Doxycycline-dependent photoactivated gene expression in eukaryotic systems

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High spatial and temporal resolution of conditional gene expression is typically difficult to achieve in whole tissues or organisms. We synthesized two reversibly inhibited, photoactivatable ('caged') doxycycline derivatives with different membrane permeabilities for precise spatial and temporal light-controlled activation of transgenes based on the 'Tet-on' system. After incubation with caged doxycycline or caged cyanodoxycycline, we induced gene expression by local irradiation with UV light or by two-photon uncaging in diverse biological systems, including mouse organotypic brain cultures, developing mouse embryos and *Xenopus laevis* tadpoles. The amount of UV light needed for induction was harmless as we detected no signs of toxicity. This method allows high-resolution conditional transgene expression at different spatial scales, ranging from single cells to entire complex organisms.

High spatial and temporal resolution of transgene expression is rarely obtained with the conditional gene expression systems currently available. We therefore decided to use light as an inducing agent because of the high spatial resolution it provides, and the ease and precision with which it can be manipulated. Previously, light had been used to regulate expression through caged transcription factors[1,2], caged RNA[3], caged plasmids[4], caged antisense oligos[5] or other caged small-molecule inducers[6–10]. Even though all of these attempts were successful in principle, there were always limitations, particularly with respect to the resolution of transgene expression or difficulties in the delivery of the caged molecule.

We established an alternative method that allows high-resolution transgene expression by irradiation with light. By combining the versatile Tet-on system with the precision of light irradiation, this is a powerful tool for targeted transgene expression in almost any biological system. Based on the Tet-on system[11], we generated photolabile ('caged') doxycycline derivatives for photoactivated gene expression allowing for precise spatial and temporal control of gene expression in various biological paradigms, including single-cell induction of transgene expression in hippocampal slices as well as topical activation in developing vertebrates.

RESULTS

Characterization of caged doxycycline derivatives

To produce a light-sensitive Tet-on system, we modified the potent tetracycline analog, doxycycline, with a caging reagent to reversibly inhibit its ability to induce tetracycline-dependent transcription[12]. In addition, we also synthesized a second analog, 2-decarbamoyl-2-cyanodoxycycline ('cyanodoxycycline') with a much reduced membrane permeability to ensure longer retention inside the cells after photoactivation. A nitrile modification on tetracycline had previously been shown to strongly reduce its membrane permeability[13] while actually increasing its induction efficiency[14]. We synthesized cyanodoxycycline by reaction of the commercially available doxycycline hyclate with *N*,*N*′-dicyclohexylcarbodiimide[15] (**Fig. 1a**).

Doxycycline and cyanodoxycycline both contain several side groups that can be derivatized. After the synthesis of caged doxycycline[12], reaction of cyanodoxycycline with 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane (DMNPE) generated a DMNPE ether of cyanodoxycycline. NMR spectroscopy analysis revealed that the DMNPE caging group was attached at the 12 position of cyanodoxycycline, similar to the caging of doxycycline with DMNPE (**Supplementary Note 1**). Some of the physicochemical properties of caged doxycycline and caged cyanodoxycycline are listed in **Table 1**. Both caged compounds are soluble in aqueous buffers, display acceptable quantum yields and have long-wavelength absorption maxima at 347 nm (**Supplementary Fig. 1**). Additionally, both compounds are very stable and can be stored in aqueous solution at −20 °C for more than a year (data not shown).

To characterize the wavelength range of efficient uncaging, we irradiated caged doxycycline at distinct wavelengths with a defined number of photons. Uncaging was most efficient around 330–350 nm (**Fig. 1b**) and there was no photolysis at visible wavelengths as irradiation with 436 and 488 nm did not lead to detectable photorelease of doxycycline. Therefore, DMNPE-caged doxycycline and cyanodoxycycline can also be used in combination with GFP excitation.

We assessed the reduction of tetracycline-dependent expression owing to the caging group by analyzing the tetracycline-dependent

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RECEIVED 28 FEBRUARY; ACCEPTED 15 MAY; PUBLISHED ONLINE 7 JUNE 2009; DOI:10.1038/NMETH.1340

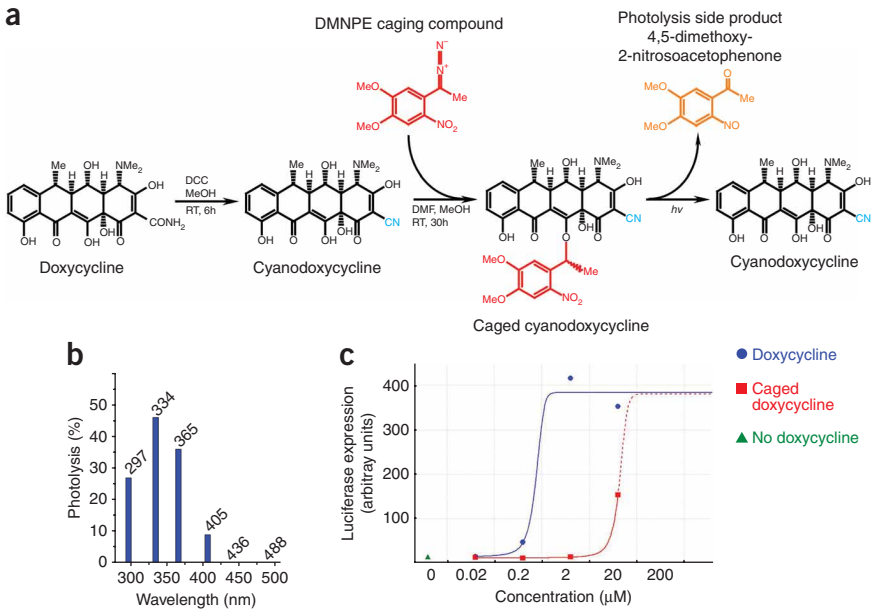

Figure 1 | Caged doxycycline or caged cyanodoxycycline and photoactivated gene expression. (**a**) Synthesis and photoactivation of caged cyanodoxycycline (**Supplementary Note 1**). Nitrile group is shown in blue and 1-(4,5-dimethoxy-2-nitrophenyl)diazoethane in red. DCC, *N,N'*-dicyclohexylcarbodiimide. RT, room temperature, 20–24 °C. (**b**) Wavelength dependence of the uncaging of caged doxycycline. Numbers above bars indicate irradiation wavelengths. (**c**) Transcriptional activity in the presence of caged, unmodified or no doxycycline was quantified with a luciferase assay. The dashed line represents the projected continuation of the dose-response curve for concentrations that could not be measured. From the inflection points of the sigmoidal fits, a 58-fold reduced affinity of the caged doxycycline compared to doxycycline was calculated.

luciferase expression in HeLa cells[16] after administration of doxycycline or caged doxycycline. This assay revealed that the modification with the DMNPE caging group yielded a caged doxycycline that only induced transcription if used in amounts at least 20-fold greater than the amount of unmodified doxycycline normally used. The dose-response curve of caged doxycycline was markedly shifted to higher concentrations (**Fig. 1c**) because of its reduced ability to induce tetracycline-dependent transcription. This assay showed that a final concentration of 2 μM doxycycline induced a full transcriptional response but the same amount of caged doxycycline did not lead to any detectable luciferase expression and thus no background in the absence of photoinduction. When we measured the activity of 2 μM caged doxycycline after photoinduction, we saw 73% of gene expression activity of 2 μM unmodified doxycycline (data not shown). Thus, there is a useful window of caged doxycycline concentrations, 0.5–5 μM, which produce no detectable background transcriptional activity and the equivalent concentration of unmodified doxycycline induced a full transcriptional response.

Photoactivation with caged doxycycline

To achieve widespread tetracycline-dependent transgene expression, we used a transgenic mouse line in which the potent and ubiquitous *CAGGS* promoter[17] controls the expression of a gene encoding a codon-optimized version of rtTA (irtTA) fused to a mutated glucocorticoid receptor ligand-binding domain (GBD*)[18] (**Supplementary Note 2** and **Supplementary Fig. 2**). To release irtTA from GBD and allow translocation into the nucleus, we administered 25 μM dexamethasone in all mouse experiments including doxycycline controls as well as irradiated and nonirradiated caged doxycycline- or cyanodoxycycline-treated samples.

We used the hippocampi of double-transgenic *CAGGS-irtTA-GBD*–GFPtetO7-lacZ* mice (*CIG-tGFP*) to obtain 300 μm thick long-term organotypic slice cultures. We performed all subsequent photoactivation experiments using the following protocol: we added either caged doxycycline or caged cyanodoxycycline directly to the medium, incubated the samples for specified amounts of time, washed them in culture medium, irradiated the samples where appropriate and quickly returned them to culture medium for at least 12 h. Irradiation was done with an upright epifluorescence microscope equipped with a standard DAPI filter set (band pass excitation filter 290–370 nm). For all hippocampal cultures, a single 0.5, 1 or 2 s pulse of DAPI excitation light (×40, ×20 or ×10 air objectives, respectively) was sufficient for photolysis of caged doxycycline or caged cyanodoxycycline and the ensuing release of active doxycycline or cyanodoxycycline. Irradiated control slices without caged doxycycline did not have any GFP fluorescence (**Supplementary Fig. 3a**). In general, there was diffuse background fluorescence that also varied across different regions within the hippocampal slices. However, this diffuse fluorescence was clearly discernible from the more intense GFP signal.

We found a scattered distribution of GFP-positive cells in brain slices after 4 μM doxycycline treatment for 16 h (**Fig. 2a**). The same concentration of caged doxycycline did not induce any transgene expression over the same time period (**Fig. 2b**). However, exposure to caged doxycycline followed by photoactivation of the entire slice produced expression of GFP (**Fig. 2c**), which was similar to treatment with unmodified doxycycline. Also, in all experimental photoactivation paradigms tested, the time course of transgene expression was identical to that after induction with unmodified doxycycline (data not shown).

To limit transgene expression to a patch of cells, we reduced the area of irradiation by confining the aperture of the microscope field stop (**Fig. 2d**). Pseudocoloring and quantitative assessment of

Table 1 | Properties of caged cyanodoxycycline and caged doxycycline

Caged compound of	Absorbance maximum[a], λ_{max} (nm)	Extinction coefficient[a], ε_{max} (M^{-1} cm^{-1})	Photolysis quantum yield[b], φ	Solubility[a], c_{sat} (μM)
Cyanodoxycycline[c]	346.5	12,800	0.068	730
Doxycycline[12]	~347	10,500	0.013	380

[a]In HEPES buffer solution (pH 7.2). [b]In HEPES buffer solution (pH7.2) with acetonitrile (80:20; vol/vol). [c]The caging reaction produced two diastereomers, compounds 2a and 2b (**Supplementary Note 1** and **Supplementary Table 1**), but we used only compound 2a for photoactivated gene expression because it produces a low transcriptional background activity and higher photosensitivity compared to that of compound 2b.

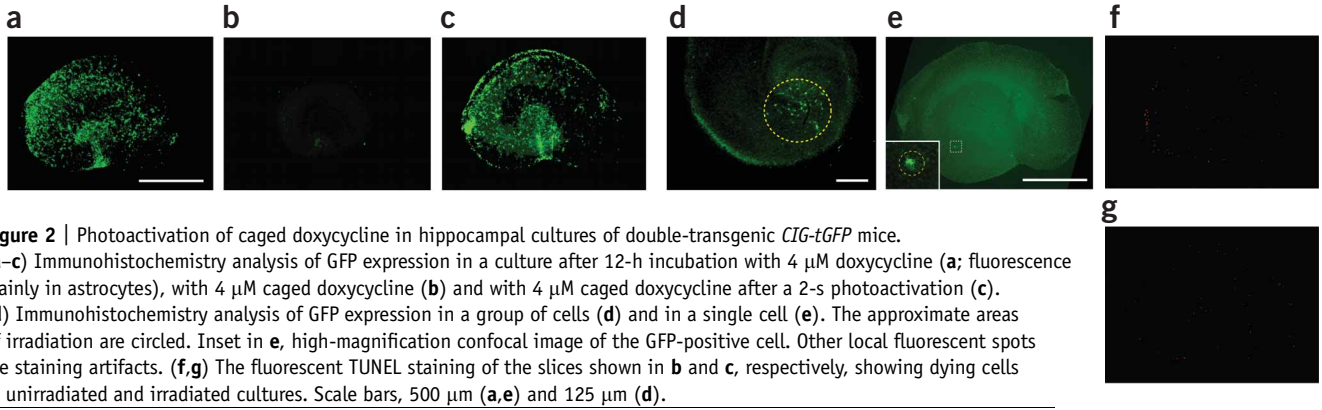

Figure 2 | Photoactivation of caged doxycycline in hippocampal cultures of double-transgenic *CIG-tGFP* mice. (**a**–**c**) Immunohistochemistry analysis of GFP expression in a culture after 12-h incubation with 4 μM doxycycline (**a**; fluorescence mainly in astrocytes), with 4 μM caged doxycycline (**b**) and with 4 μM caged doxycycline after a 2-s photoactivation (**c**). (**d**) Immunohistochemistry analysis of GFP expression in a group of cells (**d**) and in a single cell (**e**). The approximate areas of irradiation are circled. Inset in **e**, high-magnification confocal image of the GFP-positive cell. Other local fluorescent spots are staining artifacts. (**f**,**g**) The fluorescent TUNEL staining of the slices shown in **b** and **c**, respectively, showing dying cells in unirradiated and irradiated cultures. Scale bars, 500 μm (**a**,**e**) and 125 μm (**d**).

the staining intensities confirmed the specificity of photoactivated transgene expression versus tissue autofluorescence (**Supplementary Fig. 3b**). Also, we adjusted the autofluorescence in the images shown in **Figure 2d**,**e** to aid visualization of the hippocampal slice and the GFP-positive cells. We restricted GFP expression to a single cell by switching to a ×40 objective and by irradiating a spot of about 2–3 cells in diameter (**Fig. 2e**). Thus, photoactivated gene expression with caged doxycycline allows induction of transgenes with high spatial resolution, including single cells.

A major concern regarding the photoactivation approach is that either the UV-light irradiation itself and/or the side product of the uncaging reaction may be harmful to the cells. We evaluated cell viability by assessing the extent of apoptosis 24 h after photoactivation of hippocampal slice cultures using a fluorescent terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction. The extent of apoptosis in a photoactivated culture and in a nonirradiated control was similar (**Fig. 2f**,**g**). TUNEL stain quantitation revealed no significant difference between the two conditions ($n = 6$, non-paired student's t-test, $P = 0.348$; **Supplementary Fig. 4a**). Measuring the extracellular field potentials as well as the (resting) membrane potential of neurons before and after irradiation also revealed no physiological differences (**Supplementary Fig. 4**) suggesting that the amount of UV light needed for uncaging is probably innocuous.

Photoactivation with caged cyanodoxycycline

We also tested caged cyanodoxycycline in hippocampal cultures to determine its photoactivation efficiency. Because caged cyanodoxycycline has a low membrane permeability like its parent

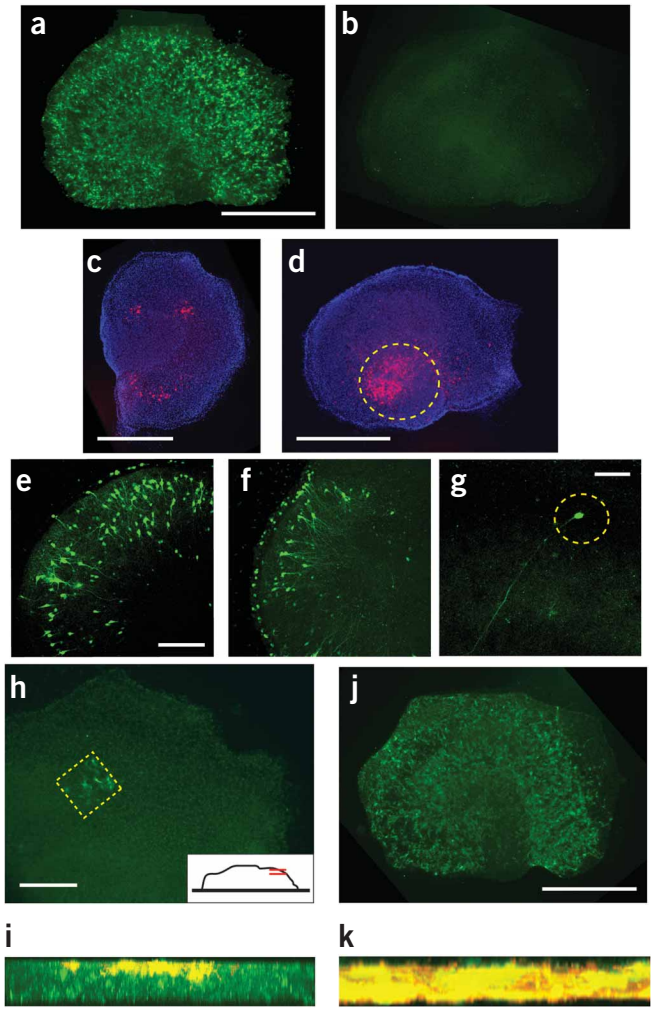

Figure 3 | Photoactivation of caged cyanodoxycycline in hippocampal cultures. (**a**,**b**) Fluorescence images of cultures of *CIG-tGFP* mice after photoactivation of 2.4 μM caged cyanodoxycycline (**a**) and without irradiation (**b**). (**c**) 'Smiley face' pattern of photoactivated GFP expression in culture of *CIG-tGFP* mice; GFP expression, red, and DAPI staining, blue. (**d**) Locally restricted photoactivated GFP expression in the dentate gyrus of a culture of *CIG-tGFP* mice. The circled area indicates the approximate area of irradiation. (**e**–**g**) Confocal fluorescence images of hippocampal slices of wild-type mice; slices were doubly transfected with rAAV viruses carrying the gene encoding human synapsin-rtTA, tet-dependent tdTomato red fluorescence was pseudocolored in green) after 48 h incubation with 3.1 μM cyanodoxycycline (**e**), after 24 h incubation with 3.1 μM caged cyanodoxycycline followed by global photoactivation (**f**), and after local photoactivation (in a single neuron) (**g**). Red fluorescence was pseudocolored in green. (**h**) Immunohistochemistry analysis in a slice showing local GFP expression after two-photon irradiation in two planes in a culture incubated with 1.9 μM caged cyanodoxycycline. Inset, approximate location of the planes (red lines) close to the surface of the culture. (**i**) 'Side view' of a confocal *z*-dimension stack of the entire slice culture in **h** shows GFP expression (red) restricted to the upper irradiated part of the culture. (**j**) Immunohistochemistry analysis of GFP expression after a single pulse UV-light irradiation of the entire culture. (**k**) 'Side view' of a confocal z-dimension stack of the entire culture in **j** reveals GFP expression (red) throughout the tissue (green background fluorescence). Cultures of *CIG-tGFP* mice are shown in **h**–**k**. Scale bars, 500 μm (**a**,**c**,**d**,**j**), 125 μm (**h**), 60 μm (**e**) and 40 μm (**f**).

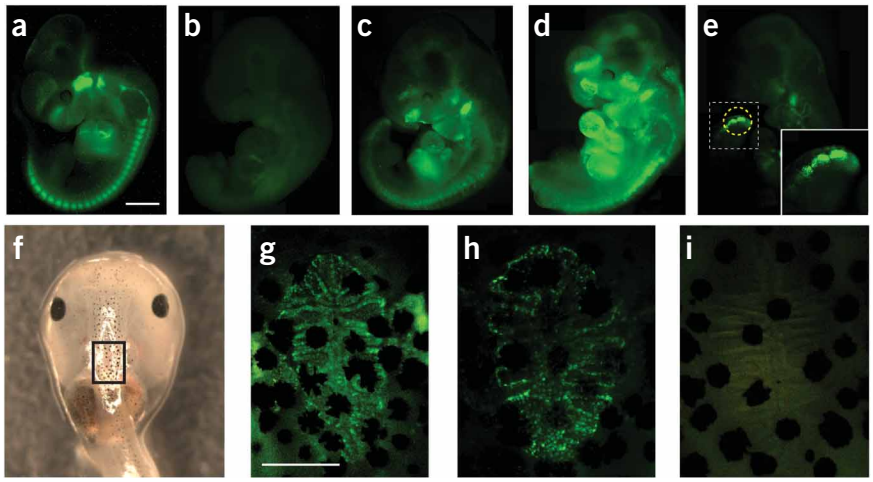

Figure 4 | Photoactivated gene expression in living organisms. (**a**) Fluorescence image of a control embryonic day 10.5 (E10.5) embryo (*CIG-tGFP*) incubated for 24 h with 20 μM doxycycline, displaying widespread GFP fluorescence particularly in neurons of the spinal cord dorsal root ganglia, the developing heart and in trigeminal ganglia of the head. (**b**) Fluorescence image of an E10.5 embryo (*CIG-tGFP*) incubated with 2.6 μM caged doxycycline without irradiation. (**c**,**d**) Fluorescence in an E10.5 embryo (*CIG-tGFP*) after a single 15-s pulse of UV-light irradiation (**c**) and after a second pulse of irradiation 3 h after the first pulse (**d**). (**e**) Highly restricted fluorescence in three dorsal root ganglia of an E10.5 embryo (*CIG-tGFP*) after localized irradiation at the tip of the tail with a spot size of about three ganglia (circle). Inset, enlargement of the irradiated area (box). (**f**) Brightfield image of a tadpole. (**g**–**i**) Fluorescence micrographs of the dorsal region of interest (boxed region in **f**) after induction with 100 μM unmodified doxycycline in the dorsal region (**g**), with 65 μM caged doxycycline after photoactivation of the whole tadpole for 15 s (**h**) and with caged doxycycline in the absence of UV-light irradiation (**i**). Scale bars, 1 mm (**a**,**g**).

compound cyanodoxycycline, it required about 5–10 times longer incubation times for full transgene expression after irradiation (data not shown).

After irradiation of the entire culture (**Fig. 3a**), we saw much more widespread transgene expression compared to doxycycline whereas the background expression in the absence of light was similar (**Fig. 3b**). Presumably, the nitrile modification of the cyanodoxycycline increased the induction efficiency similarly to what has been reported for 'cyanotetracycline'[14]. To demonstrate the enhanced photoactivation efficiency, we used a custom-made microscope insert with the shape of a smiley face to irradiate a hippocampal culture. The shape of the smiley face was reproduced by the GFP transgene expression (**Fig. 3c**) across various subregions of the slice as indicated by the DAPI counterstain. With photoactivated gene expression, the three main subregions, dentate gyrus (**Fig. 3d**), CA3 and CA1, could also easily be targeted (**Supplementary Fig. 5a**,**b**).

To circumvent the need of having to use genetically manipulated mice for every transgene, we attempted to combine photoactivation with virus-mediated delivery of transgenes. We specifically directed Tet-dependent tandem dimer (td)Tomato expression to neurons by using two adeno-associated viruses (rAAV) including one driving rtTA expression using the human synapsin promoter[19]. Many doubly transfected neurons showed strong fluorescence upon administration of 3.1 μM cyanodoxycycline for 48 h (**Fig. 3e**). Photoactivation after incubation with 3.1 μM caged cyanodoxycycline also produced many fluorescent neurons (**Fig. 3f**) whereas cultures that were not irradiated displayed no fluorescence (**Supplementary Fig. 5c**). Local irradiation of only a small spot limited expression to a single neuron (**Fig. 3g**) or two adjacent neurons (**Supplementary Fig. 5d**).

Two-photon mediated uncaging of caged cyanodoxycycline

The use of two-photon microscopy for photoactivated gene expression would extend transgene expression to less-transparent tissues with particular emphasis on *in vivo* applications. After incubation with 1.9 μM caged cyanodoxycycline, we irradiated hippocampal cultures in a plane of 100 × 100 μm with a wavelength of 710 nm. Originally, we scanned several stacks of 50–100 planes (*z*-dimension steps of 1 μm and scan time of 1 s), which did not produce any photoactivated transgene expression (data not shown). Instead, if only two planes were irradiated (about 5–8 μm separation between) with 500 scans each, transgene expression was robustly induced (**Fig. 3h**). A 'side-view' of a *z*-dimension stack through the entire slice demonstrated that photoactivated transgene expression only occurred in the upper, irradiated parts of the culture, whereas the lower, nonirradiated bottom areas did not show any GFP expression (**Fig. 3i**). For comparison, irradiation of the entire culture with a single pulse of UV light produced widespread transgene expression throughout the slice and penetrated all layers (**Fig. 3j**,**k**).

Photoactivated gene expression in living organisms

For photoactivation in entire organisms, we combined photoactivation with the mouse whole-embryo culture system, which reproduces *in utero* development of postimplantation mouse embryos at the morphological, cellular and molecular level[20–22]. For these experiments, we used the faster diffusing caged doxycycline because diffusion in the compact embryo is expected to be more limited.

To characterize the extent of inducible transgene expression in whole-embryo culture of the *CIG-tGFP* line, we removed embryonic day 10.5 embryos from the uterus, dissected them and incubated the embryos in the presence of 20 μM doxycycline for 24 h (**Fig. 4a**). Irradiation was done with a ×4 air objective (15 s) using a DAPI filter set. Irradiated embryos displayed GFP fluorescence in a pattern similar to that seen with unmodified doxycycline although the expression was somewhat reduced. Notably, nonirradiated embryos showed no detectable GFP fluorescence except for the trigeminal ganglia, in which we observed low fluorescence (**Fig. 4b**,**c**).

To determine whether transgene expression could be enhanced by repeated photoactivation, we performed each step of incubation with caged doxycycline and irradiation twice. In fact, double photoactivation produced GFP fluorescence that markedly increased compared to singly irradiated embryos (**Fig. 4d**). Finally, in one embryo, we limited UV-light irradiation to about three dorsal root ganglia, resulting in highly localized GFP fluorescence restricted to the photoactivated area (**Fig. 4e**). Pseudocoloring and quantitative assessment of the staining intensities confirmed the specificity of photoactivated transgene expression versus tissue autofluorescence (**Supplementary Fig. 3c**).

Overall, we observed no anatomical differences between untreated control embryos, nonirradiated and photoactivated embryos as all embryos had normal somite numbers, size and shape of developing organs, and limb formation (data not shown). Therefore, high-resolution photoactivated gene expression by itself does not interfere with the complex developmental programs of living organisms.

Photoactivated gene expression in *Xenopus* tadpoles

To determine whether photoactivation could be used to induce transgenes in other organisms, we tested our method in *Xenopus* tadpoles. The transgenic tadpoles contained a construct with the collagen promoter ubiquitously driving rtTA and a tetracycline-dependent nuclear GFP[23]. Doxycycline-dependent GFP fluorescence was most readily induced in a dorsal structure that appeared to be part of the spinal cord (**Fig. 4f**). We observed abundant nuclear GFP fluorescence in a 2-week-old tadpole that had been incubated with 100 μM doxycycline for 12 h (**Fig. 4g**). We observed the same pattern of GFP fluorescence after whole-tadpole photoactivation with 65 μM caged doxycycline (**Fig. 4h**). Control tadpoles that we only incubated with caged doxycycline but did not irradiate did not show any GFP fluorescence (**Fig. 4i**). Irradiated tadpoles continued to live for several days without displaying any obvious developmental defects.

DISCUSSION

The advantage of using light as the inducing agent is its ease of manipulation and the ability to identify target cells by, for example, GFP fluorescence and to then photoactivate them by simply switching excitation filters. We had previously demonstrated localized photoactivated transgene expression in cell culture and plants, and have now extended this paradigm to mice and frogs, in addition demonstrating single-cell induction of transgene expression in mouse brain cultures. Our studies indicate that the photoactivated Tet system will work wherever the Tet system works. However, Tet-responder transgenic mice do not allow doxycycline-induced, rtTA-dependent gene activation in a majority of postmitotic neurons[19]. To circumvent this problem, we introduced a Tet-responder in neurons via rAAV and successfully applied this methodology for photoactivated gene expression in neurons of wild-type hippocampal cultures[19]. In all systems tested so far, a single pulse of photoreleased doxycycline was sufficient for substantial transgene expression. In addition, photoactivation can be repeated at least once for enhanced induction. We foresee a wide range of applications for our method such as the use of two-photon uncaging to achieve high-resolution *in vivo* transgene expression deep inside the brain or other organs. One of the main applications will be in developmental biology, an area of research in which regional and pattern-specific regulation of gene expression is central to many questions. We believe that this will be a powerful approach for elucidating and manipulating the individual contributions of cells in the complex network of cell-cell interactions.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

ACKNOWLEDGMENTS

We thank I. Mansuy and O. Griesbeck for critically reading the manuscript, D. Brown and members of his laboratory for help with the photoactivation of the *Xenopus* tadpoles, V. Staiger for conducting the electrophysiological measurements, C. Huber and F. Voss for technical assistance, and P. Schmieder and M. Beerbaum for recording the NMR spectra. This work was supported by the Max Planck Society. Additional support came from the Ford Foundation, the Minorities in Neuroscience Fellowship Program and a grant of the Volkswagen-Stiftung to S.B.C., F.C. and W.B.H were supported by the Federal Ministry of Education and Research (BMBF) in the framework of the National Genome Research Network, Systematic Methodological Platform RNAi, Förderkennzeichen 5 (NGFN-2).

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ONLINE METHODS

Irradiation of caged doxycycline at different wavelengths. Caged doxycycline (25 μM) in HEPES buffer (10 mM HEPES, 120 mM KCl, pH 7.2) with acetonitrile (80:20, vol/vol) was irradiated monochromatically at wavelengths of 297, 334, 365, 405, 436 or 488 nm (cuvette, 3.8 cm × 1 cm × 1 cm; filling volume, 2.8 ml; and irradiated area, 2.8 cm^2). The monochromatic light was provided by a 500 W mercury arc lamp (Lot Oriel) equipped with metal interference filters (Lot Oriel). Using a power meter (model 1815-C, detector 818-UV, Newport Corporation), the irradiance (mW cm^{-2}) of the monochromatic light was measured at the incidence of the cuvette to determine the number of photons per second and square centimeter. Samples of caged doxycycline were exposed to 1 or 10 μmol of photons at different wavelengths. During irradiation, the solution in the cuvette was stirred by a small magnetic stir bar. The irradiated and control nonirradiated samples were analyzed by HPLC (HP 1100 series, Hewlett-Packard; column, EC 250/4 Nucleodur 100-5 C18 ec, Macherey-Nagel; eluents: 0.1% aqueous TFA (solution A) with acetonitrile (solution B); flow rate: 1 ml min^{-1}; gradient: 5–95% B in 25 min; UV-light detection wavelengths: 254 and 350 nm), and the extent of photolysis was calculated from the decrease in the peak area upon irradiation.

Luciferase assay. The luciferase assay was performed according to the manufacturer's protocol (Promega). Stably transfected HeLa cells expressing tet-dependent luciferase (line X1.6; provided by H. Bujard) were incubated with the respective concentrations of caged or unmodified doxycycline for 24 h, the cells were then washed, lysed, and finally, the lysate was mixed with the luciferase assay reagent for immediate measurements. The values represent the average of three independent samples. The luminescence was read at 555 nm. Caged doxycycline was irradiated for 1 h with a low-power hand-held UV lamp before adding it to the cells.

Photoactivation of biological specimen. Irradiations were done on standard upright fluorescent microscopes (Zeiss and Olympus) equipped with a standard DAPI fluorescent filter set using the DAPI excitation light for photoactivation of the samples. For the electrophysiological measurements, photoactivation was performed on an inverted microscope using a FURA filter set for excitation. Using 100 W mercury lamps, the shutter-controlled irradiation times were 0.5 s (×40 air objective), 1 s (×20), 2 s (×10) or 15 s (×4).

Mice. The *CAGGS-irtTA-GBD** (*CIG*) line was obtained by conventional transgenesis of ES cells (Anastassiadis, K. *et al.*, unpublished data). All protocols related to animal experiments were performed according to the German Animal Welfare legislation, and the Animal Welfare Officers appointed for the facility oversaw them. The *CAGGS-irtTA-GBD** mice were crossed with a *GFPtetO7lacZ* mouse (G3, kindly provided by R. Sprengel). Double transgenic mice were identified by PCR genotyping using the following primers: irtTA1, 5′-AGTTGGCATTGAGGGCTTGACC-3′ and irtTA2, 5′-CAACTTGGTGCTCCTGGTCCTC-3′ for the irtTA-GBD* and primer hGFP12 and hGFP13 for *GFPtetO7lacZ*[24] (**Supplementary Table 2**). Expression of the irtTA-GBD* fusion in different organs was analyzed by northern blots and RT-PCR using standard protocols. To monitor GFP expression in adult animals, mice were injected intraperitoneally with a daily dose of 0.5 mg doxycycline (50 mg ml^{-1} in 50% EtOH) and 0.2 mg dexamethasone (10 mg ml^{-1} in 50% EtOH, freshly prepared each day) for four consecutive days. Mice were sacrificed after injections and expression of GFP in different organs was analyzed by RT-PCR.

Hippocampal cultures. Postnatal day 2–7 hippocampal cultures were prepared according to standard procedures[25]. Hippocampi were excised from the brain, cut into 300 μm thick slices with a Tissue Chopper (McIlwain) and placed on a membrane filter. Photoactivation experiments were performed after 1–5 d *in vitro*. For the electrophysiological recordings, wild-type cultures were prepared from postnatal day 3 pups and the experiments were performed at 5–6 d *in vitro*.

Photoactivation procedure and staining of hippocampal cultures. Double transgenic cultures were first incubated in standard culture medium[25] with 50 mM Tris pH 7.0 plus the caged doxycycline or cyanodoxycycline, briefly (15–30 min) transferred to medium containing 50 mM Tris pH 7.0, 25 μM dexamethasone, 0.5 mM octanol, irradiated, and were then quickly returned to medium containing only 25 μM dexamethasone. The octanol was included to block gap junctions, the dexamethasone to allow translocation of irtTA-GBD* from the cytoplasm to the nucleus. GFP expression could be detected immunohistochemically within 3 h, but longer incubation of cultures (12–48 h) increased signal-to-noise. Primary antibodies: GFP 1:1,000 (RBD), glial fibrillary acidic protein 1:100 monoclonal antibody (Sigma), microtubule-associated protein 2 1:10,000 monoclonal antibody (Sigma). Double immunostainings to identify GFP positive cells were done with cultures aged 20–22 d *in vitro*, which were continuously exposed to 2 μM doxycycline and 2.5 μM dexamethasone. TUNEL stains (cell death detection kit, TMR red; Roche) were performed subsequent to the immunostaining procedure. Membranes with the antibody stained cultures were completely dried, then incubated at 37 °C for 1 h with the TUNEL reaction mixture, and finally washed twice in PBS. TUNEL stained cultures were analyzed in a blind fashion.

Two-photon illumation–mediated photoactivation of hippocampal cultures. Cultures were incubated in caged cyanodoxycycline and prepared the same way as with UV photoactivation. Cultures were irradiated while on the interface-membrane through a ×20, 0.4 NA air objective (Zeiss). Irradiations were done on a custom-built two-photon microscope with excitation light (λ = 710 nm) from a mode-locked Ti-sapphire laser (Mai Tai; Spectra-Physics). The average light power after the objective was set to 6 mW. Scanning was controlled by custom-made software written in LabView (National Instruments). The scan parameters were the following: 100 × 100 μm area, 1-s duration, 1 μm pixel size.

Recombinant adeno-associated viruses (rAAV) equipped with tetracycline-controlled genetic modules. The plasmids *rAAV-P_{hsyn}-rtTA-nM2*, *rAAV-Ptetbi-Cre/Venus* and *rAAV-Ptetbi-Cre/tdTomato* are based on the AAV expression plasmid[26]. High titer rAAVs with serotypes 1 and 2 were prepared by transfecting one of the plasmids, *rAAV-P_{hsyn}-rtTA-nM2*, *rAAV-Ptetbi-Cre/Venus*, or

doi:10.1038/nmeth.1340

rAAV-Ptetbi-Cre/tdTomato, together with helper plasmids, *pDp1* (serotype 1), and *pDp2* (serotype 2) in a ratio of 2:1:1. Titers for these viruses were almost 10^{11} genomic particles per milliliter and viruses were prepared as previously described[19].

Whole-embryo culture and photoactivation. For whole-embryo culture, embryos are typically collected between embryonic day 7 (E7) to E11 followed by removal of their decidua and Reichert's membranes. Embryos are then allowed to develop *in vitro* for up to 48 h in continuously oxygenated medium containing immediately centrifuged mouse serum[21,22]. Culture of mouse embryos was performed as previously described[21]. E10.5 embryos were removed from the uteri, the yolk sac was opened, and normal development was allowed to continue in a whole embryo culture incubator (Ikemoto) in 1.5 ml of a 2:1 mixture of immediately centrifuged, heat-inactivated mouse serum (Harlan): DMEM, and 0.1 mM octanol in a continuous-flow (50 cm^3 min^{-1}) of 60% O_2, 5% CO_2, 35% N_2 for 12 h and 95% O_2, 5% CO_2 for the following 12 h. To identify transgenic *CIG-tGFP* embryos, tail buds of each embryo were removed, incubated in 200 μM and 25 μM of doxycycline and dexamethasone, respectively, and analyzed for GFP expression 3 h later. *rtTA/tet-GFP* positive embryos were cultured 3 h in medium containing 2.6 μM caged doxycycline, washed briefly in PBS, irradiated for 15 s through a ×4 objective and were finally transferred to medium containing 25 μM dexamethasone. For double irradiations, the induction protocol was repeated. Thus, following 3 h in dexamethasone containing medium, the embryos were transferred back to 2.6 μM caged doxycycline (3 h), washed, irradiated and finally incubated again in dexamethasone. GFP fluorescence was assessed the following day.

***Xenopus* tadpole experiments.** Tadpoles contained a *rtTA-M2* construct and a tet-dependent GFP fusion with a dominant negative form of the thyroid hormone receptor α, which localizes the fusion protein to the nucleus[23]. For photoactivation, tadpoles were incubated 24 h in standard 0.1x MMR containing 65 μM caged doxycycline at room temperature. Tadpoles were then briefly washed in 0.1× MMR and were subsequently irradiated for 15 s with an ×4 air objective. GFP expression was assessed 12–16 h after irradiation.

Electrophysiology. Electrophysiological recordings were performed at 32 °C in an external solution (ASCF) containing: 125 NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 25 mM glucose saturated with 95% O_2 and 5% CO_2. The recording chamber was constantly superfused with a flow rate of 1 ml min^{-1}. Data were collected at a sample frequency of 10 kHz, low-pass filtered at 3 kHz for the patch recordings and band-pass filtered between 1–3 kHz for the extracellular recordings. Data were analyzed by programs written in LabView (National Instruments).

Whole-cell patch-clamp. Recording electrodes (~8 MΩ) were filled with internal solution containing: 125 mM potassium gluconate, 5 mM KCl, 10 mM Hepes, 1 mM EGTA, 2 mM Na-ATP, 2 mM Mg-ATP, 10 mM sodium phosphocreatine. Recordings were carried out with an Axoclamp 2B (Axon Instruments) amplifier in current clamp mode. Cells in the CA1 region were patched in a blind fashion in voltage clamp mode. After establishing a stable patch, the recording mode was switched to current clamp. Before and after 10-s irradiation of the entire culture using a ×10 objective, a current–voltage characteristic (*I/V* curve) was recorded by injecting current pulses (250 ms, 20 pA steps, −240 pA to 160 pA) through the recording electrode.

Extracellular recordings. Field EPSPs were recorded in the CA1 region with a glass electrode filled with 3 M NaCl (8–15 MΩ). The stimulating electrode was placed in the Schaffer collateral region. Stimuli were delivered at a frequency of 0.5 Hz (50–100 μA, duration 0.1 ms). After baseline recording for 15 min, a 2 s UV-light pulse (same conditions as for patch experiments) was applied and after another 15 min of recordings, a second pulse of 10 s was applied. At the end of the experiment, the light was applied continuously to test whether the signal eventually breaks down. The amplitude of the signal was measured as the difference between the minimum and maximum of the excitatory post-synaptic potential including population spikes.

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