking responses were strongly diminished (Fig. 5*A*) and were completely silenced in <3 min. The first measurement began 46 s after the start of AL application and was completed 110 s later; the second measurement began 160 s after AL and was completed in 100 s. Because no spikes were evoked during the second measurement, this suggests that complete silencing occurred within 160 s. After switching back to saline superfusion, responses rapidly recovered to baseline levels and then increased to nearly two-fold greater than baseline levels for ~10 min. The suppression of spiking responses during AL application was effective across the entire range of stimulus intensities tested (0–80 dB SPL, Fig. 6*A*) for all three +/+ mice we successfully tested (Fig. 6*B*) but not for three littermate controls (either +/- or -/- mice), which showed no effect of AL at the population level.

While these data are entirely consistent with complete, rapid and reversible silencing of sound-evoked responses in the mice

expressing AlstR in auditory cortex, there are some interesting deviations from this straightforward interpretation that warrant further investigation. Most notably, in all three +/+ mice, we observed a partial recovery of sensory-evoked responses within ~20 min despite continued superfusion of AL. In addition, the animal shown in Fig. 5 demonstrated hyperexcitability following saline washout (Fig. 5*F*). Both of these phenomena could be explained either by cell-autonomous mechanisms such as receptor desensitization or homeostatic plasticity or network phenomena such as a gradual reduction in steady-state inhibition. Future experiments, ideally involving *in vivo* whole cell recordings, will be required to determine the nature of these phenomena.

DISCUSSION

Transgenic technologies arguably have the potential to revolutionize systems neurophysiology much as the advent of the slice preparation revolutionized the cellular neurophysiology of the mammalian brain in the 1960s (Skrede and Westgaard 1971) previously dominated by recordings from intact brains. Beyond simply increasing yield, the hippocampal slice preparation (and other reduced preparations) basically brought cellular specificity to the neurophysiological study of local circuits in the mammalian brain. In the slice, one can place extracellular electrodes with sufficient accuracy to evoke and record specific synaptic potentials and with the advent of differential interference contrast techniques (Dodt and Zieglensberger 1990) directly visualize the specific neurons one records from. This enables the investigation of the electrophysiological properties of specific neuronal cell types. Moreover, reduced preparations greatly streamline pharmacological manipulations, facilitating the investigation of the molecular bases of observed physiological phenomena.

However, these enormous advantages come at great cost: typically only the most local of circuits remain intact in reduced preparations. Thus *in vivo* recordings in intact brains are required to study the neural processing of information in the mammalian CNS at the systems level. However, it is extremely difficult to distinguish between diverse classes of neurons with *in vivo* recordings and even more difficult to manipulate specific classes of neurons. The promise of mouse molecular genetics for systems neurophysiology comes from its potential to provide something approaching the access and specificity afforded by the slice preparation to recordings from intact brains, ideally even in freely behaving animals. The AlstR/tetO line of mice presented here should aid in this goal by providing the means to express a ligand-gated silencer transgene in specific populations of CNS neurons.

There are two key requirements for the successful application of transgenics to the functional analysis of neural circuits: transgene expression needs to be limited to a defined set of neurons and the functional consequences of transgene expression in a given neuron must be electrophysiologically unambiguous. The tetO/AlstR line presented here has utility on both counts. We chose to use a tTA line that broadly expresses in forebrain neurons to facilitate the characterization of our tetO/AlstR line, but many other cell-specific transactivator (tTA or rtTA) lines that these mice could be crossed with have already been described (Chen et al. 1998; Nakashiba et al. 2008; Ralph et al. 2000; Yasuda and Mayford 2006) and more are surely

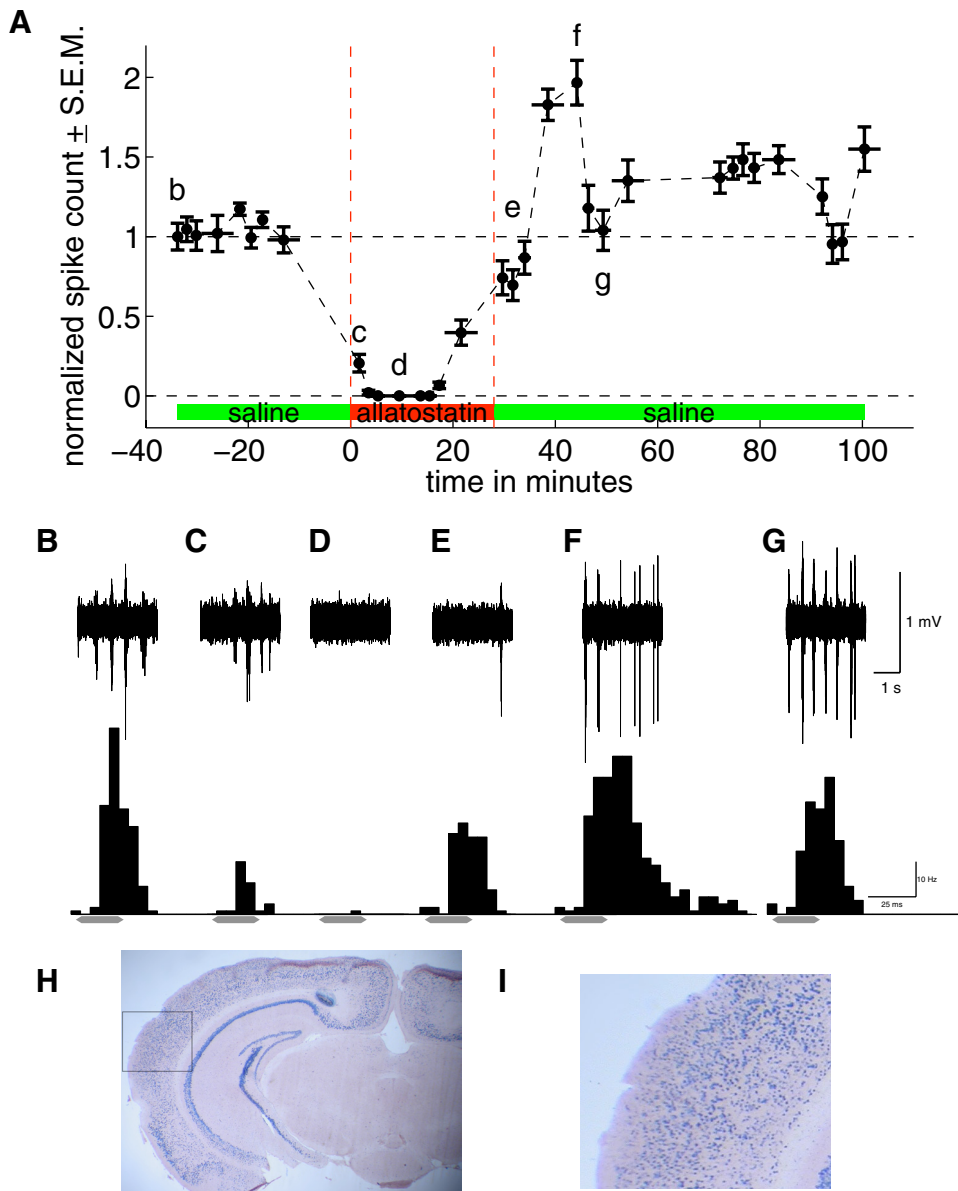


FIG. 5. A: multiunit spiking responses to white noise bursts (70 dB SPL, 25 ms) before, during, and after 10 μ M AL application. Recordings are from middle layers of primary auditory cortex. Each dot is the spike count (mean $\pm$ SE, $n = 20$ repetitions) in a 100-ms window following sound onset. The horizontal line for each dot indicates the duration of each stimulus protocol. Responses are normalized to the first measurement in the series. Note that spiking responses are markedly diminished within ~ 2 min and completely silenced shortly thereafter. Note also that evoked responses began to recover even before the end of AL application and showed a rebound effect after ~ 10 min of saline application. B–G, top row: extracellular voltage waveforms at each of the time points indicated in A. White noise bursts (interleaved intensities) were delivered at a rate 2/s. Bottom row: post-stimulus time histograms at the same time points (70 dB bursts). H and I: photomicrographs of AlstR mRNA in situ hybridization of a coronal slice from the brain of this mouse. Primary auditory cortex is located in the boxed region, which is expanded in I.

forthcoming. The modularity of the tTA/tetO system is thereby one of the line's major strengths. Each different cross could yield distinct and interesting results because silencing of neuronal cell types mainly probes circuit-level function, which may well be unique to each circuit. Recent experiments showing the change in the network properties of the spinal cord when AlstR is expressed in a particular set of spinal motoneurons by a recombinase-based approach (Gosgnach et al. 2006) provide an excellent illustration of the promise of these techniques, and the extensive silencing of auditory-evoked multiunit responses demonstrated here bodes well for AlstR's efficacy in other parts of the intact brain. Tools such as these are likely to be of considerable use in addressing questions at multiple levels of analysis, including cellular, local circuit, systems, perception, and behavioral, as they are in essence very selective molecular lesions with a granularity approaching that of the nervous system.

The silencing of neuronal activity described here is unambiguous. AL application in $+/+$ mice completely abolished

spiking activity in auditory cortex neurons. However, reversing the effect in vivo was not as straightforward as previously seen with this silencer (Tan et al. 2006) for reasons that may be biologically interesting (i.e., it may have to do with which specific subsets of cortical neurons express the transgene). We observed the first signs of recovery of neuronal activity prior to washout. In a subset of animals, we also saw hyperexcitability after washout, which declined over time back toward baseline. These results suggest that silencing and subsequent restoration of neuronal activity may show complex dynamics in vivo. This is perhaps not surprising given the complexity of cortical circuits and the homeostatic mechanisms that regulate their activity. However, it is worth noting that these interesting dynamics may more accurately portray what happens in vivo following disruption or damage to a discrete neural locus.

Our technique adds to a growing arsenal of genetically encoded systems designed to selectively and reversibly perturb the activity of specific neuronal populations in vivo. Each of these systems has considerable merit, and one may be more

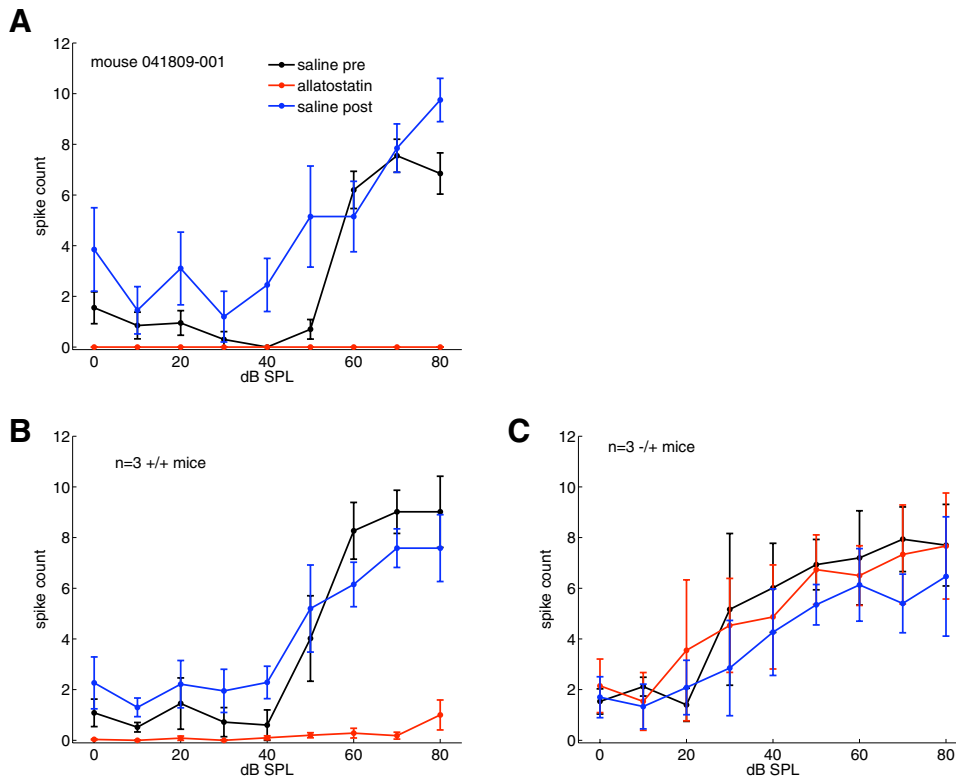


FIG. 6. *A*: multiunit spiking responses to white noise bursts across a range of stimulus intensities (0–80 dB SPL). Each dot is the spike count (mean $\pm$ SE, $n = 20$ repetitions) in a 100-ms window following sound onset. Same recording site and animal as Fig. 1. Black line represents saline superfusion 35 min prior to allatostatin; red line represents 12 min after start of AL superfusion; blue line represents 19 min after start of 2nd saline superfusion. Note that spiking responses were silenced even at the highest stimulus intensities used (80 dB SPL). *B*: multiunit spiking responses across the same stimulus intensities in 3 +/- mice (error bars indicate SE with $n = 3$ mice). Responses during AL (red line) were recorded at maximal silencing (12, 5, and 2 min after start of AL application). *C*: multiunit spiking responses across the same stimulus intensities, in 3 +/- or -/- mice ($n = 3$ mice).

appropriate than another depending on the specific application (Tervo and Karpova 2007). Two systems involve the expression of nonmammalian transmitter receptors that hyperpolarize neurons, either directly (the ionotropic GluCl system; Lerchner et al. 2007) or indirectly (the AlstR system) (Lechner et al. 2002). The onset and recovery of GluCl silencing take 1 and 4 days, respectively, which may be ideal for learning and other behavioral paradigms but may be too slow for reversibly silencing neurons when using most electrophysiological recording techniques. The time course of the AlstR system may be better suited for electrophysiology but requires that the targeted neurons express endogenous GIRK channels. AL also does not cross the blood brain barrier, whereas the GluCl ligand does. Because both systems act by hyperpolarization, they may be overwhelmed by strong synaptic drive, although we observed complete or near-complete silencing even for strong sensory stimuli (80 dB white noise bursts, see Fig. 6). A system that does not act by hyperpolarization is molecular inactivation of synaptic transmission (MIST), which instead involves the dimerization of modified synaptic proteins using an exogenous cell-permeable dimerizer. This system may therefore achieve silencing regardless of the strength of synaptic drive and indeed has been demonstrated to rapidly (~ 20 min) and reversibly block synaptic transmission in vitro and to affect behavior in vivo (Karpova et al. 2005). All of these systems require the application of a ligand (either systemically or intracerebrally); the advantage of rhodopsin-based systems is that they are instead activated by light. Channelrhodopsin-2 is a light-gated cation channel that has received much attention for its ability to optically stimulate neurons with exquisite temporal precision and reliability (Aravanis et al. 2007; Boyden et al. 2005; Huber et al. 2008; Lima et al. 2009; Tsai et al. 2009). Optical silencing of neurons using the halorhodopsin system, which activates a chlo-

ride pump, offers similarly impressive temporal precision (Zhang et al. 2007; Zhao et al. 2008). Optical silencing, however, requires optical access to the neurons in question. Surface illumination may work for superficial neurons, but deep structures require the use of fiber optics, with tissue scattering and absorption limiting the effective range to ~ 1.4 mm from the fiber tip (Aravanis et al. 2007). As with local application of silencer ligands, this can be advantageous if locally restricted silencing is desirable or can be a serious limitation if the desired target population is widely distributed. The unique advantages and limitations of each of these systems are good news for neuroscientists, as an ever-expanding toolbox is much more likely to provide the right tool for the job.

The functional consequences of AlstR activation are particularly unambiguous from an electrophysiological perspective. It is reversible, its effects can be quantitated relatively easily, and its time frame is fast enough following AL application to do the before-and-after experiments so critical for circuit-level questions. A potential issue is that because AlstR recruits endogenous GIRK channels, the target neurons must express GIRK channels to be silenced. Fortunately, these are relatively ubiquitous in the mammalian CNS (Ponce et al. 1996; Saenz del Burgo et al. 2008) and are clearly sufficient to silence the firing of CNS neurons, both in our hands and that of others (Gosgnach et al. 2006). There are lingering questions such as whether AlstR-induced activation of G-protein-mediated cascades does more than simply activate GIRK or indeed what the longer-term ramifications of sustained GIRK activation are. However, these can perhaps best be thought of as interesting questions in their own right that should not interfere with the immediate circuit-level manipulation of turning off specific neurons. Thus assuming the continuing generation of specific transactivator lines, these animals should enable a multitude of

experiments that all ask the same basic question: what happens to the rest of the system when you remove *these* neurons?

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During production, the superscript numeral 4 after Ute Hostick's name, indicating institutional affiliation at Transgenic Mouse Facility, University of Oregon, Eugene, OR, was omitted. The list of authors and their affiliations is given correctly here.

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