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Research report

Chlorotoxin-mediated disinhibition of noradrenergic locus coeruleus neurons using a conditional transgenic approach

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Abstract

The noradrenergic locus coeruleus (LC) has been implicated in the promotion of arousal, in focused attention and learning, and in the regulation of the sleep/waking cycle. The complex biological functions of the central noradrenergic system have been investigated largely through electrophysiological recordings and neurotoxic lesions of LC neurons. Activation of LC neurons through electrical or chemical stimulation has also led to important insights, although these techniques have limited cellular specificity and short-term effects. Here, we describe a novel method aimed at stimulating the central noradrenergic system in a highly selective manner for prolonged periods of time. This was achieved through the conditional expression of a transgene for chlorotoxin (Cltx) in the LC of adult mice. Chlorotoxin is a component of scorpion venom that partially blocks small conductance chloride channels. In this manner, the influence of GABAergic and glycinergic inhibitory inputs on LC cells is greatly reduced, while their ability to respond to excitatory inputs is unaffected. We demonstrate that the unilateral induction of Cltx expression in the LC is associated with a concomitant ipsilateral increase in the expression of markers of noradrenergic activity in LC neurons. Moreover, LC disinhibition is associated with the ipsilateral induction of the immediate early gene NGFI-A in cortical and subcortical target areas. Unlike previous gain of function approaches, transgenic disinhibition of LC cells is highly selective and persists for at least several weeks. This method represents a powerful new tool to assess the long-term effects of LC activation and is potentially applicable to other neuronal systems.

Keywords: Chlorotoxin; Conditional transgene; Cre-mediated recombination; Locus coeruleus; NGFI-A; Norepinephrine

1. Introduction

The locus coeruleus (LC), located in the dorsal pontine tegmentum, contains the largest number of noradrenergic neurons in the brain and is the major source of the brain's noradrenergic innervation. The LC has been implicated in the promotion of arousal, in focused attention and learning, as well as in the regulation of the sleep/waking cycle [7].

The biological functions of the LC have been investigated using several different approaches. Changes in LC firing pattern and/or in the release of brain norepinephrine have been studied as a function of circadian time, behavioral state, and nature of the task. These studies have shown that LC activity is high during the dark phase and low during the light phase [6], highest in waking, low in slow wave sleep and lowest in REM sleep [2], and highest in response to salient stimuli but low in response to non-relevant cues [4,5]. LC activity predicts behavioral performance, and changes in LC responses precede changes in behavioral responses [4,5,56].

Many studies have examined LC functions through neurotoxin-mediated lesions or pharmacological block with noradrenergic antagonists. For example, extensive depletion

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of telencephalic noradrenergic projections in animals can impair the acquisition of new behaviors [42], and pharmacological block of central noradrenergic activity increases lapses of attention in humans [47]. At the molecular level, an intact LC is necessary for the induction, during wakefulness, of several plasticity-related genes [15,16].

Fewer studies have attempted to study LC function by increasing its neural activity. Chemical stimulation of the LC produces cortical and hippocampal EEG signs of alertness [11] and electrical activation improves acquisition [57] and/or memory retrieval of specific tasks [43]. However, both electrical and chemical stimulation present several technical challenges. Electrical stimulation also activates fibers of passage, and pharmacological stimulation targets a particular receptor rather than a specific cell type. Moreover, these methods are short in duration and disrupt the physiological input patterns to the stimulated cells.

Here, we describe a novel method to stimulate LC neurons with high selectivity, for a prolonged period of time, and without uncoupling LC activity from its afferent input. We have generated transgenic mice in which LC neurons are chronically disinhibited because of a partial relief from GABAergic and glycinergic inhibition. This was obtained by the conditional activation of a transgene for chlorotoxin (Cltx) in the LC of adult mice. Chlorotoxin encodes a toxin with the ability to partially block small conductance chloride channels [18,19]. We show here that *Cltx* can be induced selectively in LC neurons and causes molecular and cellular effects in LC cells and in their target cortical and subcortical areas for at least several weeks. Although LC is the focus of this study, this strategy is potentially applicable to other neuronal systems.

2. Materials and methods

2.1. Transgene array construction and generation of transgenic mice

A synthetic gene for chlorotoxin containing a Kozak consensus sequence, ATG start codon, DNA sequence encoding the published amino acid sequence for chlorotoxin (GenBank Accession No. P45639), a stop codon, and flanking *Bam*HI restriction sites was constructed from synthetic oligonucleotides, cloned, and verified by sequencing. A cassette containing two loxP recombination sites separated by a *Stu*I restriction site, followed by a *Sna*BI restriction site, and flanked by *Bgl*II restriction sites, was also constructed from synthetic oligonucleotides, cloned into a plasmid vector, and verified by sequencing. To generate the transgene array, a lacZ reporter gene sequence containing a nuclear localization signal (J.M.S., unpublished) and a polyadenylation signal derived from SV40 was inserted via blunt ends into the *Stu*I site of the lox cassette vector, and the chlorotoxin gene was subsequently placed via blunt ends into the *Sna*BI site located 3′ to the loxP site. A fragment containing the "floxed" nlacZ reporter and the chlorotoxin gene was excised using the flanking *Bgl*II restriction sites and inserted into the *Bam*HI cloning site of an expression vector (kindly provided by Dr. Richard Palmiter, University of Washington) containing the promoter of the rat dopamine-β-hydroxylase gene [26,38], an intron of the rat preproinsulin II gene, a *Bam*HI cloning site, and a 3′-untranslated region and polyadenylation signal from the mouse protamine gene. Construct structure (see Fig. 3) was verified by sequencing. To prepare DNA for the production of transgenic mice, DNA containing the sequences of interest was purified from plasmid vector sequences by digestion with *Not*I, subjected to sucrose gradient centrifugation, dialyzed against injection buffer (5 mM Tris pH 7.50, 0.1 mM EDTA), and adjusted to a concentration of 2.5 μg/ml. Transgenic mice were generated by pronuclear microinjection of DNA [24] into fertilized oocytes of FVB mice, and subsequent fostering using B6CBAF1 mice. Transgene presence was assessed by PCR analysis of DNA samples. Transgenic mouse lines were maintained on the FVB background. Three independent lines of mice were obtained. F1 animals from each line were analyzed for correct transgene expression by staining for reporter gene activity in the brain (see below). Two lines of mice, DZC-1 and DZC-4, showed the expected lacZ reporter gene activity in LC cells and were used in all further experiments. All animal protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

2.2. Stereotaxic injection of Adenovirus vectors

Two Adenovirus vectors were used in this study. AdLacZ carries a lacZ reporter gene with a nuclear localization signal [59], and Adeno-Cre contains the gene for Cre recombinase under control of the thymidine kinase promoter [59]. Virus purification was performed as described [59], and viral suspensions were used at a concentration of 10^{11} plaque-forming units (pfu) per milliliter. Mice (average weight 23 g) were anaesthetized with pentobarbital (50 mg/kg, i.p.), and viral suspension was injected with a 33-gauge needle using a Kopf stereotaxic frame. Because LC is only 80 μm wide but extends for 450 μm in the rostro-caudal direction, 4 injections (300 nl of viral suspension each) were performed in each mouse: site I, AP −4.8, L 0.75, DV −4.2; site II, AP −4.8, L 0.75, DV −3.8; site III, AP −5.3, L 0.75, DV −4.2; site IV, AP −5.3, L 0.75, DV −3.8 [22]. At all sites, the injection needle was aimed to the medial aspect of LC, in order to avoid mechanical destruction of this nucleus and to prevent damage to fiber tracts. AdLacZ and histological staining for β-galactosidase were used as a marker for the accuracy of the injections. Mice were allowed to recover from surgery under supervision. No gross behavioral abnormalities were observed after unilateral viral injections.

2.3. Histological analysis of gene expression

All animals were deeply anesthetized with metofane inhalation and perfused transcardially with 20 ml of phosphate-buffered saline (PBS) followed by 40 ml of PBS containing 10% Sucrose and 4% Paraformaldehyde as fixative. Brains were dissected, placed in PBS/Sucrose/Paraformaldehyde for 6 h, frozen on dry ice and stored at − 80 °C. Frontal cryosections were cut at 30 μm and stored in PBS at 4 °C. Sections from the pontine area were examined to determine whether the viral injection procedure had resulted in tissue damage; animals that displayed excessive damage in LC were excluded from further analysis. For β-galactosidase staining, free-floating sections were washed three times for 15 min each in 0.1 M sodium phosphate buffer pH 7.3, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% Igepal (Sigma, St. Louis, MO). Staining was carried out in a solution containing 0.1 M sodium phosphate buffer pH 7.3, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% Igepal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 1 mg/ml X-Gal substrate (Molecular Probes, Eugene, OR) at 37 °C in the dark for up to 2 h. Immunocytochemistry was performed on frontal sections of the entire brain using a rabbit polyclonal antibody against NGFI-A (Santa Cruz Biotechnologies, Santa Cruz, CA; 1:1000). Cell counting was performed with the Image-1/metamorph imaging system by observers blind to the origin of the sections. The evaluation was based on at least three sections per region per animal. The background level was set such that only unequivocally positive, darkly stained cells were counted. The nonparametric Mann–Whitney *U* test was used for the statistical analysis of the results.

2.4. Cre-mediated recombination in cultured cells

A hemizygous transgenic male was mated to a FVB female, and embryos were obtained at 14.5 days of gestation. Primary mouse embryonic fibroblasts (MEF) were prepared from each embryo in the litter, cultured in DMEM, and expanded for DNA preparation. Each MEF line was genotyped by PCR for transgene presence. Cells from transgene-positive MEF lines were seeded in 24-well plates at a density of 50,000 cells/well, and infected with 10^6 pfu of Adeno-Cre 24 h after seeding. DNA was extracted from cells after 24 h and analyzed by PCR. A PCR product of 4 kb was indicative for the native transgene locus, whereas a PCR product of 0.5 kb was diagnostic for the recombined transgene locus.

2.5. Analysis of chlorotoxin (Cltx), tyrosine hydroxylase (TH), and norepinephrine transporter (NET) expression in vivo

To study the expression of the *Cltx* transgene after unilateral activation, RT-PCR reactions were performed on RNA extracted from hemisections of tissue. For this purpose, transgenic mice were injected unilaterally with Adeno-Cre as described above. Animals were sacrificed 4 weeks after injection. Brains were dissected, frozen on dry ice and sectioned at 30 μm thickness. Sections were thaw-mounted onto slides and stored at − 80 °C. Selected sections containing the LC area were stained for β-galactosidase activity to assess recombination efficiency as well as tissue integrity. Samples from animals that showed a 90% reduction of blue cells in LC of the injected side (indicating efficient recombination), concomitant with absence of gross tissue scarring, were selected to further analyze *Cltx* expression. A slide with a section containing LC and adjacent to a section showing positive reporter gene staining was placed on a slab of dry ice. The left and right half of the section were scraped off the slide individually and rinsed off the blade using 1 ml of TRIZOL reagent (GIBCO-BRL, Gaithersburg, MD). RNA was extracted according to instructions of the manufacturer and precipitated in the presence of 20 μg glycogen as a carrier. RNA was reverse transcribed with Superscript II (GIBCO-BRL) and used in hot-start PCR amplification reactions with Taq polymerase (Boehringer, Indianapolis, IN). Reaction conditions were 35 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 35 s. Oligonucleotide primers with the sequences 5′-CTTCTCTCCCAGTGCTGCATCTGATG-3′ and 5′-GAGAGGTTGGGGAGTAGGAAGGTGAA-3′ were used in control reactions to amplify a 243-bp fragment derived from the RNA polymerase II mRNA. Since these primers span intron AA [1], genomic DNA contamination in the RNA samples would be indicated by the presence of a fragment of 327 bp. To analyze transgene expression, primers with the sequences 5′-GAAACCATCAGCAAGCAGGTCATTG-3′ and 5′-TCCTATCACCTGCACAGG-3′ were utilized. The first primer spans the intron present in the construct to selectively amplify spliced mRNA derived from the transgene, whereas the downstream primer is derived from *Cltx* sequence. PCR reactions were analyzed by agarose gel electrophoresis. Presence of a 211-bp fragment after amplification is indicative of *Cltx* expression. To specifically detect *TH* and *NET* mRNAs, the following primers were used: *TH*: 5′-GGCCTTTGACCCAGACACAG-3′, 5′-ATCAATGGCCAGGGTGTACG-3′; *NET*: 5′-TGCCCTCTATGTCGGCTTTT-3′, 5′-ATGGATCCCACTGCTCTCGT-3′. PCR conditions were as follows: 1 cycle of denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s and annealing at 60 °C for 30 s and, finally, 1 extension at 72 °C for 7 min.

2.6. Fluoro-Jade staining

Fluoro-Jade staining was performed as described [45]. Briefly, brain sections containing LC were immersed in 100% ethanol for 3 min and rehydrated through graduated alcohol solutions. Sections were then incubated in 0.06% potassium permanganate solution for 15 min, rinsed with distilled water, and then incubated in a solution of 0.001% Fluoro-Jade (Histo-Chem, Jefferson, AR) in 0.1% acetic

acid for 30 min. Sections were then rinsed in distilled water, air-dried, cleared in xylene, coverslipped with DPX and examined under an epifluorescence microscope with a filter system designed to detect fluorescein.

2.7. Electrophysiological recordings from LC slices

FVB mice, approximately 1-month old (average weight 18.6 g ± 0.6, $n = 17$) were deeply anesthetized with metofane inhalation and decapitated. Coronal brainstem slices (350 μm thick) centered on LC were collected and maintained according to a published protocol for neocortical slices [58]. LC neurons were visualized for whole cell recording using a 40× water-immersion objective based on a visually guided patch-clamp protocol [20]. Glass micropipettes were fabricated identically for use in loose patch and whole-cell recording. They were filled with an intracellular medium consisting of (in mM): 130 potassium gluconate, 5 KCl, 5 NaCl; 0.2 EGTA, 10 HEPES, 4 ATP, 0.3 GTP. pH was 7.3–7.4 and osmolality was between 280 and 290 mOsm. Tip resistances in the bath were 3–4 MΩ. Intracellular response waveforms were recorded using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). The analog data streams were low-pass filtered at 2–5 kHz, digitized with 12-bit resolution at 10 kHz, analyzed at run time and stored for later analysis using programs written in Visual Basic. Minimal acceptance criteria for whole-cell data included a resting potential −60 mV or greater. V_ms were not corrected for liquid junction potentials. During whole-cell voltage and current clamp recordings, 400 ms depolarizing steps were provided every 4 s. The amplitude of the depolarizing step was selected to be just large enough to elicit an action current. *Cltx* (Alomone Labs, Jerusalem, Israel) was introduced into the intracellular patch solution at a concentration of 100 μM. GABA (50 μM, Tocris, Ellisville, MO) and clonidine (50 μM, RBI, Natick, MA) were bath-applied for up to 3 min prior to wash out with ACSF. GABA responses normally reversed within 15 min, while clonidine responses took over 45 min to return to baseline.

3. Results

3.1. A disinhibition strategy to increase LC activity: the use of chlorotoxin to block chloride conductances

LC neuronal activity is regulated both by excitatory and inhibitory inputs [3]. We chose to activate LC cells by using a disinhibition strategy, i.e., by decreasing the influence of their inhibitory afferents (Fig. 1). The inhibitory input to LC is predominantly GABAergic, with glycine also playing an important role [48]. GABA and glycine strongly inhibit LC firing in anesthetized [21] as well as in awake or spontaneously sleeping animals [17]. By contrast, $GABA_A$ receptor [23] and glycinergic receptor [17] antagonists increase LC

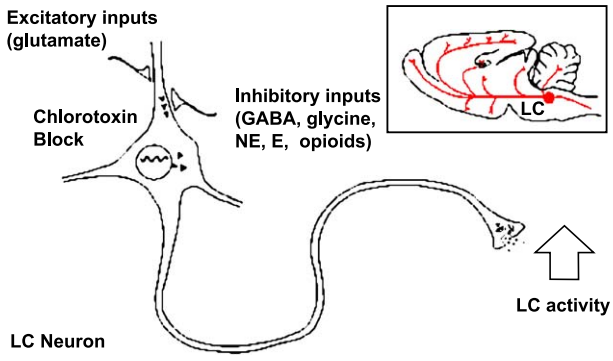

Fig. 1. Disinhibition strategy for LC neurons. The output of LC neurons, which are located in the medial pontine tegmentum (small insert), is dependent on the integration of both excitatory and inhibitory inputs, the latter being mainly mediated by GABA and glycine through an increase in chloride conductances. Chloride channels can be partially blocked by chlorotoxin (triangles), after its synthesis within LC neurons. In this manner, chlorotoxin can potentially reduce or block the efficacy of inhibitory inputs to LC neurons. This lends more weight to the excitatory inputs, with an increase in LC activity as the net result.

activity independently of behavioral state. The inhibition of LC cells by GABA and glycine is mainly mediated by an increase of the chloride conductance through activation of $GABA_A$ and glycine receptors [14,21,39]. Thus, an effective way to disinhibit LC neurons would require the direct block of the chloride conductance. For this purpose, we selected Cltx, a venom component of the scorpion *Leiurus quinquestriatus*. Cltx is a peptide of 36 amino acids that has been reported to partially block small conductance chloride channels by acting from the cytoplasmic side [18,19]. Cltx inhibits chloride channels isolated from rat epithelia, embryonic rat brain [18,19], as well as from cultured glioma cells [33,49]. Cltx appears to have two main advantages over other pharmacological agents: (i) It diminishes the overall chloride influx into a cell without blocking it completely. This is important, because a massive and complete block of such currents can lead to epilepsy and subsequent excitatory cell death (e.g., see Refs. [10,51] for recent reviews); (ii) Because it is effective intracellularly, it can act in a cell-autonomous fashion, thus favoring specific targeting of the cells of interest. For the disinhibition strategy to be effective, we first needed to determine whether Cltx is able to reduce the efficacy of inhibitory inputs to LC.

3.2. Cltx reduces the efficacy of inhibitory synaptic inputs to LC in brainstem slices

We performed electrophysiological recordings from LC neurons in vitro in brainstem slices following injection of Cltx. Previous in vitro studies have shown that LC neurons exhibit spontaneous activity between 0.2 and 5 Hz and that GABA completely inhibits this spontaneous activity [39,62]. This inhibition has been shown to be synaptically driven through $GABA_A$ receptors [14]. In the present study,

spontaneous action potentials were recorded in the loose patch configuration; we found inhibition by low concentrations of GABA (50 μM) and clonidine (50 μM): 60% of cells responded to clonidine ($n=5$), 80% responded to GABA ($n=5$); see Fig. 2A. As a test of the capacity of Cltx to block the GABA response in LC neurons, whole-cell patch clamp recordings were made in which Cltx was introduced into the cell through the recording pipette. LC neurons were stimulated intracellularly to induce action potentials and 50 μM GABA was bath-applied. In control cells, GABA had a strong inhibitory effect on the induced action currents, whereas in cells injected with 100 μM Cltx, action currents persisted in the presence of GABA (Fig. 2B). The group data revealed a significant reduction in the amount of inhibition observed after the application of GABA, as expressed as the percentage of trials in which action potentials were fired during the depolarizing step (ANOVA, $p<0.05$ at 2 min, $p<0.01$ at 3 min; Fig. 2C). Thus, the data suggested that Cltx is able to decrease the efficacy of local synaptic inhibition within LC neurons. Our next step was to generate transgenic mice in which Cltx could be expressed specifically in LC neurons under controlled temporal conditions.

3.3. Construction of the transgene array

In designing the transgene array, two criteria were considered important: (i) the transgenic modification should be silent in baseline conditions in order to circumvent any deleterious effects that might affect embryonic development and impede maintenance of a transgenic mouse line; and (ii) transgene expression should be conditional so that it could be activated under controlled temporal and spatial experimental conditions. To satisfy these criteria, we constructed a transgene array which consists of (i) the promoter of the dopamine-β-hydroxylase (*DBH*) gene for specific gene expression in noradrenergic and adrenergic neurons [26,38], (ii) an intron from the rat preproinsulin II gene to allow splicing and efficient expression, (iii) the reporter gene *nlacZ* encoding β-galactosidase, and (iv) the effector transgene *Cltx* encoding the chlorotoxin peptide (Fig. 3). The presence of *nlacZ* in the array serves two purposes. Before recombination (Fig. 3, step 1), only the reporter gene, but not the effector gene, is expressed in DBH+ cells, because the *nlacZ* polyadenylation signal normally prevents expression of *Cltx*, which is located downstream in the construct. The β-galactosidase expression per se does not

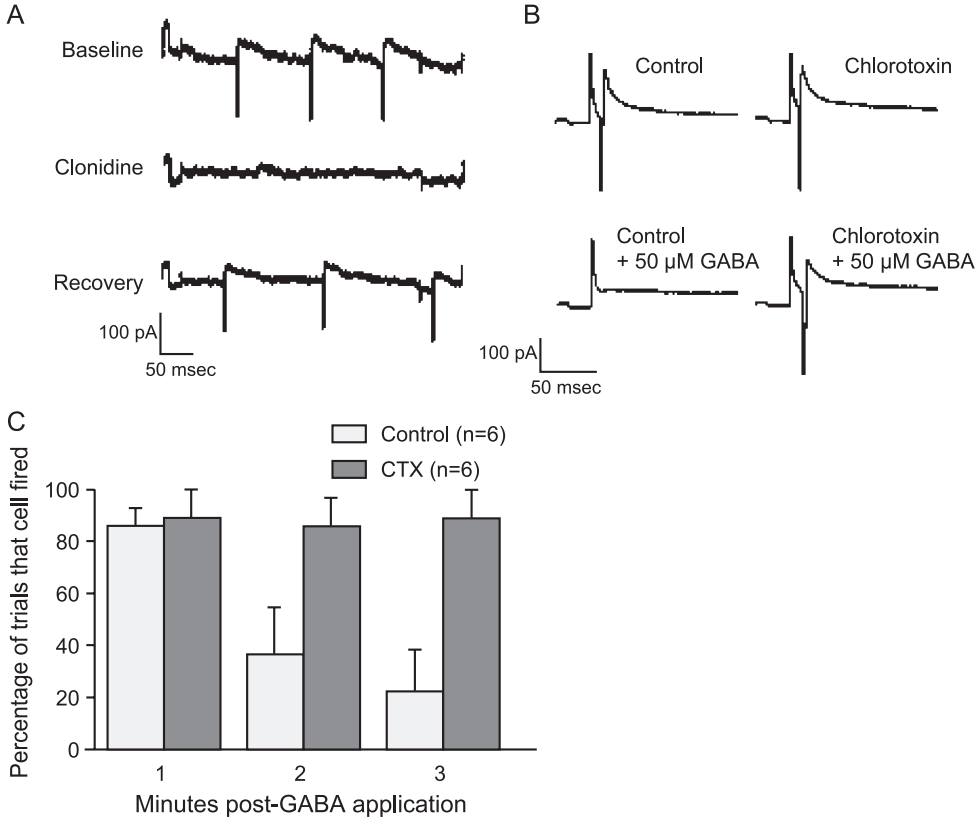

Fig. 2. Chlorotoxin reduces the efficacy of inhibitory inputs to LC neurons. (A) Bath application of 50 μM clonidine reversibly reduced firing in the depolarized LC neurons. (B) Left panels. The action potential induced by intracellular stimulation of a LC neuron (upper panel, Control) is abolished when 50 μM GABA is bath applied (lower panel). Right panels. The presence of chlorotoxin *per se* does not affect the induction of an action potential following intracellular stimulation (upper panel). Moreover, the action potential can still be induced when 50 μM GABA is bath applied in the presence of chlorotoxin (lower panel). (C) Averaged group data from patch-clamp recordings from 12 LC neurons. During the second and third minute following the application of GABA, the percentage of trials in which a LC cell fired in response to intracellular stimulation was significantly reduced in control cells relative to chlorotoxin-filled cells.

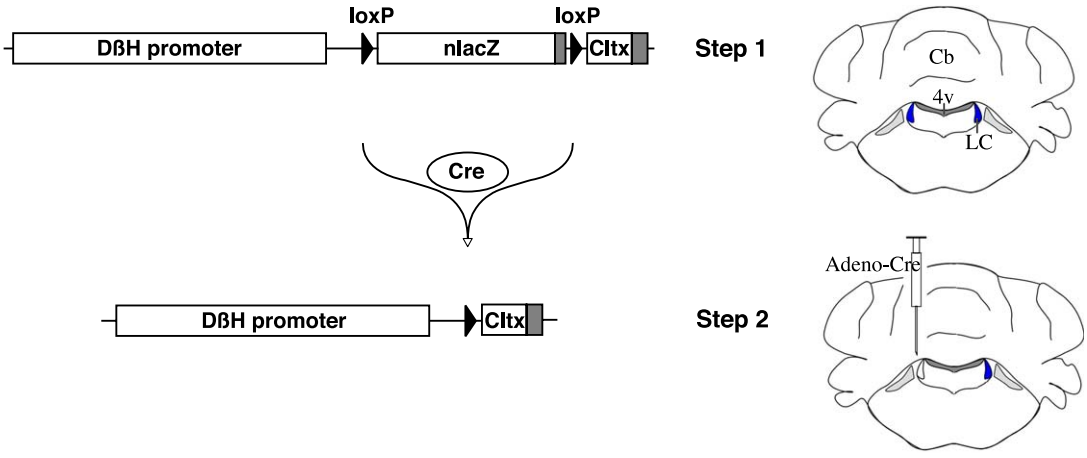

Fig. 3. Transgene array to modify LC activity. Structure of the transgene array. The expression of both the reporter gene (*nlacZ*, a *lacZ* gene with a nuclear localization signal) and of the effector gene (*chlorotoxin*, *Cltx*) is under the control of the promoter of the rat dopamine-β-hydroxylase (*DβH*) gene. Before recombination (step 1), *nlacZ* silences the effector gene in the array, *Cltx*, so that only *nlacZ* is expressed in the LC. The reporter gene activity visualized using β-galactosidase histochemistry is indicated by the blue staining inside the LC. Gray boxes indicate polyadenylation sites for *nlacZ* and *Cltx*. In step 2, a Cre-mediated recombination following the injection of Adeno-Cre virus in the LC of one side (left in the figure) excises the reporter gene from the array at loxP recombination sites (triangles) and brings the effector gene *Cltx* close to the promoter, thus allowing its effective expression. As a consequence of the switch in expression between reporter and effector gene, the blue staining associated with the reporter gene activity is no longer present in LC on the side of the injection. 4v, forth ventricle; Cb, cerebellum; LC, locus coeruleus.

affect the host cells. Moreover, it can be visualized by using β-galactosidase histochemistry, thus allowing an easy method to screen mouse lines for spatially correct transgene expression. The *nlacZ* gene is flanked by loxP sites, and therefore can be removed from the array upon delivery of a Cre recombinase through an Adenovirus vector. To induce the switch from reporter to effector gene expression, a trigger Adenovirus, Adeno-Cre, is injected in the midbrain pontine area of transgenic mice (Fig. 3, step 2). Adeno-Cre infection causes Cre recombinase to be efficiently synthesized inside LC cells, with subsequent excision of *nlacZ*. This recombination removes the transcription stop from the *Cltx* gene and places the *Cltx* gene in close proximity to the *DBH* promoter, resulting in the induction of *Cltx* gene expression. The success of this transgene array relies on two main factors: (1) the ability of adenoviral vectors to infect LC neurons; (2) the ability of Cre-mediated recombination to cause an effective DNA rearrangement, i.e., the loss of *nlacZ* reporter gene activity and the concomitant activation of effector gene *Cltx*.

3.4. LC neurons can be infected with Adenovirus vectors in vivo

Adenoviral vectors have been shown to strongly infect neurons and glial cells after intracerebral or intraventricular injection (e.g., Ref. [31]). To determine whether LC is a suitable target for genetic manipulations with Adenovirus vectors, normal FVB mice were stereotaxically injected in the dorsal pontine tegmentum with an Adenovirus vector that carried a *LacZ* reporter gene (Adeno-nlacZ). Five days later, mice were sacrificed and brains were processed for β-galactosidase staining. We found that LC neurons expressed *LacZ* very effectively, together with a few scattered cells in the parabrachial area (Fig. 4). By con-

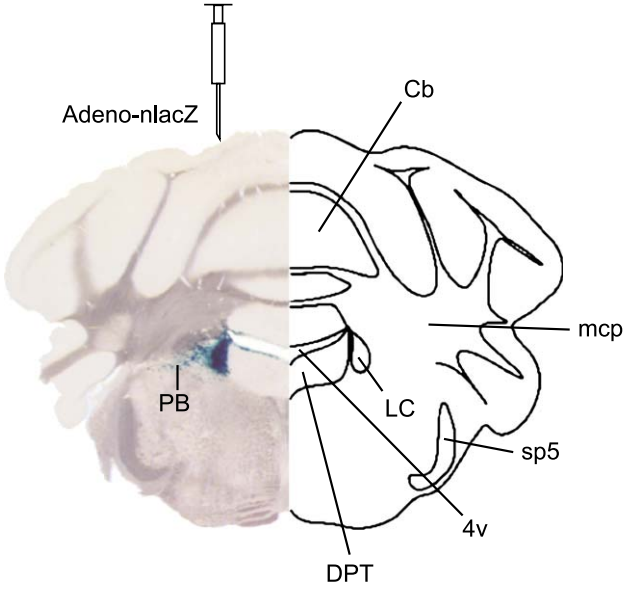

Fig. 4. LC neurons can be infected with an adenoviral vector at high efficiency. A mouse brain section stained for β-galactosidase reporter gene activity after stereotaxic injection of the reporter vector Adeno-nlacZ in the LC area of the left side. Infected neurons express the reporter gene *LacZ*, whose activity can be visualized as a dark nuclear staining when brain sections are processed with β-galactosidase histochemistry. Note the absence of staining in most cells of the dorsal pontine tegmentum (DPT) outside LC. 4v, fourth ventricle; Cb, cerebellum; LC, locus coeruleus; mcp, medial cerebellar peduncle; PB, parabrachial nucleus; sp5, spinal trigeminal tract.

trast, the great majority of cells situated in the dorsal pontine tegmentum, even if located just outside LC, were not infected (Fig. 4). Some reporter gene activity was also present in a few cells along the injection pathway and in the ventricular epithelium around the point where the needle had passed through the fourth ventricle. These cells were probably labeled after leakage of the viral suspension during the injection procedure.

To exclude potential damage to LC neurons due to the stereotaxic injection and/or the infection with Adenovirus, pontine sections were reacted histochemically with Fluoro-Jade, a dye that stains cell bodies, dendrites and axons of degenerating neurons but does not label healthy neurons [45]. Fluoro-Jade staining was never observed in LC neurons in any of the injected mice (data not shown). By contrast, in agreement with the original description [45], some staining was seen in cells of the meninges and the choroid plexus, and in a few cases, some minor damage was also observed around the path of the injection needle. Thus, it appears that LC neurons can be efficiently infected with Adenovirus without showing visible signs of permanent damage. Moreover, the infection seems to target noradrenergic neurons and to spare neighboring cells in the dorsal pontine tegmentum.

3.5. Effects of Cre-mediated recombination in vitro and in vivo: reporter gene loss and effector gene activation

After screening F1 transgenic mice by β-galactosidase histochemistry, we identified two independent lines, DZC-1 and DZC-4, in which *LacZ* was strongly expressed in LC neurons (Fig. 5A). We compared consecutive sections of the dorsal pontine tegmentum from these mice and found that,

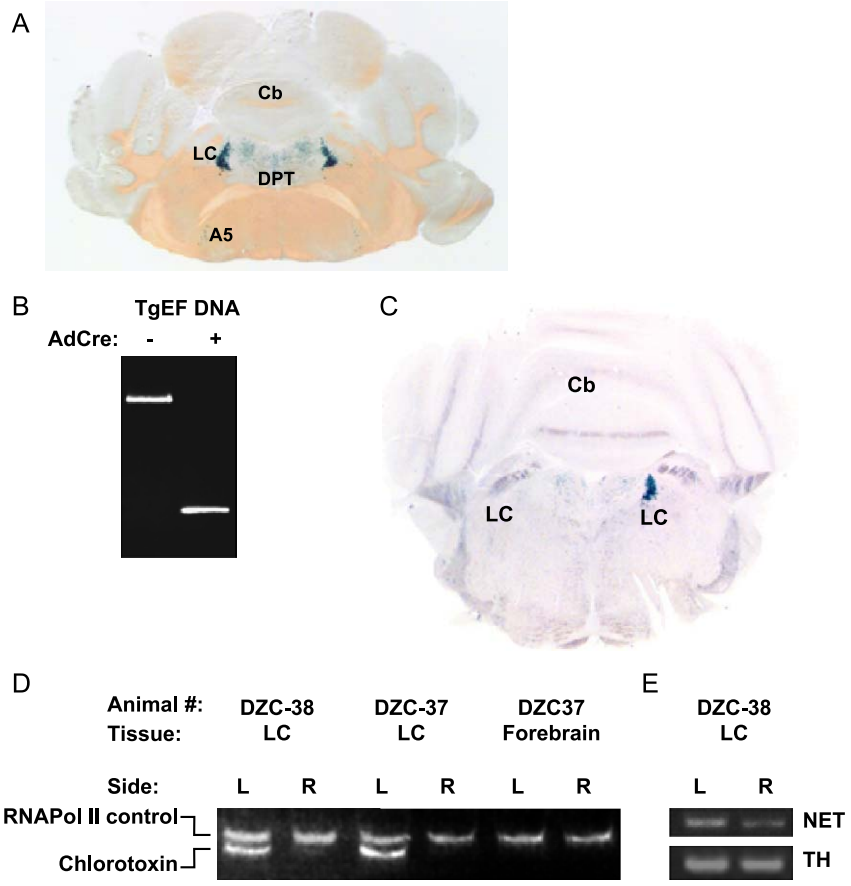

Fig. 5. Cre-mediated recombination in vitro and in vivo. (A) Pontine section from a transgenic mouse. β-galactosidase histochemistry shows strong *nlacZ* expression in LC of both sides. Some weak reporter gene activity can also be seen in several cells of the dorsal pontine tegmentum (DPT) and in the ventral groups of A5 noradrenergic cells (A5). Cb, cerebellum. (B) DNA from Adeno-Cre-infected transgenic embryonic fibroblasts was assayed for evidence of recombination. (−) Uninfected cells; (+), infected cells. Absence of the 4-kb product and presence of a 0.5-kb product indicated that the transgene locus was accessible for Cre-mediated recombination. (C) Unilateral stereotaxic injection of the Adeno-Cre vector leads to recombination in the transgene array and to subsequent loss of β-galactosidase activity on the injected (left) side. (D) Analysis of *Cltx* expression by RT-PCR. Presence of a 211-bp band indicates that chlorotoxin was expressed specifically on the Adeno-Cre-injected side (left, L) of a pontine section containing LC (two animals, DZC-38 LC and DZC-37 LC), but neither on the right side (R) of the same pontine section nor in a section spanning the forebrain area. Reactions to amplify a fragment from the RNA Pol II gene as a positive control display a 243-bp fragment in all samples, indicating efficient reverse transcription and amplification of all samples. (E) Analysis of *TH* and *NET* expression by RT-PCR in the LC of one of the animal shown in D (DZC-38). Both genes are expressed at higher level on the left side (L), where chlorotoxin was expressed, than on the right side (R).

as expected, the number of *LacZ*-positive cells in LC closely corresponded to that of TH+-positive cells (data not shown). Animals from these two transgenic lines were used in all further experiments. We also observed some weak ectopic activity of the *DBH* promoter outside noradrenergic neurons, for instance in several cells of the dorsal pontine tegmentum medial to LC (Fig. 5A). This is in agreement with other studies (see Ref. [27], and references therein). However, as shown below, the combination of regionalized promoter activity and spatial restriction of the Adenovirus trigger resulted in the desired spatial and cellular specificity required to affect only LC neurons.

Administration of Adeno-Cre, an Adenovirus vector carrying the gene for Cre recombinase, should lead to loss of *LacZ* activity and to concomitant activation of *Cltx*. We first examined whether successful DNA rearrangement through Cre-mediated recombination would occur in vitro. We isolated primary embryonic fibroblasts from transgenic mouse embryos. In uninfected control cells, using a PCR assay with primers that flank the ''floxed'' reporter gene, we detected a large DNA band that reflected the presence of the *LacZ* sequence in the unrecombined locus (Fig. 5B). By contrast, after infection of fibroblasts with Adeno-Cre, we detected a small DNA band, consistent with a properly recombined locus, whereas the large band representing the unmodified locus was absent (Fig. 5B).

To test Cre-mediated recombination in vivo, we unilaterally injected Adeno-Cre into the pontine area of transgenic mice and measured *LacZ* activity after 2–5 weeks. No increased morbidity or mortality was observed after the injection. Since Cre-mediated recombination should excise the reporter gene, we expected that *LacZ* activity would disappear on the injected side but not on the control side (see Fig. 3). β-Galactosidase histochemistry performed on brain sections containing LC showed that this was indeed the case: The number of lacZ-positive LC cells was unchanged on the uninjected side, but was greatly diminished on the side injected with Adeno-Cre (Fig. 5C). A successful virus injection resulted on average in a 90% reduction of *LacZ*-positive LC cells, with cases where no labeled cells could be detected at all.

Finally, we examined whether *Cltx* expression could be induced in LC neurons after a unilateral Adeno-Cre injection. We prepared RNA from the left and the right half of individual unfixed cryosections, reverse transcribed it into cDNA, and assayed for the presence of a *Cltx* transgene-specific transcript by PCR amplification. We found that a band representing *Cltx* expression could only be detected in RNA samples derived from the injected side of pontine sections containing LC (Fig. 5D). *Cltx* expression could not be detected in the contralateral side of a pontine section, or in either side of a section spanning the forebrain area. Thus, Cre-mediated recombination of our transgene array in vivo can both silence *LacZ* activity and induce *Cltx* expression. The effects are region-specific and under full temporal control.

3.6. Chlorotoxin expression leads to an increase of markers for LC activity

Intracellular injection of *Cltx* demonstrated that chlorotoxin is able to reduce the efficacy of GABAergic inhibition in LC neurons. These results are consistent with previous evidence showing that this toxin is able to block chloride channels [19,49,54,55,]. However, it remained to be determined whether the disinhibition of LC neurons by *Cltx* induction would result in an increase in their activity. To address this question, we measured mRNA levels of TH, the rate-limiting enzyme in catecholamine synthesis, and of the NET, which is responsible for the uptake and the maintenance of synaptic norepinephrine stores. TH and NET mRNA levels in LC have been shown to increase and decrease in response to increases and decreases in LC activity and in norepinephrine release, respectively. For instance, TH and/or NET mRNA levels are increased in LC following sleep deprivation (which increases norepinephrine release and metabolism [8,40]), after treatment with tricyclic antidepressant (which increases availability of norepinephrine at the synapse [50]), after acute methamphetamine administration (which increases norepinephrine release in the forebrain [46]), during acute stress (which increases norepinephrine release from LC terminals [13]), and after angiotensin II administration (which stimulates catecholamines release [64]). On the other hand, TH and/or NET mRNA levels are reduced in LC after brain depletion of norepinephrine by alpha-methyl-*p*-tyrosine [63] and in major depression [30]. Thus, we used TH and NET mRNA levels as indirect indices of LC noradrenergic activity. As before, we prepared RNA samples from the left side of brainstem sections containing the injected LC as well as from the right side of the same sections where LC was not injected. TH and NET mRNA levels were measured by RT-PCR. We found that both TH and NET mRNA levels were increased in the left LC relative to the right LC (*t* test, NET $60 \pm 11\%$, $P = 0.021$; TH $28 \pm 5\%$, $P = 0.037$; Fig. 5E).

3.7. Increased expression of the immediate early gene NGFI-A after chlorotoxin induction

In previous studies, we have shown that LC activity plays a major role in the induction of brain gene expression during wakefulness [15,16]. The expression of immediate early genes and transcription factors such as *c-fos*, *NGFI-A*, *P-CREB*, as well as that of other plasticity-related genes such as *Arc* and *BDNF*, is high in the cerebral cortex during wakefulness and low during sleep. However, if tested 2–3 weeks after, the noradrenergic innervation from LC to the cerebral cortex has been destroyed by a neurotoxic lesion, the expression of all these genes during wakefulness is as low as during sleep. This occurs despite the fact that LC-lesioned rats show normal electroencephalographic markers of brain activity, as well as normal amount of sleep and wakefulness. Thus, the noradrenergic system is not crucial

for electroencephalographic activation during wakefulness, but is essential for the induction of gene expression.

Having determined that *Cltx* induction increases the expression of markers of LC activity, we wanted to determine whether we would detect changes in gene expression during wakefulness in brain areas targeted by LC. More specifically, in past experiments, we demonstrated that a chronic decrease in LC activity is sufficient to cause a decrease in cortical gene expression. Here, by contrast, we wanted to determine whether *Cltx* induction in LC was enough to increase gene expression in LC projection areas. With this aim in mind, we measured brain expression levels of the immediate-early gene *NGFI-A* in transgenic mice injected with Adeno-Cre on the left LC. *NGFI-A* (also called *Egr1* or *Zif268*) has been extensively used as a marker of brain physiological activity because the up- or downregulation of its expression reliably reflects increases or decreases in neuronal activity, respectively (see e.g., Ref. [35], and references therein). In contrast to *c-fos*, another immediate early gene extensively used as marker of neuronal activity, *NGFI-A* expression persists at increased levels for as long as the inducing stimulus is present. Adeno-Cre injection did

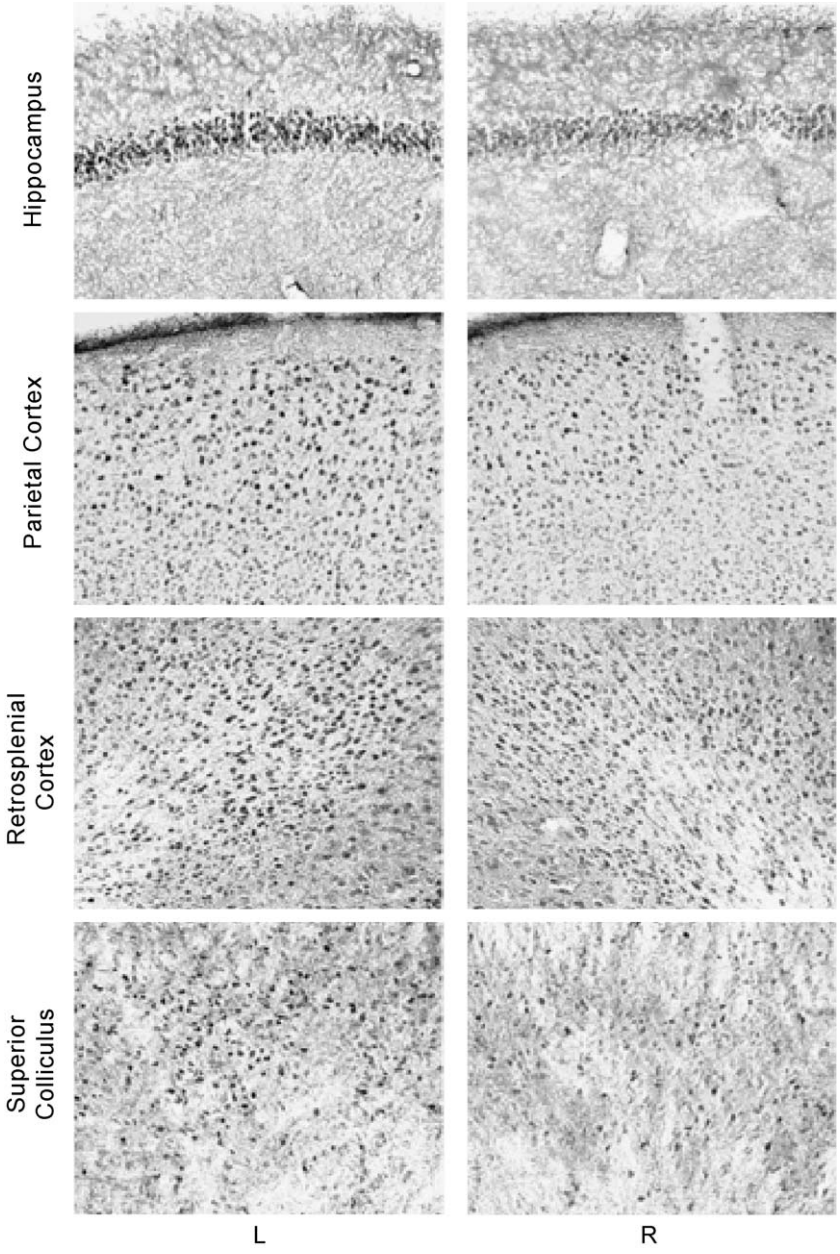

Fig. 6. NGFI-A expression is increased unilaterally in LC projection areas. Four weeks after adenoviral injection in the left LC, mice were sleep deprived for 4–6 h and then sacrificed. Brain sections were reacted with an antibody against NGFI-A. As expected since NGFI-A is a transcription factor, immunostaining is localized to the nucleus. In the CA1 region of the hippocampus, parietal cortex, retrosplenial cortex, and in the superior colliculus, NGFI-A staining is higher on the left side (L), which receives input from the LC in which *Cltx* was induced, relative to the right, uninjected side (R).

not affect locomotion, grooming, eating and drinking, or the responses to auditory stimuli and to the presentation of novel objects. One to three weeks after the injection, mice were kept awake for 4–6 h starting from the beginning of their resting period (light period; light on at 10:00) by presenting them with novel objects and then sacrificed. Brains were processed for immunocytochemistry with an antibody against NGFI-A and the number of NGFI-A-positive cells was counted in coronal sections at all rostro-caudal levels (from olfactory bulbs to the medulla oblongata). We found that NGFI-A expression was significantly higher on the side ipsilateral to the Adeno-Cre injection relative to the other side in both cortical and subcortical areas (Fig. 6). Quantification of the nuclear staining revealed a 25–40% increase in the number of NGFI-A-positive cells on the injected side relative to the contralateral control side, depending on the brain region (*t* test, parietal cortex $33 \pm 5\%$, $P = 0.031$; hippocampus CA1 $29 \pm 7\%$, *t* test = 0.015; superior colliculus $40 \pm 8\%$, $P = 0.011$). This increase was clearly correlated with the degree of recombination observed in LC by lacZ staining, since viral injections that failed to induce noticeable levels of Cre-mediated recombination did not lead to asymmetrical expression of NGFI-A in any LC target area (data not shown). Thus, the conditional activation of *Cltx* in LC is sufficient to produce downstream effects in LC target areas such as an increase in the expression of the gene *NGFI-A*.

4. Discussion

We have described a new method aimed at selectively stimulating noradrenergic LC neurons in a highly selective manner. Through the conditional expression of a chlorotoxin transgene in the LC of adult mice, we have partially disinhibited LC neurons from their GABAergic and glycinergic inhibitory inputs, while leaving excitatory inputs unaffected. LC disinhibition is highly specific, persists for at least several weeks, and is associated with molecular and cellular effects in LC neurons as well as in several LC target areas.

While Cltx was shown before to be effective in blocking Cl^- channels in immature neurons and in tumor cells [18,19,49], here, we have shown for the first time its efficacy in blocking GABA inhibitory effects in mature LC neurons. Moreover, the increase in *TH* and *NET* expression in LC, which has been used as an indication of the level of activity of noradrenergic cells, is the first demonstration of the ability of Cltx to affect neuronal activity in vivo. Finally, we have shown that the induction of *Cltx* in the LC of one side is associated with the ipsilateral increase in the expression of *NGFI-A* in several cortical and subcortical areas. Since the great majority of noradrenergic projections from LC are unilateral, these results are a further indication of the ability of Cltx to specifically affect LC activity. It remains to be determined, however, how the firing of LC neurons in vivo is affected by the induction of *Cltx*. This can only be demonstrated directly by performing single-unit recordings in behaving animals.

4.1. Site- and time-specific transgene expression

With the advent of techniques that permit the generation of transgenic mice or gene knockouts, molecular genetic manipulations have provided new ways to create animal model systems to understand brain functions at all levels, from anatomy, neurochemistry, cellular, and systems physiology to behavior. Two major avenues have been pursued with these technologies: Gene knockouts have been applied to generate loss-of-function models, where the function of a single gene could be deduced from consequences that such a mutation invoked for processes ranging from cell biology to animal behavior. Transgenic gain-of-function approaches, on the other hand, have been employed to investigate the function of genes that were causal, for instance, to neurodegenerative disorders such as Alzheimer's disease (e.g., Ref. [25]). In many studies of gene manipulations, two limitations are apparent: (i) genetic manipulations can dramatically affect development, often precluding investigation of a particular function in the adult animal, and (ii) regulatory elements to control gene expression are often found to be limited in their cellular, spatial, and temporal specificity. Conditional control of gene expression can solve the first problem, and technologies such as the Cre–lox recombination system [9,44] or substance-inducible genetic regulatory elements (see Ref. [32] for a recent review) are being developed to ensure survival of a model animal to adulthood. We utilized a system of conditional transgene expression from an array construct to achieve effector gene expression in a highly specific, controlled manner. Two features were essential to ensure developmental viability and specificity of action. First, we utilized effector transgene silencing through a reporter gene, which renders any potentially deleterious effector transgene dormant during development. Second, we were able to solve the problem of expression specificity by the combinatorial use of a cell-type specific promoter and through the local microinjection of the trigger virus to the target area. Moreover, Cltx is effective from inside the cell, and, therefore, its effects can be restricted to the transgene-expressing cells, thus further contributing to the specificity of the experimental approach.

Adenovirus vectors have a wide host cell range, but certain preferences for infection may exist. In fact, our own study demonstrated that neurons of the dorsal tegmentum are less likely to be subject to adenoviral infection compared to LC neurons. However, delivery of the Cre recombinase gene is by no means limited to Adenovirus vectors and can be achieved by all means of modern gene transfer into the brain, including liposome transfection with DNA or other viral vectors such as those based on Herpes simplex virus HSV [41] or Adeno-associated virus AAV [28]. Viral

vectors delivering Cre recombinase are becoming an essential tool for genetic manipulation of the brain.

Neuronal firing, in conjunction with connectivity and plasticity, forms the basis for essentially all processes of brain function. Therefore, if one wants to investigate brain functions at systems level, it is necessary to gain experimental control over neuronal activity. Traditionally, this has been achieved through electrophysiological or pharmacological interventions (see Introduction), and only in a few cases through genetic manipulations [34,36,37,52,53,60,61]. We have developed a tool to affect neuronal activity in a specific cell group under strict spatial and temporal control. Although our effort focused on the noradrenergic system, this approach is in principle applicable to the study of other neurochemical systems. Indeed, since essentially every neuron receives a certain degree of inhibitory input, our strategy can in theory be applied to every region or system of the brain. In practice, the main limitation is the availability of cell-type specific promoters to drive Cltx expression.

4.2. Transgenic LC disinhibition and changes in gene expression

We have developed our model system to increase neuronal firing based on a disinhibition strategy, with the rationale that reduced efficacy of inhibitory inputs would elevate the influence of excitatory inputs and thereby increase the probability of a manipulated neuron to elicit an action potential. However, a massive block of chloride conductances mediated by $GABA_A$ and glycine receptors could lead to epilepsy and subsequent excitatory cell death [10,51]. Therefore, Cltx was considered an ideal candidate because it diminishes the overall chloride influx into a cell without blocking it completely. Indeed, our in vitro data show that Cltx decreased, but did not completely abolish, the ability of GABA to inhibit LC neurons.

As discussed in the Introduction, previous studies that aimed at increasing LC activity have relied on electrical stimulation or pharmacological approaches with agonist substances specific for adrenergic receptors. Difficulties with conventional stimulation approaches include limited specificity for the target neuronal population and short duration of the effects. Moreover, electrical or chemical stimulation uncouple the activity of the target population from its normal inputs and thereby limit system-level analysis. Here, we provide evidence that Cltx-induced disinhibition of LC neurons persist for at least several weeks after *Cltx* induction. Moreover, our in vitro data, in agreement with previous evidence showing a selective action of Cltx on chloride channels [18,19,49], strongly suggest that Cltx affects inhibitory inputs to LC without affecting the ability of noradrenergic cells to be excited. Thus, an elevated noradrenergic activity in our transgenic mice is still coupled to the input patterns to LC, which is important considering that norepinephrine release may signal ‘‘value’’ or ‘‘saliency’’ of a particular event [4,5].

In previous studies, we had shown that the chronic inactivation of the noradrenergic system greatly reduces the expression of *NGFI-A* and several other genes in the cerebral cortex during wakefulness [15,16]. Here, we provide further evidence for an essential role of LC in regulating gene expression in its target areas. Consistent with the fact that transgenic disinhibition increased, rather than decreased, LC activity, we observed an upregulation, rather than a downregulation, of *NGFI-A* expression in LC target areas. It will be important to determine in the future whether other genes whose expression depends on intact LC activity, such as *Arc* and *BDNF*, are also affected by LC disinhibition. Our previous results also demonstrated a dissociation between the role of the LC in EEG activation and in activation of gene expression during the waking state. A quantitative analysis of EEG waking pattern with and without *Cltx* induction would be important to determine whether such dissociation is also present in this transgenic animal model.

The new animal model presented here may help to elucidate the long-term effects of LC activation in the promotion of arousal, in learning and memory, and in the regulation of sleep. This study provides proof-of-concept that increases in neuronal activity can be engineered in a chronic fashion by means of molecular genetics. We were able to detect molecular and cellular effects due to unilateral LC activation. Yet, overt changes in behavior, for instance, in the response to stimuli, were not detected, and are perhaps not to be expected after unilateral LC modulation. A more accurate and quantitative analysis is certainly warranted before excluding behavioral effects after unilateral LC disinhibition. However, it may be that behavioral effects can only be observed after bilateral activation of the LC. This may be achieved by bilateral injection of Adeno-Cre, or though approaches where Cre expression from a second transgene or a modified genomic locus can be induced by systemic drug administration [12,29]. As more mouse strains with Cre expression in specific brain areas become available, the approach to activate neurons by Chlorotoxin-mediated disinhibition will become a powerful new tool to dissect brain function ranging from the molecular level of gene expression to animal behavior.

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