New Mouse Lines for the Analysis of Neuronal Morphology Using CreER(T)/loxP-Directed Sparse Labeling

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Abstract

Background: Pharmacologic control of Cre-mediated recombination using tamoxifen-dependent activation of a Cre-estrogen receptor ligand binding domain fusion protein (CreER(T)) is widely used to modify and/or visualize cells in the mouse.

Methods and Findings: We describe here two new mouse lines, constructed by gene targeting to the Rosa26 locus to facilitate Cre-mediated cell modification. These lines should prove particularly useful in the context of sparse labeling experiments. The R26rtTACreER line provides ubiquitous expression of CreER under transcriptional control by the tetracycline reverse transactivator (rtTA); dual control by doxycycline and tamoxifen provides an extended dynamic range of Cre-mediated recombination activity. The R26IAP line provides high efficiency Cre-mediated activation of human placental alkaline phosphatase (hPLAP), complementing the widely used, but low efficiency, Z/AP line. By crossing with mouse lines that direct cell-type specific CreER expression, the R26IAP line has also been used to visualize the full morphologies of retinal dopaminergic amacrine cells, among the largest neurons in the mammalian retina.

Conclusions: The two new mouse lines described here expand the repertoire of genetically engineered mice available for controlled in vivo recombination and cell labeling using the Cre-lox system.

Introduction

Altering the mouse genome by Cre-mediated recombination is one of the central technologies of modern mammalian genetics [1]. An important adjunct to this technology has been the development of a method for pharmacologic control of Cre-mediated recombination by fusing Cre with a mutant estrogen receptor ligand-binding domain that recognizes 4-hydroxytamoxifen (4HT) rather than estrogen [CreER(T); referred to hereafter as CreER; 2,3]. The CreER protein is sequestered in the cytosol until 4HT exposure releases it for nuclear migration.

For typical applications of the CreER technology - for example, experiments involving timed inactivation of conditional alleles - highly efficient recombination is considered desirable. By contrast, relatively inefficient recombination is essential for experiments in which the goal is to monitor the behavior, morphology, or function of individual genetically modified cells in an environment populated by unmodified neighbors. In extreme cases - for example, experiments using genetically encoded reporters to study the morphologies of very large CNS neurons - the efficiency of recombination must be lowered to fewer than 20 cells per mouse [4]. Other applications have used sparse labeling with Cre/loxP recombination to study neuronal morphologies in the retina [5], to define the role of the Frizzled5 receptor in the survival of thalamic neurons [6], and to compare the roles of transcription factors Brn3a and Brn3b in retinal ganglion cell (RGC) development [7].

One limitation of the mouse lines currently available for sparse labeling relates to the efficiency of Cre-mediated recombination, which varies substantially with different combinations of CreER source and loxP target. For example, we observed that the Z/AP line, which expresses human placental alkaline phosphatase (AP) upon excision of a loxP-flanked beta-geo/transcriptional stop cassette [8], recombines with an efficiency ~1,000 lower than similarly designed conditional AP knock-in alleles of the Brn3a and Brn3b genes (Brn3aCKOAP and Brn3bCKOAP), when each target gene is tested with a ubiquitously expressed CreER knock-in at the Rosa26 locus.

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sequences are transcribed as part of the ubiquitously expressed DNA-binding protein that activates transcription upon binding reverse tetracycline trans-activator (rtTA), a sequence-specific Rosa26 expression, we generated the (Figure 1A,B). In this allele, CreER number of AP + 200 postnatal exposure to 4HT, with a single IP injection of 100–200 µg in the early postnatal period producing several dozen AP+RGCs per R26CreER;Z/AP retina and many thousands of AP+RGCs per R26CreER;Bm3a/CreloxP/+ or R26CreER;Bm3a/CreloxP/+ retina [7,10].

For sparse labeling of neurons without selection for a particular subtype, the R26CreER;Z/AP combination with an early postnatal injection of 4HT generates a frequency of labeling that is close to optimal for morphologic analyses [0.001%–0.01% of neurons labeled; 5,10]. When the Z/AP reporter is used in conjunction with a set of cell-type selective CreER lines – produced by knocking an IRES-CreER into the 3’ untranslated region (UTR) of the genes coding for choline acetyl transferase (ChAT-IRES-CreER) or tyrosine hydroxylase (TH-IRES-CreER) – the low level of CreER production, together with the relatively small number of catecholaminergic or cholinergic neurons, results in extremely sparse cell labeling [4].

In the present work we have enhanced the sparse labeling technique by developing two Rosa26 knock-in mouse lines: one line increases the dynamic range of CreER action, and the second line exhibits Cre-mediated activation of AP with high efficiency and with undetectable background AP activity in unrecombined cells. The R26rtTACreER line uses the reverse tetracycline transactivator (rtTA) and doxycycline to control the expression of CreER, with the result that 4HT-induced Cre recombinase activity can be set at a level that is substantially lower than that typically obtained with constitutively expressed CreER lines. Thus the R26rtTACreER line permits precisely timed sparse recombination of target loci with a high efficiency of Cre-mediated recombination. Because the Rosa26 locus is widely expressed, the R26rtTACreER line can be used to generate sparse genetic mosaics to test the behavior of individual mutant cells in a wide variety of tissues and/or in situations in which the conventional null allele is lethal. The R26IAP line complements the popular but inefficiently recombining Z/AP line by providing an AP reporter that exhibits high efficiency Cre-mediated recombination in virtually any cell. The R26IAP line should prove especially useful when paired with CreER loci that exhibit low levels of 4HT-induced Cre activity. We demonstrate the utility of the latter line by using it to: (a) assess the efficiency of Cre-mediated recombination following either direct uptake of Cre protein or infection by an adenovirus vector expressing Cre, (b) generate brain atlases of sparsely labeled cholinergic and catecholaminergic neurons, and (c) characterize the morphologies of type 1 dopaminergic retinal amacrine cells, which have axon-like arbors that are among the largest of any cells in the mammalian retina.

Results

R26rtTACreER: Dual Control of Cre Action by Doxycycline and 4-Hydroxytamoxifen (4HT)

To construct a ubiquitously expressed CreER(T) allele (hereafter referred to as ‘CreER’; 2) with a large dynamic range of CreER expression, we generated the Rosa26 knock-in allele R26rtTACreER (Figure 1A,B). In this allele, CreER is under the control of the reverse tetracycline trans-activator (rtTA), a sequence-specific DNA-binding protein that activates transcription upon binding tetracycline derivatives such as doxycycline [11]. The rtTA coding sequences are transcribed as part of the ubiquitously expressed Rosa26 transcription unit and are joined by splicing to the 5’ exon of this transcript. Upon administration of doxycycline (in food pellets and/or drinking water), rtTA activates transcription of the CreER coding region by binding to seven tandem copies of the Tet operator located immediately 5’ of a minimal promoter.

To systematically explore the relationship between the level and timing of doxycycline and 4HT exposure and the efficiency of Cre-mediated recombination, we analyzed the expression of AP in RGCs using R26rtTACreER;Bm3a/CreloxP/+ and R26rtTACreER;Bm3a/CreloxP/+ mice (Figure 2; see Figure 1 of [reference 7] for a schematic of these reporters). Doxycycline was delivered in food pellets at 1.75 mg/g and/or in drinking water at 0.3, 1, or 2 mg/ml. 4HT dissolved in vegetable oil was delivered to pregnant females or to early postnatal mice as a single IP injection. Retina flat mounts were prepared from adult mice, stained histochemically for AP activity, and scored as...
having high/medium labeling (>50 AP+ RGCs per retina; Figure 2E, F), sparse labeling (1–50 RGCs per retina; Figure 2C), or no labeling (Figure 2B). Various dosing regimens were tested, of which 33 are shown diagrammatically in Figure 2A. For each of the two genotypes tested, the diagrams have been ordered with respect to recombination efficiency, presented in the histograms on the right side of Figure 2A.

From these experiments several patterns emerge. First, initiating doxycycline exposure after approximately embryonic day (E) 5 gives low or no recombination.
delivered subsequently [e.g., experiments 1–8, 22, and 23]; (Figure 2B). Second, continuous exposure to doxycycline beginning prior to or at E9 produces recombination in a dose-dependent manner, with some recombination seen even in the absence of 4HT [e.g., experiments 13, 18–21, 28, 32, and 33]; (Figure 2F). Third, initiating doxycycline exposure between E2 and E4 and delivering 4HT between E10 and E12 gives dose-dependent recombination that results in a relatively sparse collection of AP+ RGCs suitable for the analysis of single cell morphologies [e.g., experiments 2, 3, 11, 12, 24–27]; (Figure 2CE). In particular, in experiments 2, 3, and 11 (with the Brn3aCKOAP/+ target), two experiments showed nobe l neun and one experiment showed sparse labeling in more than half the retinas. Similarly, in experiments 24, 25, and 27 (with the Bm3.zCKOAP/+ target), one third of the retinas have no labeled neurons and two thirds of the retinas have a sparse distribution of labeled neurons.

Interestingly, several retinas with sparse labeling exhibited clusters of AP+ RGCs indicative of early clonal activation or repression [e.g., Figure 2C]; an analogous clustering of AP+ neurons and their processes were observed in the brains of several of these mice, as seen in the coronal section through the superior colliculus in Figure 2D. Importantly, clustering of labeled RGCs was observed in retinas that received 4HT after E11, a time when there are already many hundreds of retinal progenitors and lateral migration of cells within a single retinal clone is severely restricted [10]. Thus, the data suggest that the clonal restriction reflects events that occurred earlier in development, such as epigenetic silencing of rTA expression and/or rTA-dependent activation of the CreER target in a subset of cells. It seems plausible to suppose that the spatial patterns of clonal restriction might reflect the same epigenetic process that silences CreER expression when doxycycline is not delivered in early gestation. The weak AP staining of all axon bundles in Figures 2B and C arises from a low-level background of AP expression in RGCs from the Bm3.zCKOAP and Bm3.zCKOAP alleles in the absence of Cre-mediated recombination.

In sum, by using an appropriate regimen of doxycycline exposure, the R26rtTA.CreER locus can be used, even with highly recombinogenic target loci, to generate a sparse set of labeled cells in a developmental time point determined by 4HT injection.

**R26IAP: A Sensitive Cre-Dependent AP Reporter for a Broad Array of Tissues and Cell Types**

To create an AP reporter that is activated by Cre-mediated recombination with an efficiency higher than the relatively inefficient Z/αAP locus and to eliminate background AP activity in unrecombined cells, a Rosa26 knock-in allele was constructed with an AP coding region in which the 3’ half is inverted (R26IAP; Figure 1C). Upon Cre-mediated recombination, the 3’ half of the coding region, which is flanked by loxP sites in head-to-head orientation, is flipped into the correct orientation in a subset of cells. On the assumption that a variable number of DNA inversion events occurs before dissociation of the Cre/loxP complex, we expect that the recombination process would produce a mixture of correct and inverted orientations in those cells in which recombination has occurred. Therefore, AP expression from the R26IAP allele should persist in a subset of recombinated cells, with virtually no background AP activity in unrecombined cells. Cre-mediated inversion has been used previously in experiments in which extremely tight control of gene expression was essential, for example in the in vivo analysis of oncogenic fusion proteins [12].

As an initial test of the efficiency of Cre-mediated recombination at the R26IAP locus, we examined AP expression by histochemical staining of adult tissues in R26CreER;R26IAP mice that had not been exposed to 4HT (Figure 3A,B). This analysis revealed 4HT-independent recombination at a frequency of hundreds of cells per retina and ~1,000 cells per 300 μm vibratome section of brain, roughly similar to the levels observed with R26CreER;Brn3αCKOAP/+ and R26CreER;Brn3βCKOAP/+ mice in the absence of 4HT. As noted in the Introduction, R26CreER;Z/αAP mice average 〈1 AP+ cells per retina or 300 μm brain section in the absence of 4HT [10]. In R26CreER;R26IAP mice, the number of AP+ cells increased by several orders of magnitude following a single IP injection of 200 μg 4HT at P3, resulting in AP-stained retina flat mounts that are completely opaque (not shown). Recombination in R26CreER;R26IAP mice is observed in a wide variety of tissues, as expected for expression of both CreER and AP from the Rosa26 locus. For example, Figure 3C and D shows scattered AP+ cells in the lens and cornea following intracocular delivery of 6 μg 4HT in adult mice.

**Using R26IAP to Assess the Efficiency of Different Methods for Cre Delivery**

The relatively high recombination efficiency of the R26IAP locus, together with the high sensitivity of AP histochemistry, suggested that the R26IAP locus might be generally useful for monitoring the efficiency of Cre-mediated recombination in a variety of experimental contexts. To test this idea, we have used R26IAP to compare Cre-mediated recombination following: (a) direct uptake of purified Cre protein fused to a poly-basic peptide (His-NLS-Cre; 15) injected into the peritoneal cavity or into the eye (Figure 3E–H), or (b) infection with a defective adenovirus vector expressing Cre (Ad-Cre) delivered by intravenous or intraocular injection (Figure 3I–O). Each of these methods of Cre administration induced recombination at the R26IAP locus, with the cell type, location, and efficiency of recombination reflecting the delivery route and vehicle.

Intraperitoneal (IP) injection of His-NLS-Cre in adulthood led to recombination predominantly in epithelial cells lining the peritoneal cavity, as seen on the surface of the liver, stomach, and abdominal wall (Figure 3E–G); the only AP+ cells not lining the surface epithelium were occasional AP+ abdominal wall muscle fibers (Figure 3F). Similarly, intraocular (i.e. intravitreal) injection of His-NLS-Cre at P5 induced recombination principally in retinal cells in close proximity to the vitreal surface: RGCs, astrocytes, and superficial vascular cells (Figure 3H). Intraocular injection of Ad-Cre in adult mice resulted in transduction of Cre into retinal and corneal cells (Figure 3I and J), and tail vein injection of Ad-Cre in adult mice resulted in efficient recombination in the liver (Figure 3O) and scattered recombination in the kidney, with the highest efficiency in the renal pelvis (Figure 3K–N). Intraocular injections of His-NLS-Cre or Ad-Cre gave highly variable frequencies of recombination within the retina, ranging from few to no recombination events to nearly confluent AP staining across the retina, a variability that most likely reflects variation in needle placement, volume injected, and the fraction of the injected volume retained within the eye. In the absence of His-NLS-Cre protein or Ad-Cre virus or a genetically introduced Cre or CreER locus, no AP+ cells were observed in any tissues examined from R26IAP mice, including brain, liver, stomach, kidney, eye, and abdominal wall.

**The Utility of Brain Atlases Composed of Sparsely Labeled and Genetically Defined Neuronal Subtypes**

The role of cholinergic and catecholaminergic (dopaminergic, noradrenergic, and adrenergic) neurotransmission in brain function and disease has been an object of long-standing scientific...
interest, and, as a result, the anatomy of the cholinergic and catecholaminergic systems has been intensively studied in both normal and diseased brains in a wide variety of species. In most studies, cholinergic and catecholaminergic neurons have been visualized by immunostaining for the biosynthetic enzymes choline acetyl transferase (ChAT) and tyrosine hydroxylase (TH), respectively, a method that reveals the cell bodies and processes of all neurons of the particular neurotransmitter type. Due to the high density and wide coverage of immunoreactive processes, this approach generally reveals only minimal information about the morphologies of individual neurons. In the present study, we have developed a complementary method for visualizing systems of neurons defined by neurotransmitter type. In this method, cell-type specific, but relatively low frequency, Cre-mediated recombination randomly labels a subset of neurons within the population of interest to generate an atlas that reveals representative soma locations, projections, and individual morphologies for all of the neurons of that class.

Using ChAT-IRES-CreER/R26IAP Mice to Produce an Atlas of Cholinergic Neurons

The application of this sampling method to the cholinergic system is illustrated in Figure 4. In this experiment, a ChAT-IRES-
A CreER;R26IAP mouse was injected IP with 200 μg 4HT at P8, and one month later a complete series of 300 μm coronal brain sections was histochemically stained for AP (Figure 4N). The distribution of AP+ cell bodies and processes was found to be in excellent agreement with the distribution of cholinergic neurons and processes observed in mouse and rat brains by ChAT immunostaining [14, 15]. In the following paragraph, we summarize the distribution of the AP+ neurons and processes in the ChAT-IRES-CreER;R26IAP brain, proceeding in a rostral to caudal direction.

AP+ neurons with well-defined processes were observed in the medial septum (Figure 4A,B) and in the basal forebrain in and near the substantia innominata (Figure 4C,D). In the striatum, AP+ neurons had both thick tortuous dendrites and a halo of much finer (presumably axonal) processes (Figure 4A,C,E). A large number of fine AP+ processes were found throughout the cortex and hippocampus, as expected for diffuse cholinergic projections originating in the basal forebrain (Figure 4F,G); an especially dense plexus of AP+ processes was present in the basolateral nucleus of the amygdala (Figure 4F-H). Near the pons/midbrain junction, AP+ neurons were localized to the laterodorsal tegmental nucleus and the pedunculopontine tegmental nucleus (Figure 4I,J). In the caudal medulla, AP+ neurons were seen in the dorsal motor nucleus of the vagus nerve (Figure 4K,L). In retina flat mounts, AP labeling was confined to starburst amacrine cells, the only cholinergic cell type within the mammalian retina, confirming the specificity of ChAT-IRES-CreER expression (Figure 4M).

Using TH-IRES-CreER;R26IAP Mice to Produce an Atlas of Catecholaminergic Neurons

To further extend the approach of labeling a subset of neurons within a given neurotransmitter system, we have also generated an atlas of labeled neurons for the catecholaminergic systems by targeting R26IAP recombination with TH-IRES-CreER following IP injection of 100 μg 4HT at P3. Figure 5 shows a series of 300 μm coronal sections from a TH-IRES-CreER;R26IAP brain that was histochemically stained for AP (Figure 5N). The distribution of AP+ cell bodies and processes was found to be in excellent agreement with the distribution of catecholaminergic

Figure 4. Survey of cholinergic neuronal morphologies and projections in the brain and retina of R26IAP;ChAT-IRES-CreER mice. Animals were injected with a single IP injection of 200 μg 4HT at P8. (A,C,F,I,K) 300 μm coronal sections at Bregma positions 1.2, 0, −2.5, −5.0, and −7.8, respectively, as shown schematically in (N). (B,D,E,G,H,J,L) enlargements of boxed regions. (M) In a flat mounted retina, AP-expression is present exclusively in starburst amacrine cells. In the absence of 4HT, retinas were devoid of AP+ cells. BNA, basolateral nucleus of the amygdala; DMN(VN), dorsal motor nucleus of the vagus nerve; LDTN, laterodorsal tegmental nucleus; MS, Medial septum; PPTN; pedunculopontine tegmental nucleus; SI, substantia innominata; St, striatum.
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neurons and processes observed in mouse and rat brains by TH immunostaining [16–21]. Below we summarize the distribution of AP+ neurons and processes in the TH-IRES-CreER;R26IAP brain, proceeding in a rostral to caudal direction.

In the TH-IRES-CreER;R26IAP forebrain, dense AP+ processes, but very few AP+ cell bodies, were seen in the nucleus accumbens, striatum, ventral pallidum, and the central nucleus of the amygdala (Figure 5A–D), as expected for these principal targets of innervation by midbrain dopaminergic and noradrenergic neurons. At the level of the hypothalamus, scattered AP+ cell bodies with a small number of thick processes were present in the arcuate nucleus (Figure 5E and F). Intense AP staining was seen in the median eminence, and AP+ neurons with numerous finer processes were seen in the zona incerta (Figure 5E and F). The greatest number of AP+ neuronal cell bodies were found in a contiguous zone encompassing the substantia nigra pars reticulata and the ventral tegmental area and extending caudally as far as the pedunculopontine tegmental nucleus, nuclei that give rise to the principal dopaminergic projections to the forebrain (Figure 5G–J). At the junction of the midbrain and pons, large numbers of AP+ cells populate the dorsal raphe nucleus, a major source of projections to the nucleus accumbens (Figure 5I). The locus coeruleus, the site with the highest concentration of central noradrenergic neurons, was densely populated with AP+ cells (Figure 5K). Finally, AP+ neurons were observed in the caudal medulla in the locations predicted for A1 and A2 noradrenergic cell groups (Figure 5L and M).

Using TH-IRES-CreER;R26IAP Mice to Visualize the Morphologies of Wide-Field Tyrosine Hydroxylase-Expressing Amacrine Cells

As a further test of the utility of the R26IAP line, we have asked whether we could use it to characterize the unusually large arbors of dopaminergic amacrine (DA) cells in the mouse retina. DA cells have been found in the retinas of a wide variety of vertebrates. Both rodent and primate retinas have a distinctive type of TH+ amacrine cells (type 1 cells) characterized by large cell bodies with two groups of neurites: one group forms a simple tree composed of relatively thick dendrites, whereas the second is composed of extremely long, thin, and sparsely branching axon-like processes [22–24]. The dendritic and axon-like arbors are confined to the outermost sublamina of the inner plexiform layer. In the primate retina, the dendritic arbors of DA cells have diameters of ~400 μm near the fovea and ~600 μm in the periphery, whereas the axon-like arbors encompass a diameter of at least 6 mm, the exact value being difficult to ascertain due to uncertainty in the extent of diffusion of the injected label [24]. Type 1 DA cells are unusually sparse. In the primate retina they have a density of ~50/mm² near the fovea and ~600/mm² in the periphery, whereas in the mouse and rabbit they have an average density of ~20/mm², equivalent to ~450 and ~6,000 cells per retina, respectively [24–27]. Despite their relatively small numbers, dopamine released by DA cells plays a central role in modulating retinal signaling in response to changes in ambient illumination or circadian cues [28–32].

Figure 5. Survey of catecholaminergic neuronal morphologies and projections in the brain of TH-IRES-CreER;R26IAP mice. Animals were injected with 100 μg 4HT at P3. (A–E,G,I,K,L) 300 μm coronal sections at Bregma positions 1.7, 1.1, 0, −1.6, −2.2, −3.2, −4.7, −5.5, −8.2, respectively, as shown schematically in (N). (F,H,J,M) enlargements of boxed regions. A1, A1 noradrenergic cell group; A2, A2 noradrenergic cell group; AcN, accumbens nucleus; AN, arcuate nucleus; CNA, central nucleus of the amygdala; DRN, dorsal raphe nucleus; LC, locus coeruleus; ME, median eminence; PPTN, pedunculopontine tegmental nucleus; SN(Pr), substantia nigra (pars reticulata); St, striatum; VP, ventral pallidum; VTA, ventral tegmental area; ZI, zona incerta.

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In most mammalian retinas, including the mouse retina, TH immunostaining can be used to identify DA cells, but the extensive intermingling of TH+ processes from different cells precludes the tracing of individual immunolabeled arbor. A similar challenge exists for transgenic approaches in which the TH promoter drives expression of a histochemical or fluorescent reporter [27,33,34].

In the present study, we have used TH-IRE5-CreER,R26LIAP mice injected with 100–300 μg 4HT between P3 and P6 to produce, on average, fewer than one AP+ DA cell per retina. This window of injection times was chosen to roughly match the initial appearance of TH+ amacrine cells in the rodent retina [35–37]. Among 120 adult retinas histochemically stained for AP and examined as flat mounts, a total of 147 AP+ cells were observed, and all were amacrine cells. Twenty-five of the AP+ cells exhibited morphologies indicative of type 1 DA cells: a large soma, a relatively simple dendritic arbor, and an extremely large and sparsely branching axon-like arbor, with both arbor confined to the outermost lamina of the inner plexiform layer (Figure 6A–C). This narrow lamination is in contrast to the diverse lamination of AP+ type 1 DA cells among the 120 retinas was consistent with a Poisson process: the number of retinas with one, two, or more than two type 1 DA cells was 97, 21, two, and zero, respectively. Fourteen of the type 1 DA cells were traced (Figure 6A and B): 12 from retinas with a single type 1 DA cell, and two from one retina that had a pair of type 1 DA cells. In the retina with two type 1 DA cells (Figure 6B and C), the extensive overlap between the two axon-like arbors precludes an accurate reconstruction and cell labeling using the Cre-lox system. The R26rtTA/CreER line extends the dynamic range of Cre-mediated recombination efficiencies relative to that of other loci with a constitutively expressed CreER, and the R26LIAP line complements the widely used Z/IP line by providing substantially higher efficiency Cre-mediated activation of AP. We note that Que and colleagues [40] have recently described the construction of a Rosa26 knock-in mouse line in which a hPLAP coding region is transcribed from a CMV enhancer/beta-actin promoter after Cre-mediated excision of a loxp-stop-loxp cassette.

It seems likely that for applications requiring low efficiency recombination, the dynamic range and temporal control offered by R26rtTA/CreER represents an improvement over pre-existing lines in which the Tet system controls a constitutively active Cre recombinase [41–44]. It will be interesting to extend the rtTA/CreER strategy by targeting this cassette to loci that confer cell-type specific expression. Based on our observations with the R26rtTA/CreER locus, we would predict that this two-stage pharmacologic strategy will also confer tighter control of Cre activity relative to the standard CreER cassette at other sites in the genome.

Factors Affecting the Efficiency of Cre-Mediated Recombination

The roughly one thousand-fold difference between Z/IP and R26LIAP recombination frequencies emphasizes the large effect of chromosomal location and/or target gene sequence on the efficiency of Cre-mediated recombination. This effect is substantially larger than the variation typically observed among target loci [45]. A second variable that affects the efficiency of Cre-mediated recombination is developmental age. With multiple target loci for which the efficiency of Cre-mediated recombination can be easily quantified with AP histochemistry - including Bm3a, Bm3b, Z/IP, and Fc5 - we have consistently observed a decline in efficiency with increasing postnatal age when tested with a variety of CreER lines, including R26CreER, NFL-IRE5-CreER, and ChAT-IRE5-CreER (our unpublished observations). As the expression of the Rosa26, NFL, and ChAT genes persists in adulthood, the observed decline in recombination efficiency with age is unlikely to arise from the loss of CreER expression. With the R26rtTA/CreER system we observe an age dependent silencing that can be inhibited by initiating doxycycline exposure – and presumably rtTA activated transcription – in early embryonic development.

These age-dependent phenomena likely represent progressive epigenetic changes in gene structure. A recent comparison of recombination efficiencies among the Z/IP, Z/EG, and R26R-EYFP loci in hematopoietic cells found a similar decline in recombination efficiency with age, as well as a strong correlation between lower recombination efficiency in adulthood and increased cytosine methylation, suggesting that age-dependent changes in chromatin structure can render loxp targets less accessible to Cre recombinase [46]. Consistent with our experience with the R26rtTA/CreER locus, studies of rtTA activation of target genes reveals a progressive silencing in neurons that can be overcome either by maintaining a basal level of target gene expression throughout development or by providing very high levels of rtTA [47].

In studies in which highly efficient Cre-mediated recombination is desirable, the use of a constitutively active Cre recombinase - even in combination with an inefficient loxp target such as Z/IP - usually suffices to produce complete or nearly complete recombination [8]. When temporal control of recombination is also required and the CreER system is used, obtaining highly efficient

Discussion

The two new mouse lines described here add to the growing list of genetically engineered mice available for controlled in vivo recombination and cell labeling using the Cre-lox system. The R26rtTA/CreER line extends the dynamic range of Cre-mediated recombination efficiencies relative to that of other loci with a constitutively expressed CreER, and the R26LIAP line complements the widely used Z/IP line by providing substantially higher efficiency Cre-mediated activation of AP. We note that Que and colleagues [40] have recently described the construction of a Rosa26 knock-in mouse line in which a hPLAP coding region is transcribed from a CMV enhancer/beta-actin promoter after Cre-mediated excision of a loxp-stop-loxp cassette.
recombination is more difficult and can require near-toxic doses of 4HT administered over multiple days [3].

By contrast, for sparse cell labeling experiments, the challenge is reversed: the problem is now to reduce the efficiency of 4HT-dependent Cre-mediated recombination and eliminate any background of 4HT-independent recombination. As shown in Figure 3A and B, when using CreER alleles with relatively high levels of expression (e.g. R26CreER) in combination with relatively efficient loxP targets (e.g. R26IAP), background recombination is substantial even in the absence of 4HT. Using a less efficient loxP target, such as Z/AP, reduces the recombination level so that there are few or no labeled cells in the absence of 4HT [10]. However, in our experience, the Z/AP locus is unusual in this respect, and most loxP target loci more closely resemble R26IAP in recombination efficiency. Long and Rossi [46] have suggested that the unusually inefficient recombination efficiencies of the Z/AP and

Figure 6. Morphologies of individual AP+ type 1 DA cells from the retinas of TH-IRESCreER;R26IAP mice. A single 100–300 μg 4HT injection was administered IP between P3 and P8; retinas were analyzed —6 weeks later. Retina flat mounts were processed for AP histochemistry and clarified in BBBA. Axon-like processes (green) and dendrites (blue) were traced with Neuromantic software. The soma is indicated by a red arrow. The outline of the flattened retina and the location of the optic disc are shown in red. (A) Twelve retinas, each of which had a single type 1 DA cell. (B) A retina with two type 1 DA cells. (C) Image of the boxed region of the retina shown in (B). The dendrites (lower right) are uniformly thicker and more darkly stained than the axon-like processes. (D) A putative type 2 DA cell. Scale bars in B and D, 500 μm. Scale bar in B applies to panels A and B. BBBA causes ~30% tissue shrinkage. doi:10.1371/journal.pone.0007859.g006
Z/EG loci reflect the high CpG content of the E. coli lacZ coding region, which is present as part of the beta-geo coding region in both loci and may promote somatic cytosine methylation and epigenetic silencing.

A second strategy for reducing recombination efficiency focuses on reducing CreER expression, either by using an IRES-CreER knock-in allele, which likely decreases translation inefficiency, or, as in the R26rtTAcreER locus, by overlaying a second level of transcriptional regulation. Both of these approaches for reducing CreER can eliminate background recombination in the absence of 4HT. Finally, combining both an inefficient loxP target (Z/AP) and low CreER expression reduces recombination to extremely low levels, such that fewer than one hundred neurons can be reproducibly labeled per brain depending on the IRES-CreER knock-in and the time and dose of 4HT [4].

Sparse Labeling of Defined Neuronal Types as a Tool to Survey Changes in CNS Structure

A method for visualizing the morphologies of a large and representative sampling of neurons of a genetically defined type has the potential to fill a hitherto vacant niche in neuro-anatomical analyses of brain structure. Historically, the analysis of neuronal morphology has been performed either by sparse labeling methods that are not cell type specific (e.g. the Golgi stain) or by filling individual neurons with a tracer using a whole cell patch electrode, a method that is extremely laborious and not easily adaptable as a survey tool [e.g. ref. 48]. These classic methods also suffer from the problem of incomplete filling of large and/or complex neuronal processes. At the other end of the spectrum with respect to the number of labeled cells, immunostaining for markers such as ChAT or TH reveals all of the cells of a given type, but the high density of labeled cells and their interdigitating processes generally preclude an assessment of individual morphologies. Moreover, the immunostaining approach is limited to those antigens that are relatively abundant, cytosolic, and cell-type specific. Thus, many molecular markers that distinguish neuronal types, such as transcription factors, or various low abundance or transiently expressed proteins cannot be used directly to reveal the morphologies of the cells in which they are [or were] expressed. While this last challenge is being partially addressed by the creation of BAC transgenic lines that express GFP or Cre in defined subsets of cells [e.g. refs 49,50], the challenge of visualizing individual neuronal morphologies can only be met by a method that generates a relatively sparse collection of labeled neurons.

The strategy of generating a sparsely labeled sampling of a single neuronal type – what we refer to here as an atlas of morphologies - is illustrated here for cholinergic and catecholaminergic neurons. The method takes advantage of the flexibility afforded by 4HT dosing to noninvasively generate a collection of several dozen to several hundred labeled neurons throughout the entire CNS. Current evidence suggests that the 1–2 month delay between Cre-mediated recombination and histologic analysis provides sufficient time for AP to uniformly populate the plasma membrane of all dendritic and axonal processes even among the largest neurons [4]. This approach could be used to survey the survival and morphology of defined classes of neurons in mouse models of a variety of CNS diseases or insults, including inherited disease, stroke, infection, drug toxicity, and sensory deprivation. For unilateral perturbations – such as a surgical lesion, localized drug injection, or recombinant virus injection – labeled cells ipsilateral and contralateral to the site of the manipulation can be compared.

The Morphologies of Type 1 DA Cells in the Mouse Retina

DA cells, first defined as dopamine-accumulating neurons and subsequently shown to express TH, have been extensively studied in a variety of species [22–25,51]. Their wide arbors and diffuse coverage likely reflect the role of dopamine as a neuromodulator that acts on a broad and still incompletely enumerated group of target cells to modulate retinal sensitivity. The best-characterized effect of dopamine in the retina is in mediating the light-dependent uncoupling of horizontal and amacrine cell gap junctions [reviewed in ref. 28]. Recent work suggests that retinal dopamine release is regulated by both ambient light level and by the circadian clock [29–32].

To the best of our knowledge, the full morphology of individual type 1 DA cells has not been previously reported in any species owing to uncertainties regarding the completeness of tracer filling. Dacey has estimated the diameter of the type 1 primate DA cell axon-like arbor at ~8–10 mm based on a quantitative analysis of arbor density, cell density, and the morphology of HRP filled cells [24]. The cell morphologies determined here indicate that the typical type 1 DA cell in the mouse retina has axon-like processes that extend both radially and circumferentially, forming a sparse arbor that generally encompasses a substantial fraction of the retinal area and has a total length of ~6.5 cm in a retina with a flat mounted diameter of ~5 mm (Figure 7; values corrected for tissue shrinkage in BBA). If the arbors were artificially straightened and directed radially, they would extend to a distance of at least several millimeters from the cell body (Figure 6). On the assumption that signals received by the dendrites of type 1 DA cells are transmitted along the full length of their axon-like arbors, then it would appear that activation of even a small number of these cells would produce a signal with a relatively uniformly spatial output across the retina, a property consistent with the known neuromodulatory functions of retinal dopamine.

In relation to the cell biological mechanisms that control the development of neuronal morphology, the large and irregularly shaped axonal arbors of the type 1 DA cells would appear to rule out any constraints on axonal trajectories based on homotypic signaling, as observed for axonal and dendritic arbors in Drosophila [52,53] and as postulated for a variety of cell types in the mammalian retina [54]. However, an interesting observation regarding type 1 DA cells is the very narrow length distributions of the dendrites and axon-like processes (Figure 7). These distributions suggest that there is a stringent control mechanism that regulates the total length of each class of neuritic process, even among cell types in which the branching pattern and coverage geometry is highly variable. The hypothesized control mechanism could, for example, reflect a genetic program that

![Figure 7. Quantification of axon-like processes and dendrite lengths for type 1 DA cells](image-url)
precisely controls the production of one or more molecules that are limiting for process formation.

**Methods**

**ES Cells and Mouse Husbandry**

Knock-in constructs were designed with inserts at the Xho I site at the Rosa26 locus as indicated in Figure 1A. ES cells were electroporated and colonies were selected in G418 and screened by Southern blot hybridization with the 5’ flanking probe shown in Figure 1A. Karyotypically normal ES cells were used for blastocyst injection. Following germline transmission of the targeted allele, the PGK-ino cassette of R26rTACreER was excised by crossing to mice expressing Flp in the germline. The R26rTACreER and R26IAP lines have been maintained as homozygous stocks on a mixed Sv129 x C57BL/6 background. Both lines have been deposited at the Jackson Laboratories and are freely available. Mice were handled and housed in accordance with the Johns Hopkins University Animal Care and Use Protocols and IACUC guidelines.

**Genotyping**

PCR primers for genotyping are as follows. R26IAP [sense strand, located in the first exon of AP: GGCAACGAGGT-CATCCTCCGTGATGAA; antisense strand, located in the intron in the inverted configuration: GTTGGCCTGGGGCTGACG-TAGTGCC; product size: 290 bp]; R26rTAoeCreER [sense strand: CCCTGGTGATCTGCCAATCCTAGTCGTC (= TB29); antisense strand: TAGAACGCGGTTAGTGCTG; product size: 700 bp]; WT [i.e. the untargeted] Rosa26 locus [sense strand: TB29, listed above; antisense strand: GGAGCGGGAGAATGATGAA; product size: 500 bp]. PCR was performed with Advantage 2 Polymerase (Clontech, La Jolla, CA) using 35 cycles with a 10 minute elongation at 72°C.

**His-NLS-Cre and Ad-Cre**

An E. coli expression plasmid encoding His-NLS-Cre [13] was a kind gift of Dr. H. Earl Ruley (Vanderbilt University). His-NLS-Cre expressed in E. coli was purified to >90% homogeneity by nickel chelate affinity chromatography. The protein was eluted in 500 mM imidazole, and concentrated and desalted by ultrafiltration against PBS supplemented with 150 mM NaCl. For IP injection of His-NLS-Cre, 100 μl of a 1.84 μg/μl stock was injected per adult mouse; for intraocular injection 2 μl of a 3.52 μg/μl stock was injected per eye at P5 using a 30 gauge needle. For IV injection into the tail vein, 400 μl of an Ad-Cre stock (3×10^7 plaque forming units/ml; Virenz, Baltimore, MD) was injected per adult mouse; for intraocular injection, 1.5 μl of the same virus stock was injected per eye at >P21 with a glass micro-needle. Tissues were analyzed >14 days later for IP His-NLS-Cre and IV Ad-Cre injections, and >10 days later for intraocular injections.

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**Tissue Processing**

Tissue processing and AP staining were performed essentially as described in ref. 10. For tissues other than eyes, mice were anesthetized and subjected to transcardiac perfusion with 4% paraformaldehyde in PBS. The brain, liver, or kidney was set in a block of 3% low-melting point agarase in PBS and a series of 300 μm sections were cut with a vibrotome. Segments of abdominal wall and stomach were processed intact. Retinas were dissected from eyes that had been immersion fixed in 4% paraformaldehyde in PBS, and were gently flattened with a plastic mesh. Tissues in PBS were heated in a water bath at 69°C for 90 minutes to inactivate endogenous AP and, for those embedded in low melting point agarase, to melt the agarase surrounding the tissue; heat-inactivated tissues were then reacted in NBT/BCIP for several hours to overnight at room temperature with gentle agitation. After washing and post-fixation, tissues were dehydrated with a graded ethanol series, and clarified in 2:1 benzylbenzoate:benzyl alcohol (BBBA). For long-term storage, samples were returned to ethanol.

**Microscopy and Neurite Tracing**

Bright-field images were captured on a Zeiss dissecting microscope equipped with OpenLab software, and bright-field and DIC images were captured on a Zeiss Axio Imager Z1 microscope equipped with a motorized stage and AxioVision software. For retina flat mounts, a montage of 5X images was obtained with the Zeiss Axio Imager Z1 microscope using an X-Y stage and the mosaics module and assembled using AxioVision software. For type 1 DA cells, in which dendrites and axon-like processes are confined to a single sublamina of the IPL, the AP-labeled neurites were traced in the X–Y plane without loss of information using Neuromantic software (www.rdg.ac.uk/neuromantic/). For coronal brain sections, Bregma positions and neuroanatomic structures were assigned as indicated in ref. 15.

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**Author Contributions**

Conceived and designed the experiments: TCB JN. Performed the experiments: TCB ZLH. Contributed reagents/materials/analysis tools: TCB ZLH PMS JW TR XY JN. Analyzed the data: TCB JN. Wrote the manuscript: TCB JN.
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