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A CreER Mouse to Study Melanin Concentrating Hormone Signaling in the Developing Brain

Staci E. Engle[1], Patrick J. Antonellis[1], Logan S. Whitehouse[1], Ruchi Bansal[1], Michelle R. Emond[2], James D. Jontes[2], Robert A. Kesterson[3], Kirk Mykytyn[4], and Nicolas F. Berbari[1*]

[1]Department of Biology, Indiana University-Purdue University Indianapolis, Indianapolis, IN 46202 USA.

[2]Department of Neuroscience, College of Medicine, Ohio State University, Columbus, OH 43210, USA.

[3]Department of Genetics, University of Alabama at Birmingham, Birmingham, AL 35294 USA

[4]Department of Biological Chemistry and Pharmacology, College of Medicine, Ohio State University, Columbus, OH 43210, USA.

*Correspondence:
Nicolas F. Berbari, Ph.D.
Indiana University-Purdue University Indianapolis
Department of Biology,
723 W. Michigan Street, Indianapolis, IN 46202
Phone: (317) 274-2896
FAX: (317) 274-274-0577
Email: nberbari@iupui.edu

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Abstract

The neuropeptide, melanin concentrating hormone (MCH), and its G protein-coupled receptor, melanin concentrating hormone receptor 1 (Mchr1), are expressed centrally in adult rodents. MCH signaling has been implicated in diverse behaviors such as feeding, sleep, anxiety, as well as addiction and reward. While a model utilizing the Mchr1 promoter to drive constitutive expression of Cre recombinase (Mchr1-Cre) exists, there is a need for an inducible Mchr1-Cre to determine the roles for this signaling pathway in neural development and adult neuronal function. Here, we generated a BAC transgenic mouse where the Mchr1 promotor drives expression of tamoxifen inducible CreER recombinase. Many aspects of the Mchr1-Cre expression pattern are recapitulated by the Mchr1-CreER model, though there are also notable differences. Most strikingly, compared to the constitutive model, the new Mchr1-CreER model shows strong expression in adult animals in hypothalamic brain regions involved in feeding behavior but diminished expression in regions involved in reward, such as the nucleus accumbens. The inducible Mchr1-CreER allele will help reveal the potential for Mchr1 signaling to impact neural development and subsequent behavioral phenotypes, as well as contribute to the understanding of the MCH signaling pathway in terminally differentiated adult neurons and the diverse behaviors that it influences.

Introduction

Originally discovered in fish as a pituitary secreted peptide involved in skin pigmentation (Kawauchi, Kawazoe, Tsubokawa, Kishida, & Baker, 1983), melanin concentrating hormone (MCH) in mammals has been found to be involved in a wide range of physiological processes including feeding behavior, energy homeostasis, sleep, anxiety, addiction and reward (Monzon, Varas, & De Barioglio, 2001; Qu et al., 1996; Tyhon et al., 2006; Willie, Sinton, Maratos-Flier, & Yanagisawa, 2008). The neuropeptide ligand, MCH, is produced solely by neurons in the lateral hypothalamus and zona incerta (Bittencourt et al., 1992), and in rodents acts on its only known G protein-coupled receptor, Mchr1, which is expressed in several regions throughout the brain (Saito, Cheng, Leslie, & Civelli, 2001).

One well-established phenotype of altered MCH or Mchr1 signaling is modulation of feeding behavior and energy homeostasis. Generally, overactivation of the pathway either pharmacologically or through genetic overexpression of MCH leads to an increase in food intake (Gomori et al., 2003; Ludwig et al., 1998; Ludwig et al., 2001; Qu et al., 1996; Rossi et al., 1997). In contrast, inactivation of the pathway through loss of ligand (Alon & Friedman, 2006; Shimada, Tritos, Lowell, Flier, & Maratos-Flier, 1998), receptor (Chen et al., 2002; Marsh et al., 2002), or antagonism leads to decreases in food intake and weight loss in rodents (Gennemark et al., 2017; Ito et al., 2010; Ploj et al., 2016; Shearman et al., 2003). Mchr1 is also expressed in the nucleus accumbens

and has the ability to modulate the response to drugs of abuse like cocaine (Chung et al., 2009; Tyhon et al., 2006) and alcohol (Duncan et al., 2007; Karlsson et al., 2016). In addition, MCH pathway antagonism can decrease sleep (Ahnaou, Dautzenberg, Huysmans, Steckler, & Drinkenburg, 2011; Willie et al., 2008), while MCH pathway activation can facilitate memory (Monzon et al., 1999; Varas, Perez, Monzon, & de Barioglio, 2002) and has anxiolytic effects in animal models (Kela, Salmi, Rimondini-Giorgini, Heilig, & Wahlestedt, 2003; Monzon et al., 2001).

Here we generated an inducible mouse Mchr1-CreER allele in order to assess the potential roles for Mchr1 signaling in neural development, as well as to have a means to acutely manipulate Mchr1 expressing neurons in adult animals.

Results and Discussion

The same BAC transgenic strategy (Lee et al., 2001) that was employed to generate the constitutive Mchr1-Cre allele (Chee, Pissios, & Maratos-Flier, 2013) was used to create the inducible Mchr1-CreER allele (**Supplementary Figure 1**). The transgene positive founder Mchr1-CreER mice were crossed with $ROSA^{LacZ}$ Cre reporter mice **(Supplementary Figure 1)** to assess inducibility and expression patterns. Offspring from founders #2 and #3 displayed active Cre with similar expression patterns (**Figure 1 and Supplementary Figure 2**). To assess adult neuronal populations expressing Mchr1-CreER activity, adult mice were induced with a five-day course of intraperitoneal injections of tamoxifen and then the reporter expression pattern in the brain was examined 3 days after the final injection. Table 1 summarizes the distribution and intensity of LacZ Cre reporter expression. Mchr1 expression was seen in several regions of the brain, most notably, the arcuate nucleus and the paraventricular nucleus

of the hypothalamus (**Figure 1a and b**). No staining in whole mount peripheral tissues was observed (data not shown). The expression pattern of adult induced Mchr1-CreER was directly compared with constitutively active Mchr1-Cre using the same LacZ reporter. Overall, a broader expression pattern was observed in Mchr1-Cre brains. However, the expression pattern in many nuclei, such as the zona incerta (**Figure 1c**), hippocampus (**Figure 1d**), pons (**Figure 1e**), and cerebellum (**Figure 1f**) remained similar in both lines. Interestingly, there was a greater amount of LacZ reporter expression in the arcuate nucleus (**Figure 1a**) and paraventricular nucleus (**Figure 1b**) of adult induced Mchr1-CreER mice compared to Mchr1-Cre mice. Furthermore, expression was nearly undetectable in the nucleus accumbens of Mchr1-CreER mice but present in Mchr1-Cre mice (**Figure 1g**). No reporter activity was observed in the brain of uninduced Mchr1-CreER mice or Cre negative littermates of Mchr1-Cre mice (**Figure 1h**).

To assess whether differences in expression pattern of the ROSA^{LacZ} reporter in Mchr1-CreER versus Mchr1-Cre mice may be due to changes in Mchr1 expression in perinatal development, we induced CreER activity at an earlier timepoint. On their day of birth, or postnatal day zero (P0), Mchr1-CreER mice were induced with a single tamoxifen injection followed by expression pattern analysis after they reached adulthood. The pattern of LacZ reporter present appeared similar to adult induced Mchr1-CreER mice (**Figure 1 and 2**) with one notable difference found in the nucleus accumbens. While not to the extent of Mchr1-Cre mice, expression was observed in the nucleus accumbens following P0 induction of Mchr1-CreER mice (**Figure 1g and 2g**), suggesting that perhaps expression of this receptor plays a role in early postnatal

development of this brain region. Alternatively, expression levels may be lower in adult accumbens. Another difference found between the P0 and adult paradigm was staining observed in the median eminence (**Figure 2a**). These findings raise the possibility that there could be a critical developmental window in which Mchr1 is produced in certain brain regions transiently during development and defects in this process may lead to behavioral phenotypes in adult animals.

To confirm the expression pattern analysis observed in the LacZ reporter line we utilized another Cre reporter by crossing Mchr1-CreER mice to a ROSA^{tdTomato} reporter mouse line. Mchr1-CreER/ROSA^{tdTomato} mice were induced as adults **(Supplementary Figure 1)**. TdTomato reporter expression was observed in similar regions as the LacZ reporter with one exception being strong tdTomato reporter expression in the nucleus accumbens **(Figure 3)**. Variability in labeling between different Cre reporter lines has been observed in other studies (Padilla, Reef, & Zeltser, 2012). The discrepancy we observe could be due to either poor Cre recombination efficiency or poor ROSA26 locus expression in the nucleus accumbens for the LacZ line. These types of observations have previously been observed in the brain (Casper, 2006) and this variability highlights the importance of understanding the expression pattern of new alleles.

Finally, to verify that Mchr1-CreER was expressed in a pattern consistent with Mchr1 expression, we compared reporter activity to Mchr1 mRNA using *in situ* hybridization with Mchr1 specific probes (**Figure 4**, left and middle panels). All regions showing tdTomato reporter expression were positive for Mchr1 mRNA, however the cerebellum and pons showed minimal labeling (**Figure 4e and f**), suggesting that these may be ectopic areas of expression in the Mchr1-CreER allele. Similar observations were also

reported and observed for the Mchr1-Cre allele (Chee et al., 2013). A probe for a non-mammalian gene (dihydrodipicolinate reductase, DapB) was used as a negative control **(Figure 4 a-g,** right panels**)**. A probe for a common housekeeping gene (Peptidylprolyl Isomerase B, PPIB) was used as a positive control probe and produced labeling throughout the brain, including the corpus callosum, a region not labeled by the Mchr1 probe (**Figure 4h**) and where no reporter staining was noted in either Mchr1-CreER or Mchr1-Cre mice.

In conclusion, the new Mchr1-CreER allele's inducible recombinase activity can be observed in several brain regions consistent with behavioral phenotypes associated with the pathway and expression pattern data. For the first time, we report novel brain regions showing Cre activity when induced on the day of birth, suggesting the potential for this pathway to be involved in neural development. Furthermore, these results suggest that some behavioral phenotypes observed in constitutive knockout and transgenic alleles of MCH or Mchr1 may be due to developmental perturbations in signaling. This model will be useful to determine both adult and developmental contributions to the diverse set of behavioral phenotypes associated with Mchr1 signaling.

Methods

Transgene Construct and Mouse Allele Generation

To produce the Mchr1-CreER transgene construct, the ~198 kb C57BL/6J mouse BAC clone (RP23-202N16) containing ~108 kb upstream of the Mchr1 open reading frame and ~86 kb after the stop codon was used. The $CreER^{T2}$ open reading frame was

inserted downstream of the Mchr1 promoter via homologous recombination/BAC recombineering as previously described (Lee et al., 2001), replacing nucleotides 4-41 from exon 1 of the Mchr1 gene. The targeting construct was generated using the following primers: GCAGCCTGCGTGGGTGGACGGGCGCTCCACTCCAGGGAGCAGGCGACCTGCAC CGGCTGCATGTCCAATTTACTGACCGT and GGACTCCAACTCGACTCACCCGCC AATGTGAAATTATCCTGGCCATCGGAGATGTTGCTGCCGCGTGTAGGCTGGAGCT G. An frt-flanked kanamycin selection cassette used during recombineering was removed by FLP expression. Proper recombination was confirmed by sequencing. The BAC was purified for subsequent male pronuclear injection. Mice were genotyped for Mchr1-CreER using 5'-GCAAACGGACAGAAGCATTT and 5'-GCGGTAGAGGAAGACCCTTT primers in the following PCR program: 95℃ for 2 minutes; 35 cycles of 95℃ for 15 seconds, 57℃ for 30 seconds, and 72℃ for 1 minute; 72℃ for 1 minute. The resulting founder lines were maintained as hemizygotes for the Mchr1-CreER BAC transgene. As such it is unknown if there are any phenotypes associated with mice homozygous for the Mchr1-CreER BAC transgene. The new Mchr1-CreER mouse will be available upon request.

Mice

All procedures were approved by the Institutional Animal Care and Use Committee at Indiana University Purdue University Indianapolis. Mice were housed on a standard 12-hour light dark cycle and given food and water ad libitum. Mice were weaned and housed with same-sex littermates after postnatal day 21. Ear punches were taken for genotype analysis by polymerase chain reaction.

Mchr1-CreER founders were compared to Mchr1-Cre mice (C57BL/6J-Tg(Mchr1-cre)1Emf/J, stock number 021582). Both Mchr1-CreER and Mchr1-Cre mice were crossed to Cre reporter lines, ROSA^{LacZ} (Gt(ROSA)26Sortm1Sor/J, stock number 003309 | R26R) or tdTomato (Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J, stock number 007909) (Jackson Labs; Bar Harbor, ME). Both ROSA^{LacZ} and ROSA^{tdTomato} only express the reporter upon Cre mediated recombination. Two founders showed robust reporter expression and were further characterized. Experiments utilized both male and female mice and no differences between sexes were noted.

CreER Induction

To induce CreER recombinase, cohorts were given intraperitoneal injections of 20 mg/ml tamoxifen (Sigma Aldrich, St. Louis, MO) dissolved in corn oil (Sigma Aldrich, St. Louis, MO). For adult induction, six-week old cohorts were given injections on 5 consecutive days at a dose of 150 mg/kg. For induction at P0, mice were given a single 50 µL injection.

Fixation and Tissue Processing for LacZ Staining

Samples were harvested when mice were 7 weeks old, 24-72 hours after the final tamoxifen injection for adult induced cohorts or 7 weeks after tamoxifen injection for P0 induced cohorts. Mice were anesthetized with 0.1 ml/ 10 g of body weight dose of 2.0% tribromoethanol (Sigma Aldrich, St. Louis, MO) and transcardially perfused with PBS followed by 4% paraformaldehyde (Affymetrix Inc., Cleveland, OH). Brains were postfixed in 4% paraformaldehyde for 4 hours at 4℃ and then cryoprotected by submersion in 30% sucrose in PBS for 16–24 hours. Cryoprotected brains were

embedded in Optimal Cutting Temperature compound (Fisher Healthcare, Houston, TX) and sectioned in a freezing cryostat at a thickness of 50 µm.

LacZ staining was carried out as previously described (Berbari et al., 2011). Sections were washed twice in PBS prior to three 10-minute washes in LacZ wash buffer (2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% nonidet-P40 in 100 mM sodium phosphate buffer, pH 7.3). Following washes, sections were incubated overnight at 37° in LacZ stain (2 mM MgCl2, 5 mM K-ferrocyanide, 5 mM K-ferricyanide, 2.45 mM x-Gal in PBS) Sections were then washed twice with PBS and mounted onto Superfrost Plus Microscope Slides (Fisher Scientific) prior to nuclear counterstaining.

Nuclear counterstaining was performed with Nuclear Fast Red. Slides were rinsed in MilliQ deionized water and then stained in 0.1% Nuclear fast red-aluminum sulfate solution (Merck, Darmstadt, Germany) for four to five minutes. Next, slides were washed with MilliQ deionized water and dehydrated through a series of ethanol and xylene washes (70% EtOH, 96% EtOH, 100% EtOH and xylene, 5 minutes each). Finally, the sections were coverslipped using Permount Mounting Media (Fisher Chemical, Pittsburg, PA).

Fixation and Tissue Processing for tdTomato

Brains were harvested when mice were 7 weeks old, 24-72 hours after the final tamoxifen injection. Mice were anesthetized by a 0.1 ml/10 g of body weight by i.p. injection of 2.0% tribromoethanol (Sigma Aldrich, St. Louis, MO) and transcardially perfused with PBS followed by a 1:1 mixture of 4% paraformaldehyde (Affymetrix Inc., Cleveland, OH) and Histochoice (Sigma Aldrich, St. Louis, MO). Brains were postfixed

in paraformaldehyde/histochoice overnight at 4℃ and then cryoprotected by submersion in 30% sucrose in PBS for 16–24 hours. Cryoprotected brains were embedded in Optimal Cutting Temperature compound (Fisher Healthcare, Houston, TX) and sectioned in a freezing cryostat at a thickness of 50 µm. Sections were stained with Hoechst nuclear stain (1:1000 in PBS) for 5 minutes at room temperature, washed with PBS, mounted onto slides, and coverslipped with Fluoro-Gel (Electron Microscopy Sciences, Harfield, PA).

In situ hybridization

Brains from C57BL/6J mice were harvested and fixed as described for LacZ staining. Sections were cut at a thickness of 15 µm and mounted directly on slides then post-fixed with 4% paraformaldehyde for 16 hours at 4℃.

Detection of transcripts in brain sections was performed using the RNAscope 2.5 HD Assay – BROWN kit (ACD). Tissue pretreatment was performed according to user manual no. 320534 and probe hybridization, counterstaining, and mounting of slides was performed according to user manual no. 322310-USM. Slides were assayed using probes to either positive control (Ppib), negative control (dapb) or MCHR1 transcripts (ACD). Sections were counterstained with Hematoxylin, dehydrated, and mounted using Cytoseal (Thermo Scientific).

Imaging

All samples were imaged using a Nikon Eclipse 90i microscope with Nikon Elements Advanced Research software v4.13.

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Figure Legends

Figure 1: LacZ reporter activity in the brains of adult Mchr1-CreER and Mchr1-Cre mice.

Representative images of Mchr1-CreER/ROSA^{LacZ} brain sections (a-g, left column) compared to constitutive Mchr1-Cre/ROSA^{LacZ} brain sections (a-g, right column). For Mchr1-CreER animals, adult induced activation of CreER was achieved via 5 days of tamoxifen injections starting when mice were 6 weeks old and brains were harvested when mice were 7 weeks old, 24-72 hours after the last tamoxifen injection. For Mchr1-Cre animals, brains were also harvested at 7 weeks old. 50 µm brain sections were cut and stained for LacZ. Negative controls include a section from an uninduced Cre positive Mchr1-CreER mouse (h, left panel) and Cre negative Mchr1-Cre littermate (h, right panel).

Neuroanatomical regions annotated include the third ventricle (V3), Cornu Ammonis 3 subfield of hippocampus (CA3), fourth ventricle (V4), granule layer (GL), anterior

commissure (ACO), and lateral ventricle (LV). LacZ staining is in blue and nuclei were counter stained with nuclear fast red. Scale bar indicates 100 µm. (induced Mchr1-CreER Cre positive n=8; induced Mchr1-CreER Cre negative (not shown) n=8; uninduced Mchr1-CreER Cre positive n=2; Mchr1-Cre Cre positive n=6; Mchr1-Cre Cre negative n=2)

Figure 2: Analysis of LacZ reporter activity in the brains of adult Mchr1-CreER mice after perinatal induction.

Representative images of Mchr1-CreER/ROSA^{LacZ} brain sections following Cre induction via a single tamoxifen injection at P0. Brains were harvested when mice were 7 weeks old and 50 µm brain sections were cut and stained for LacZ. Inset in (a) shows magnification of region inside box. Negative control represents a Cre negative Mchr1-CreER littermate (h).

Neuroanatomical regions annotated include the third ventricle (V3), Cornu Ammonis 3 subfield of hippocampus (CA3), fourth ventricle (V4), granule layer (GL), anterior commissure (ACO), lateral ventricle (LV), and median eminence (ME). LacZ staining is in blue and nuclei were counter stained with nuclear fast red. Scale bar indicates 100 µm, scale bar of inset indicates 10 µm. (induced Mchr1CreER Cre positive n=2, injected Mchr-CreER Cre negative n=2)

Figure 3: Analysis of tdTomato reporter expression in adult Mchr1-CreER mice.

Representative images of Mchr1-CreER/ROSA^{tdTomato} brain sections following adult induced activation of CreER via 5 days of tamoxifen injections starting when mice were 6 weeks old. Brains were harvested when mice were 7 weeks old, 24-72 hours after the last tamoxifen injection. 50 µm brain sections were cut and analyzed for tdTomato expression. Inset is a magnified image of the annotated box. Negative control represents a Cre negative Mchr1-CreER littermate (h).

Neuroanatomical regions annotated include the third ventricle (V3), Cornu Ammonis 3 subfield of hippocampus (CA3), fourth ventricle (V4), granule layer (GL), anterior commissure (ACO), and lateral ventricle (LV). TdTomato expression is in red. Scale bars indicate 100 µm. (induced Mchr1-CreER Cre positive n=5; induced Mchr1-CreER Cre negative n=1)

Figure 4: *In Situ* Hybridization for Mchr1 in adult mouse brain

Representative images of brain sections from adult wild-type mice following *in situ* hybridization using a Mchr1 specific probe (a-h, left and middle columns; middle panel represents a magnified image of the annotated box, insets in the middle column are from individual cells in that panel). A dapB probe was used as a negative control (a-g, right column) and a Ppib probe was used as a positive control probe (h, right).

Neuroanatomical regions annotated include the third ventricle (V3), Cornu Ammonis 3 subfield of hippocampus (CA3), fourth ventricle (V4), granule layer (GL), anterior commissure (ACO), and lateral ventricle (LV). Probe staining in brown with haematoxylin counter stain. Scale bar in left column indicates 100 µm, scale bars in middle and right column indicate 10 µm. (n=3)

Supplementary Figure 1: Allele schematic

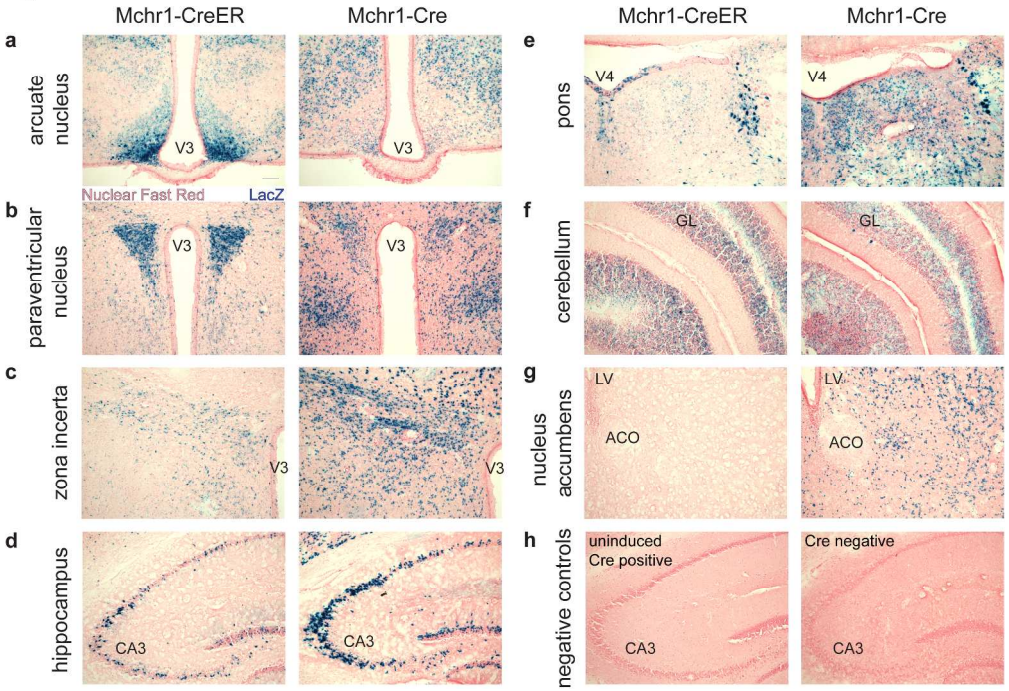
Schematic of Cre and Cre reporter alleles used throughout the paper including (a) inducible BAC transgenic Mchr1-CreER, (b) constitutive BAC transgenic Mchr1-Cre, (c) LacZ Cre reporter ROSA^{LacZ}, and (d) fluorescent tdTomato Cre reporter ROSA^{dTomato}.

Supplementary Figure 2: Adult LacZ reporter activity of a second Mchr1-CreER founder line.

Representative images illustrating that offspring of Founder 2 have a similar expression pattern as Founder 3 used in other figures. Mchr1-CreER/ROSA^{LacZ} brain sections following adult induced activation of CreER via 5 days of tamoxifen injections starting when mice were 6 weeks old. Brains were harvested when mice were 7 weeks old, 24-72 hours after the last tamoxifen injection. 50 µm brain sections were stained for LacZ reporter. Negative control is from a Cre negative Mchr1-CreER littermate (h).

Neuroanatomical regions annotated include the third ventricle (V3), Cornu Ammonis 3 subfield of hippocampus (CA3), fourth ventricle (V4), granule layer (GL), anterior commissure (ACO), and lateral ventricle (LV). LacZ staining is in blue with nuclear fast red counter stain. Scale bar indicates 100 µm.

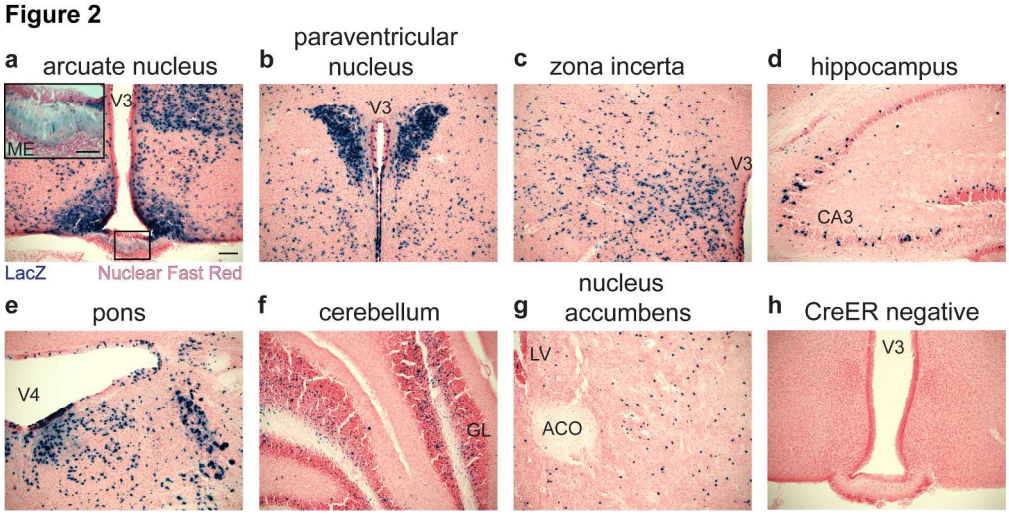
Figure 1



LacZ Reporter Activity in Brains of Adult Mchr1-CreER and Mchr1-Cre Mice

266x190mm (300 x 300 DPI)

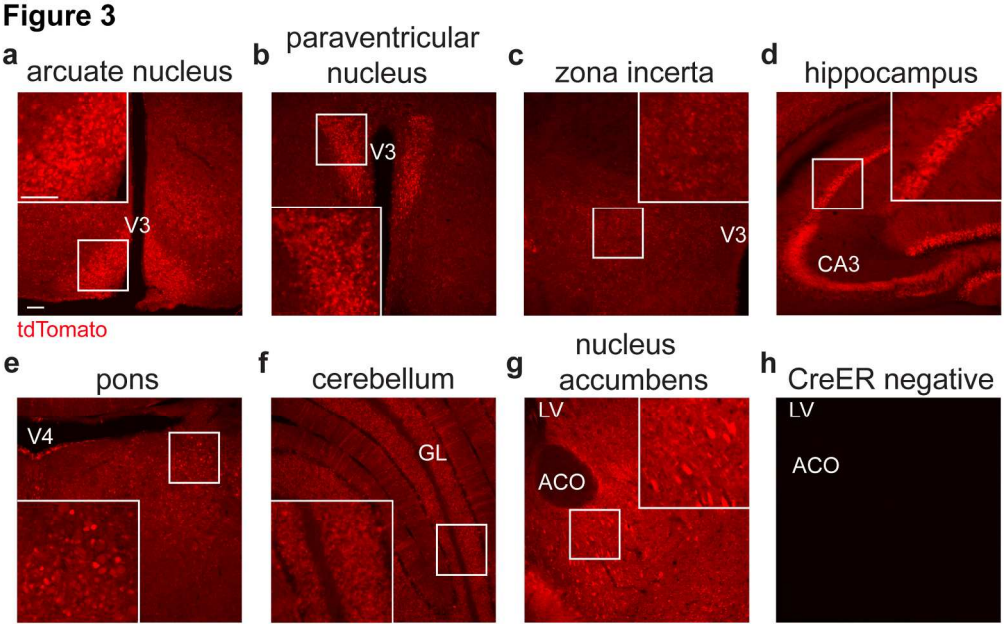
Figure 2



Analysis of LacZ Reporter Activity In Brains of Mchr1-CreER Mice After Perinatal Induction

227x115mm (300 x 300 DPI)

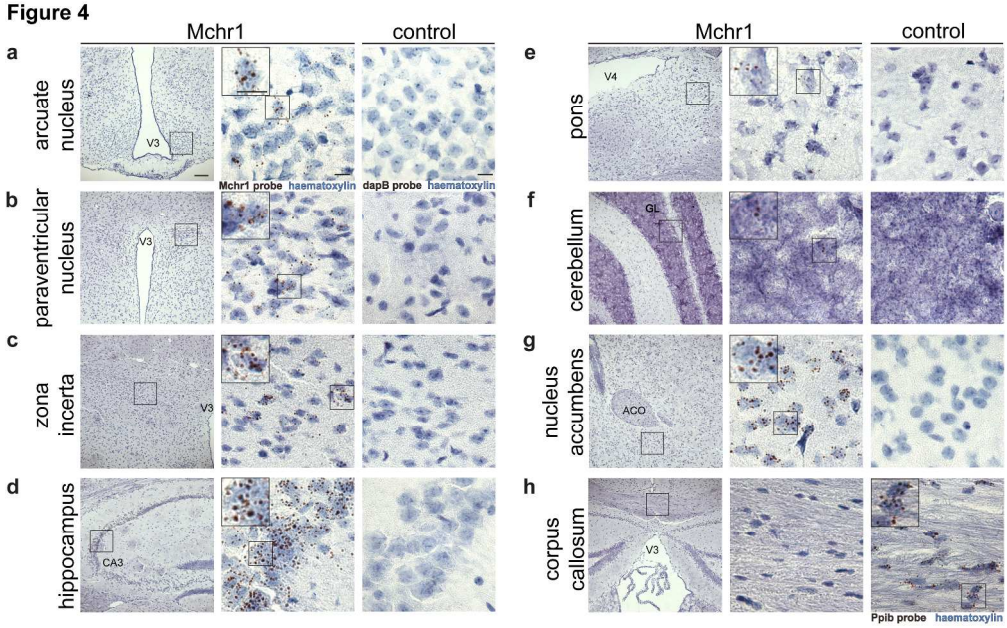
Figure 3



Analysis of tdTomato reporter expression in adult Mchr1-CreER mice

193x120mm (300 x 300 DPI)

Figure 4



In Situ Hybridization for Mchr1 in adult mouse brain

265x169mm (300 x 300 DPI)

Table 1: Distribution of LacZ Reporter Expression

Brain Region	Relative abundance of LacZ reporter		
	Mchr1-creER		Mchr1-cre
	adult induced	P0 induced	constitutive
Cerebral cortex			
Isocortex	+	+	++
Olfactory areas	+	+	++
Hippocampus			
CA1 pyramidal cell layer	++	+	++
CA2 pyramidal cell layer	+++	++	+++
CA3 pyramidal cell layer	+++	++	+++
Dentate gyrus granule cell layer	++	+	++
Striatum			
Caudoputamen	+/-	+	+++
Globus pallidus	+/-	+/-	+
Nucleus accumbens	+/-	+	++
Thalamus	+/-	+/-	++
Hypothalamus			
Arucate nucleus	+++	+++	+
Paraventricular nucleus	+++	+++	+
Median eminence	-	+	-
Zona incerta	+	+	+
Ventromedial nucleus	+/-	+/-	++
Dorsomedial nucleus	++	++	++
Lateral hypothalamic area	+	+	+
Periventricular hypothalamic nucleus	++	+	+
Mammilary nucleus			
Supramammillary nucleus	+	+	+/-
Medial mammillary nucleus	-	+/-	++
Lateral mammillary nucleus	+/-	+/-	++
Midbrain			
Ventral tegmental area	+	+	++
Substantia nigra	-	+	++
Periaqueductal gray	+/-	+	++
Interpeduncular nucleus	+/-	+	+/-
Pons and Medulla			
Dorsal tegmental nucleus	++	++	++
Laterodorsal tegmental nucleus	+	+	++
Locus ceruleus	++	++	++
Motor nucleus	++	++	++
Facial motor nucleus	++	++	++
Cerebellum			
Granule layer	++	++	++
Molecular layer	-	-	-

The relative amount of LacZ reporter in each brain region designated by:
-, no LacZ reporter expression
+/-, sparse LacZ reporter expression
+, low density of LacZ reporter expression
++, moderate density of LacZ reporter expression
+++, high density of LacZ reporter expression