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TECHNOLOGY REPORT

Generation of a *KOR-Cre* Knockin Mouse Strain to Study Cells Involved in Kappa Opioid Signaling

Xiaoyun Cai,[1] Huizhen Huang,[1,2] Marissa S. Kuzirian,[1] Lindsey M. Snyder,[1] Megumi Matsushita,[3] Michael C. Lee,[1] Carolyn Ferguson,[4] Gregg E. Homanics,[4] Alison L. Barth,[3] and Sarah E. Ross[1]*

[1]Department of Neurobiology and the Pittsburgh Center for Pain Research, University of Pittsburgh, Pittsburgh, Pennsylvania

[2]Tsinghua University School of Medicine, Beijing, China

[3]Department of Biological Sciences and Center for the Neural Basis of Cognition, Carnegie Mellon University, Pittsburgh, Pennsylvania

[4]Departments of Anesthesiology and Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania

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Summary: The kappa opioid receptor (KOR) has numerous important roles in the nervous system including the modulation of mood, reward, pain, and itch. In addition, KOR is expressed in many non-neuronal tissues. However, the specific cell types that express KOR are poorly characterized. Here, we report the development of a *KOR-Cre* knockin allele, which provides genetic access to cells that express KOR. In this mouse, *Cre recombinase* (*Cre*) replaces the initial coding sequence of the *Opkr1* gene (encoding the kappa opioid receptor). We demonstrate that the *KOR-Cre* allele mediates recombination by embryonic day 14.5 (E14.5). Within the brain, *KOR-Cre* shows expression in numerous areas including the cerebral cortex, nucleus accumbens and striatum. In addition, this allele is expressed in epithelium and throughout many regions of the body including the heart, lung, and liver. Finally, we reveal that *KOR-Cre* mediates recombination of a subset of bipolar and amacrine cells in the retina. Thus, the *KOR-Cre* mouse line is a valuable new tool for conditional gene manipulation to enable the study of KOR. genesis 54:29–37, 2016. © 2015 Wiley Periodicals, Inc.

Key words: genetics; opioid; OPRK1; KOPr; Cre-loxP system

INTRODUCTION

The kappa opioid receptor (KOR; also known as KOPr, Oprk1) and its endogenous ligand dynorphin are a modulatory system with numerous roles in the nervous system, including the regulation of mood, reward, aversive somatosensation, and cognitive function (Butelman *et al*., 2012; Cahill *et al*., 2014; Crowley and Kash, 2015; Lalanne *et al*., 2014). Like other members of the opioid subfamily, KOR is a seven transmembrane receptor that couples to the inhibitory G-protein, Gαi/o. As a result, KOR signaling generally inhibits cell activity by decreasing cyclic AMP, reducing calcium currents, modulating kinase pathways, and/or increasing potassium conductance (Bruchas and Chavkin, 2010; Piros *et al*., 1996). In neurons, this signaling results in decreased cell firing and reduced neurotransmitter release. There is now good evidence that modulating the dynorphin/KOR system has clear therapeutic potential. In particular, kappa agonists have therapeutic efficacy for the treatment of itch and pain (Inui, 2015), whereas kappa antagonists are currently being investigated for their role in the treatment of addiction and relapse (Carroll

Additional Supporting Information may be found in the online version of this article.

X. Cai and H. Huang contributed equally.

*Correspondence to: Dr. S.E. Ross, Department of Neurobiology, University of Pittsburgh, 200 Lothrop Ave. Pittsburgh, PA 15217.
E-mail: saross@pitt.edu

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and Carlezon, 2013). In addition, the plant derivative Salvinorin A, a recreational drug that causes spatiotemporal dislocation, mediates its effects via activation of KOR (Chavkin *et al*., 2004; Roth *et al*., 2002). Thus, KOR signaling is pertinent to many aspects of human health. However, the cellular targets and circuit-based mechanisms underlying the effects of kappa opioids are poorly understood. This gap in our understanding highlights the need for the development of new tools to visualize and manipulate KOR-expressing cells.

Previous work from our lab and others′ has shown that one of the key roles of kappa agonists is the inhibition of itch (Inan *et al*., 2009; Kardon *et al*., 2014; Togashi *et al*., 2002). In particular, we characterized a population of dynorphin-expressing inhibitory interneurons in the dorsal horn of the spinal cord, and provided evidence that these cells function to inhibit itch in part through the release of dynorphin (Kardon *et al*., 2014; Ross *et al*., 2010). Moreover, we found that modulation of KOR signaling in the spinal cord bidirectionally alters itch tone—kappa agonists reduce itch—whereas antagonists exacerbate itch. Finally, we provided evidence that kappa agonists can selectively reduce itch and not pain (Kardon *et al*., 2014). Both spinal interneurons and primary afferents (which innervate the spinal cord) are known to express KOR (Arvidsson *et al*., 1995). However, the specific interneurons and afferent subtypes that express this receptor are unknown. As a result, the cellular target through which dynorphin inhibits itch remained unclear. To begin addressing this question, we pursued a genetic approach—the generation of a *KOR-Cre* allele—with the hope of labeling KOR-expressing cells in mice.

In addition to the inhibition of itch, KOR signaling has numerous other roles that have been studied in detail. For instance, dynorphin promotes stress-induced anxiety and dysphoria, and blocking KORs with antagonists can reduce these stress-induced emotional responses (Chavkin, 2011; Land *et al*., 2008). Also, KORs in the ventral tegmental area (VTA) regulate locomotion, as inhibition of KORs in the VTA decreases locomotion (Vardy *et al*., 2015). Kappa agonists act on the pituitary to trigger the release of prolactin. KOR signaling is also important in the periphery, and KOR is expressed in the heart, vasculature, and gut (Pugsley, 2002; Yamamizu *et al*., 2015; Zhu *et al*., 1998). *KOR* and *dynorphin* null mice have an increase in vascular formation during embryogenesis (Yamamizu *et al*., 2015).

Given the diverse nature of KOR functions, we thought that tools to study KOR function would be of broad interest. Thus, we developed a *KOR-Cre* knockin mouse with the goal of visualizing and manipulating cells that express KOR. Here, we describe the generation of this mouse line and we characterize the Cre-mediated recombination that is observed when this mouse is crossed with a Cre-responsive reporter, which is predicted to mark all of the cells with current or a history of KOR expression.

RESULTS AND DISCUSSION

To gain genetic access to cells that express (or have expressed) KOR, we targeted Cre recombinase into the endogenous *KOR* locus (*Oprk1*). Cre is a recombinase that enables conditional expression of reporter or effector alleles using the Cre/loxP system. To target the KOR locus, fusion PCR was used to replace the coding region of the *OPRK1* gene in exon 2 with that of *Cre Recombinase* followed by a strong polyadenylation sequence. The targeting vector PL450-nCrepA (Taniguchi *et al*., 2011), featured a FRT-flanked PGK-neomycin positive selection cassette (*Neo*) downstream to the 3'UTR (Fig. 1A). Linearized targeting vectors were electroporated into Sv129 mouse ES cells and G418-resistant clones were screened for homologous recombination at the *KOR* locus by PCR (Fig. 1B). The neomycin cassette was removed *in vivo* via Flp recombinase. Finally, Southern blotting was performed to confirm correct targeting into the genome (Fig. 1C). *KOR-Cre* heterozygous mice gave rise to offspring of the expected ratio, as confirmed by genotyping PCR (Fig. 1D).

To analyze the pattern of recombination in *KOR-Cre* mice, we used the Cre-dependent allele $Rosa^{lsl\ tdTomato}$ (also known as *Ai14*; Jax Labs). Since KOR mRNA is known to be expressed in the brain at embryonic day 14 (E14) (Zhu *et al*., 1998), we began our analysis of Cre-mediated recombination at E14.5. At this time there is recombination in many regions of the embryo (Fig. 2A). We also analyzed several regions at higher magnification, where we observed recombination in subsets of cells including the liver (Fig. 2B,C), dorsal root ganglia (DRG; Fig. 2D), heart (Fig. 2E), lung (Fig. 2F), epithelium of the branchial arches (Fig. 2G), and eye (Fig. 2H). We find that the tdTomato signal is specific since it is not observed in a control littermate that lacks the *KOR-Cre* allele (liver; Fig. 2C). Previously, we reported that kappa opioid signaling in the spinal cord inhibits itch. We find that *KOR-cre* causes recombination in ~30% of spinal neurons in the dorsal horn (data not shown) as well as ~25–30% of primary somatosensory neurons in developing (Fig. 2D) and adult (data not shown) mice. Which, if any, of these cells is responsible for the inhibition of itch by kappa agonists is unknown.

To better visualize *KOR-Cre* mediated recombination in the nervous system, we took serial sections of the brain of a *KOR-Cre;* $Rosa^{lsl\ tdTomato}$ mouse in the coronal plane. Some representative sections are shown (Fig. 3) and the entire set is available in the supplementary data. We observe very robust recombination in both the caudate putamen and the nucleus accumbens (Fig. 3A). Higher magnification of the caudate putamen suggests that KOR is expressed in many, but not all cells in this region. *KOR-Cre* causes recombination in a layer and region specific manner in neocortex (Fig. 3). For instance, in the somatosensory

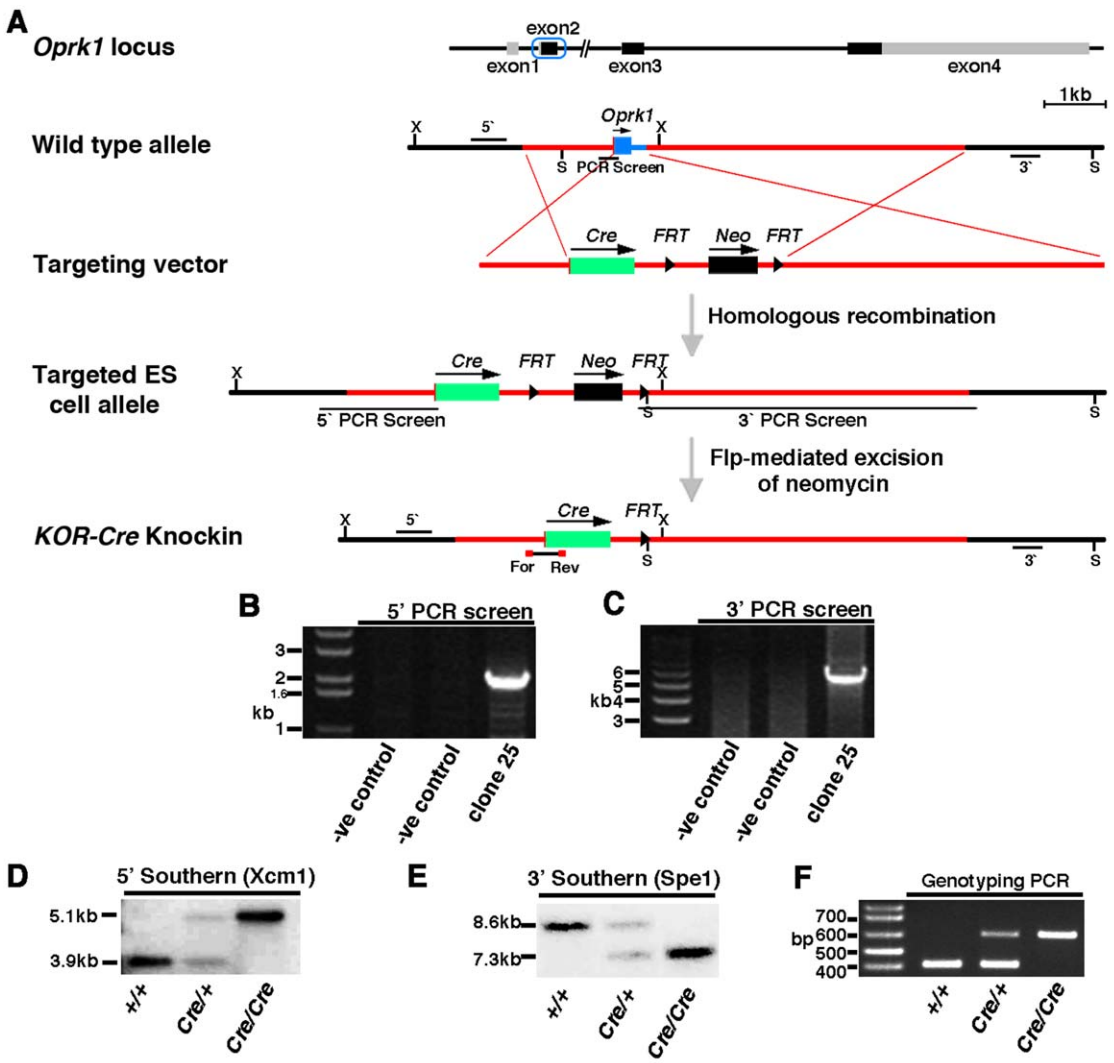

FIG. 1. Generation of a *KOR-Cre* knockin mouse. A. Targeting of *Oprk1*. The *KOR-Cre* KI targeting vector was constructed by subcloning ~1.6 kb 5' of exon 2 translation initiation ATG and ~5.2 kb 3' of exon 2 into the PL450-nCrepA vector (Taniguchi *et al*., 2011), containing the Cre recombinase gene and a FRT-flanked neomycin (neo) cassette. Open reading frames are indicated with arrows and dark shaded regions within rectangles for *Oprk1* (blue) and Cre (green). FRT sites are indicated with black triangles. PCR products are marked with black bars. Forward (For) and Reverse (Rev) primers used for genotyping are marked by red boxes, as indicated, on either side of the PCR product. S: SpeI restriction site; X: XcmI restriction site; Neo: neomycin positive selection cassette. B-C. PCR-based screening for homologous recombination at the 5' (B) and 3' arms (C). PCR products or appropriate size are observed upon screening clone 25. D-E. Southern blot analysis of XcmI-digested (D) or SpeI-digested (E) genomic DNA from mice that are wild type (+/+), heterozygous (*KOR-Cre*/+) or *KOR-Cre* mutant (*KOR-Cre*/*KOR-Cre*) using the 5' or 3' probes, respectively, results in fragments of the predicted sizes, indicating correct targeting. F. PCR to amplify the junction between the 5' or 3' UTR and the coding region of either the *Oprk1* gene or the *KOR-Cre* KI gene. Genotyping PCR products of genomic DNA isolated from tails of mice that are wild type (+/+), heterozygous (*KOR-Cre*/+), or mutants (*KOR-Cre*/*KOR-Cre*).

cortex, tdTomato is observed in layers 4 and 6 [Fig. 3B, Bi (somatosensory cortex 20x)] in neurons that have apical dendrites characteristic of pyramidal neurons (Fig. 3Bii; somatosensory cortex 60x). *KOR-cre* also causes recombination in the insular cortex (Fig. 3B; asterisk), consistent with previous reports. The role of KOR signaling in the neocortex is not well understood, but these receptors may underlie the altered perception of space and time that is induced by Salvinorin A (Chavkin, 2011; Roth *et al*., 2002). Another region that shows pronounced expression of tdTomato is the substantia nigra (Fig. 3C, Ci, Cii, and D), which is a brain structure in the midbrain that plays important roles in both reward and movement (Isomura *et al*., 2013). Here, tdTomato is expressed in the pars reticulata of the substantia nigra, which serves as an output from the basal ganglia (Isomura *et al*., 2013). Interestingly, *KOR-Cre* mediated recombination in the substantia nigra is observed in axon tracts that are likely from cell bodies in the basal ganglia (Fig. 3Ci and Cii).

In general, we observe *KOR-cre* mediated recombination in brain regions that previously have been reported to express KOR. Thus, we find tdTomato expression in

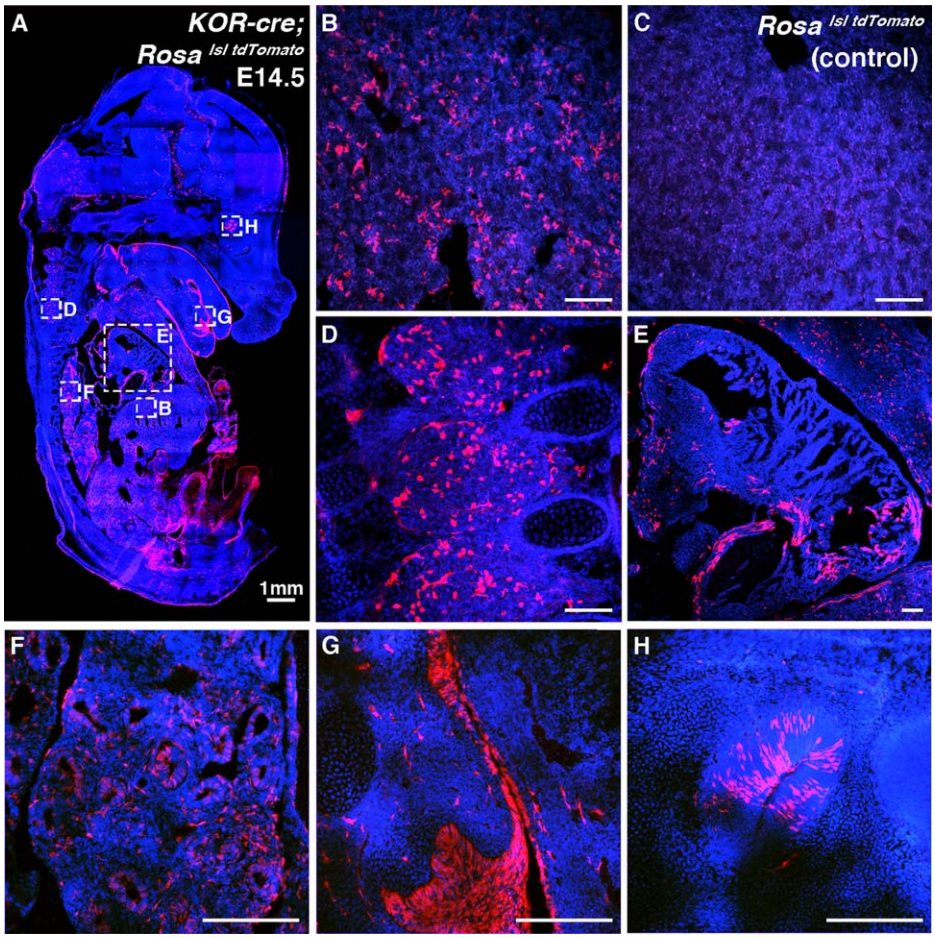

FIG. 2. *KOR-Cre* mediated recombination in the mouse embryo at E14.5. A. Sagittal section of a *KOR-Cre; Rosa*$^{lsl\ tdTomato}$ mouse at E14.5 (tiled 20x images; scale bar = 1 mm). Regions in boxes are shown at higher magnification in B-H. B-C. Magnification of tdTomato expression in liver of *KOR-Cre; Rosa*$^{lsl\ tdTomato}$ (B) compared to the same region in a control littermate lacking *KOR-cre* (C). D-H. TdTomato expression in dorsal root ganglia (D), heart (E), lung (F), epithelium of the branchial arches (G), and eye (H). All images were acquired with a 20x objective with a confocal microscope. For B-H, scale bar = 100 μm.

the anterior olfactory nucleus, olfactory tubercle including the islands of Calleja, caudate putamen, the lateral septum, the thalamus, the claustrum, the hypothalamus, the amygdala, nucleus accumbens, superior colliculus, substantia nigra, central gray, the ventral tegmental area, the brainstem including the raphe, the dorsal cochlear nucleus, and the trigeminal nucleus (Fig. 3 and Supporting Information). These findings are consistent with previous reports describing the distribution of KOR mRNA and protein (Arvidsson *et al.*, 1995; Mansour *et al.*, 1994; Zhu *et al.*, 1998). However, it should be noted that we see a somewhat broader distribution of *KOR-cre* mediated recombination than one would expect based on the mRNA transcript expression seen in an adult mouse. For instance, whereas KOR mRNA was reported in a limited number of cortical areas (Mansour *et al.*, 1994), we find *KOR-cre* mediated recombination in many regions of the cortex, including the anterior cingulate, motor, somatosensory, visual and insular cortices. Whether this discrepancy reflects the recombination of cells with very low levels of KOR expression or cells with a developmental history of KOR expression remains unknown.

Opioids also play an important role outside of the nervous system. Previous work suggests that KOR acts to inhibit the growth of ventricular myocytes and the heart during development (Wang *et al.*, 2004). Consistent with this, we observed that *KOR-Cre* mediates recombination in the neonatal heart (Fig. 4A). In adult animals, KOR regulates arrhythmias and protects against severe ischemia (Schultz and Gross, 2001; Wu *et al.*, 1999). Our knowledge of KOR expression in the heart is based primarily on studies using agonist-binding assays, which reveal strong KOR expression in the right atrium (Krumins *et al.*, 1985; Pugsley, 2002; Tai *et al.*, 1991; Zhang *et al.*, 1996). Here, we show that the *KOR-Cre* allele causes recombination in subsets of cells throughout the heart. In both neonatal (P1) and adult mice, the

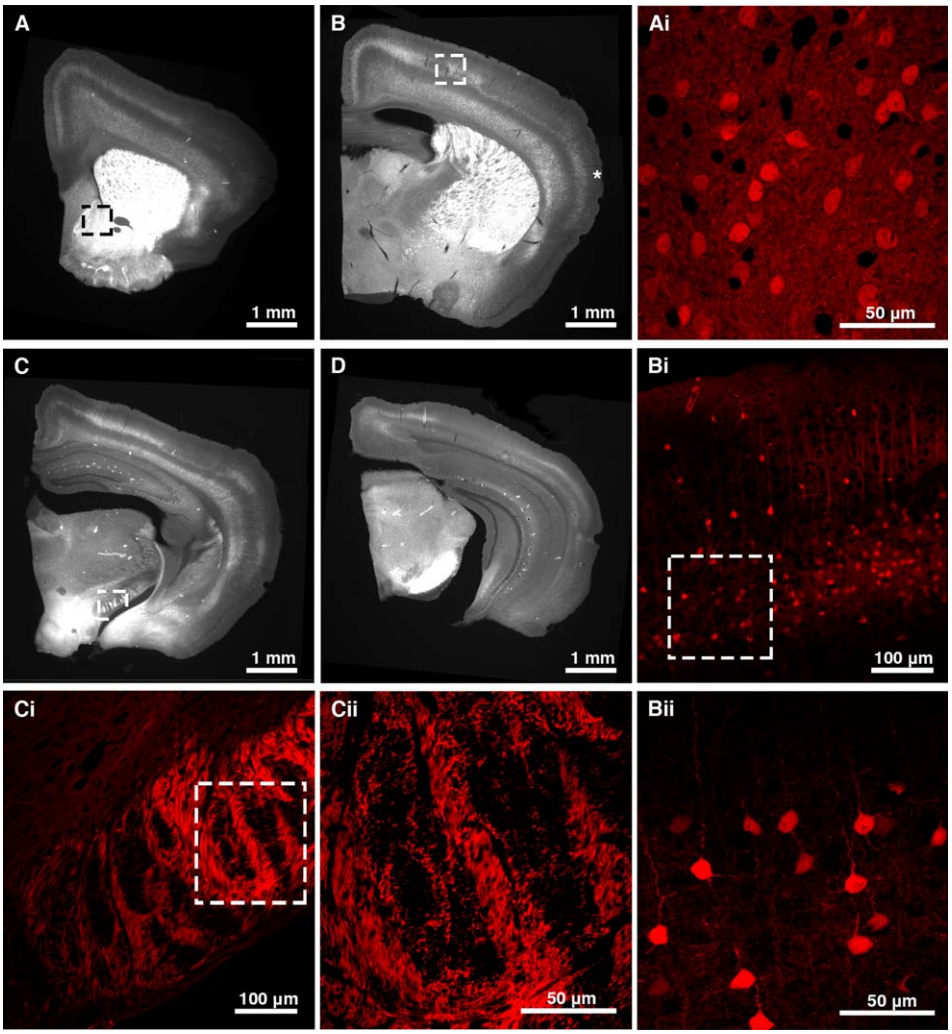

FIG. 3. *KOR-cre* mediated recombination in the adult mouse brain. Coronal sections through the brain, moving anterior (A) to posterior (D) in an adult *KOR-Cre; Rosa*$^{lsl\ tdTomato}$ mouse. Scale bar = 1 mm. * marks insular cortex. Ai. 60x confocal image of the region highlighted in A by the dashed box, visualizing cells in the nucleus accumbens. Scale bar = 50 µm. Bi. 20x confocal image from the region highlighted, in B, by the dashed box, showing tdTomato expression in the somatosensory cortex. Scale bar = 100 µm. Bii. Further magnification (60x) of the region in Bi showing tdTomato expression in pyramidal neurons. Scale bar = 50 µm. Ci. 20x confocal image of the substantia nigra as seen in C. Scale bar = 100 µm. Cii. *KOR-cre*-positive fibers in the substantia nigra (60x; scale bar = 50 µm). Sections through the entire mouse brain are available in the Supporting Information.

Cre is expressed the myocardium and endocardium of both the right and left ventricles (Fig. 4). Cre is also expressed in the atrioventricular valve (Fig. 4 Ciii).

Kappa agonists are known to be diuretics. Some of this effect is likely mediated via inhibition of vasopressin release (Floyd *et al*., 2009). However, bilateral adrenal demedullation significantly attenuates the diuretic response to KOR agonists (Ashton *et al*., 1989; Blackburn *et al*., 1986; Borkowski, 1989), raising the possibility that kappa agonists act, at least in part, on cells in the adrenal medulla. Autoradiography binding assays (Quirion *et al*., 1983) as well as RT-PCR and Northern analysis (Wittert *et al*., 1996) suggest KOR expression in the kidney and adrenal gland. Consistent with this, we see *KOR-Cre* mediated recombination in the kidney as well as adrenal glands, both in the cortex and medulla (Fig. 5A, B).

Previous work suggested that KOR is expressed in the retina (Husain *et al*., 2009), but the specific cell types were unknown. To address this question, we analyzed the expression of a ChR2-eYFP fusion reporter (*Rosa*$^{lsl\ ChR2\text{-}eYFP}$; also known as *Ai32*; Jax lab) upon *KOR-Cre* mediated recombination. Because eYFP is fused to channel rhodopsin, this protein is localized to the membrane, enabling a clear view of the neuronal processes. We find that *KOR-Cre* causes recombination in several retinal cell types, giving rise to two prominent bands of innervation in the inner plexiform layer (Fig. 6A). To identify which sublaminae are stratified by *KOR-Cre* expressing cells, we co-staining with the layer-specific marker, ChAT.

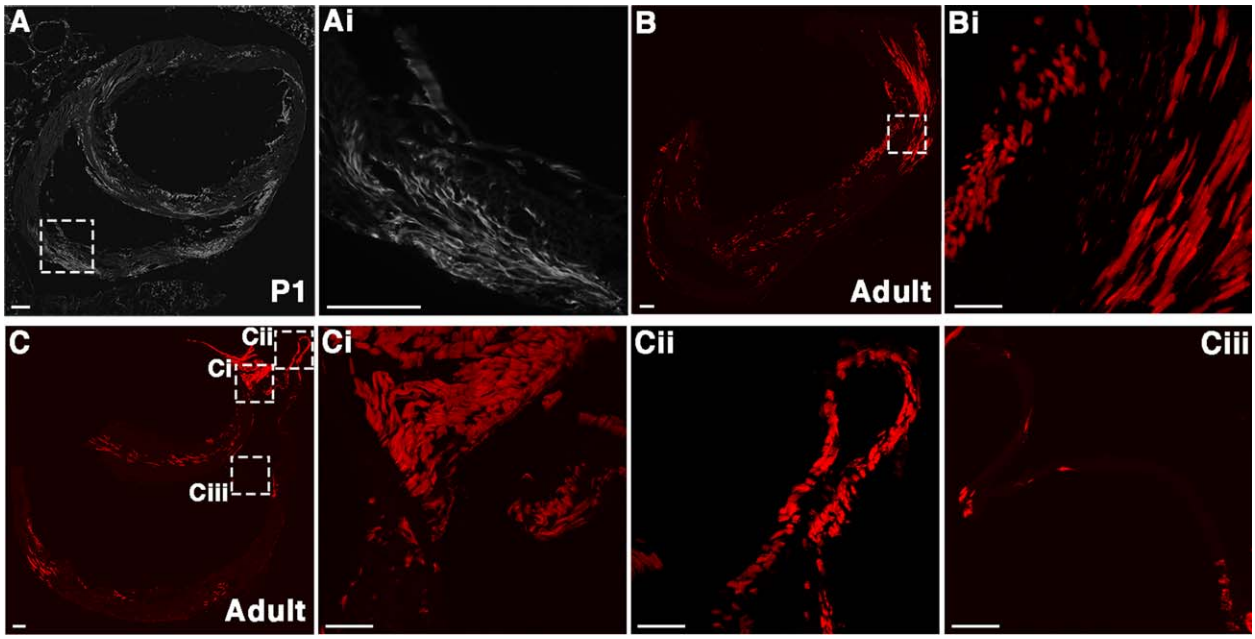

FIG. 4. *KOR-cre* mediated recombination in the heart. (A) *KOR-cre* mediated recombination in the heart at post-natal day 1 (P1; *KOR-Cre; Rosa*$^{lsl\ tdTomato}$). Ai. Magnification of the wall of the right ventricle (20x). TdTomato is expressed in the myocardium and endocardium. (B) TdTomato is expressed in the heart wall of the adult *KOR-Cre; Rosa*$^{lsl\ tdTomato}$ mouse. Bi. Magnified view of the wall of the left ventricle in B (20x). (C) TdTomato is expressed in the wall of atrium in adult mouse. Ci, Cii. Magnified view of right atrium wall and Ciii. Atrioventricular valve from C (20x). Scale bar = 50 μm in all images.

This analysis revealed that *KOR-Cre*-recombined cells target predominantly layers 1 and 5 of the IPL (Fig. 6B). Based on their location within the inner plexiform layer, *KOR-Cre* appears to cause recombination of both bipolar cells and amacrine cells. To investigate this idea further, we co-stained with the amacrine cell marker, Pax6. These co-labeling experiments revealed that numerous *KOR-Cre* recombined cells are Pax6-positive, indicating they are amacrine cells (34% of amacrine cells show *KOR-cre* mediated recombination; Fig. 6C–E). Thus, it is likely that Cre expressing cells in the retinal ganglion layer are displaced amacrine cells, rather than retinal ganglion cells. Together these findings indicate that *KOR-Cre* causes recombination in at least two retinal subtypes: bipolar cells and amacrine cells, which primarily stratify in layers 1 and 5 of the inner plexiform layer.

In summary, we show that the *KOR-cre* knockin mouse line causes recombination in a number of cell types throughout the body, including many brain regions that have been reported to express KOR (Mansour *et al.*, 1994). Thus, this allele may be useful for conditional manipulation of cells that mediate KOR signaling.

MATERIALS AND METHODS

Animal Husbandry

KOR-Cre mice were generated as described below and genotyped using the following primers: F: GGTGAATCC AAGCAAGATAAACGG; R: CAGCACAGGTAGGTCAGCTC.

Mice were given free access to food and water and housed under standard laboratory conditions. The use of animals was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Generation of *KOR-Cre* Knockin Mouse

The targeting vector was constructed from 129s6/SvEvTAC mouse genomic fragments, which were amplified by PCR and sequenced. The 5′ and 3′ homologous arms were PCR amplified from genomic DNA using the following primers: 5′ arm (F: TAAAAGACCCGTCCCT TGTG and R: GTTCACGATGTCGAAGCTCA) and the 3′ arm (F: GGGGAACTTCCTGACTAGGG and R: CTTGTG AAGCCTGCAAAACA). To replace the coding region of the *OPRK1* gene in exon 2 with that of *Cre Recombinase* followed by a strong polyadenylation sequence. This manipulation was confirmed by restriction digest and sequencing. These arms, along with the fusion product, were subcloned into the targeting vector (PL450-nCrepA) (Taniguchi *et al.*, 2011). The targeting construct was sequenced in its entirety prior to its use in gene targeting. Linearized targeting vectors were electroporated into Sv129 (J1) mouse ES cells (Li *et al.*, 1992) and G418-resistant clones were screened for homologous recombination at the *KOR* locus by PCR. Confirmed ES cells were injected into C57BL/6 blastocysts and implanted into pseudopregnant females to generate chimeric offspring. Mice homozygous for the

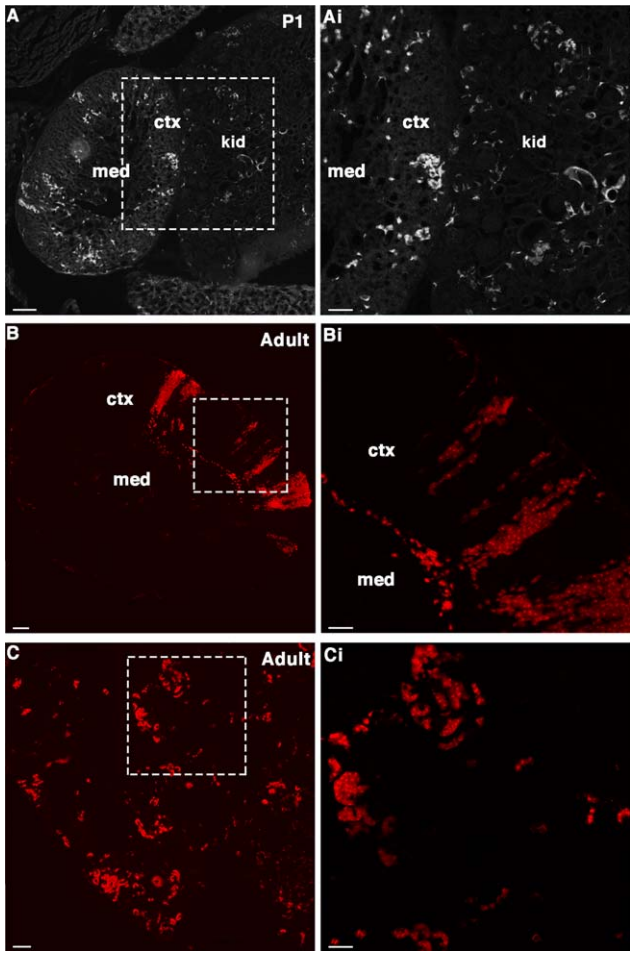

FIG. 5. *KOR-cre* mediated recombination in the adrenal gland and kidney. (A) *KOR-cre* mediated recombination in adrenal gland and kidney (P1; *KOR-Cre; Rosa*$^{lsl\ tdTomato}$). Ai. Magnified view of adrenal gland and cortex of the kidney from A (20x). tdTomato expression was found mainly in the cortex of adrenal gland (ctx) and the kidney (kid). (B–C) TdTomato expression in adrenal gland (B, Bi) and cortex of the kidney (C, Ci) in the adult *KOR-Cre; Rosa*$^{lsl\ tdTomato}$ mouse. TdTomato expression was primarily found mainly in cortex of adrenal gland (ctx) and the cortex of the kidney. Note the clusters of small cells in the medulla (med). Scale bar = 50 μm.

KOR-cre allele are viable. Mice were bred for at least three generations onto a C57BL/6 background. Mice are available upon request.

Genotyping PCR

For routine genotyping, the primers were as follows: Forward Primer (common): CTAGTGCTTCTTGGGGTT GC; WT reverse primer: AAGCACTGGGAGAGCAGGTA, *KOR-cre* reverse primer: CATGTTTAGCTGGCCCAAAT. These primers give PCR products of 403 bp for the WT allele and 648 bp for the KOR-cre knockin allele.

Immunohistochemistry

For immunohistochemistry, heterozygous mice were fixed with 4% paraformaldehyde in PBS by intracardial perfusion. Tissues were post-fixed for 1 h to overnight at 4°C. E14.5 mice were dated with E0.5 at time of plug detection. Embryos were drop fixed in 4% paraformaldehyde immediately after dissection. After post-fix, all tissue was washed extensively with PBS, cryopreserved in 30% sucrose in PBS overnight, embedded in OTC (Sakura Finetek, Torrance, CA), and frozen. Sections were cut at 20 μm on a cryostat and placed on slides. For immunostaining, sections were blocked in 10% goat serum and 0.25% triton-X in PBS for 1 h at RT. Sections were incubated with primary antibodies in block overnight at 4°C. Slides were washed 4 × 5 min in PBS containing 0.1% triton-X. Detection of antibodies was carried out using Alexa-fluor secondary antibodies (Life Technologies) diluted 1: 500 in blocking solution. Sections were washed, as above, and coverslipped. The following primary antibodies used were: rabbit anti-GFP (1:1,000, Life Technologies), goat anti-ChAT (1:500,

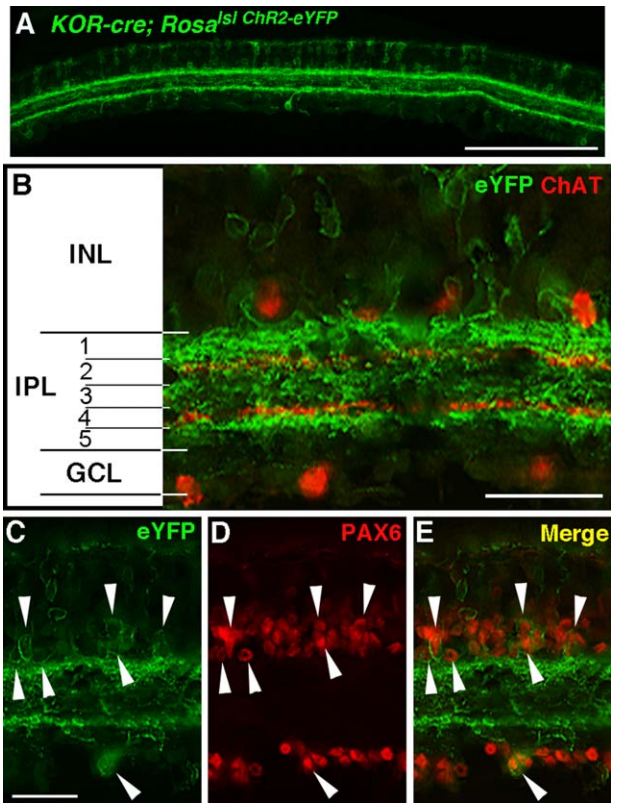

FIG. 6. *KOR-cre* mediated recombination in the retina. (A) *KOR-Cre* causes recombination in many cells in the retina, as observed by expression of the ChR2-eYFP reporter, which is membrane localized (transverse sections; KOR-Cre;Rosa$^{lsl\ ChR2\text{-}eYFP}$; scale bar = 100 μm). (B) Left, cartoon stratification of retina based on ChAT immunostaining. Right, ChAT expression (red) and ChR2-eYFP expression (green) in the inner plexiform layer (IPL). (C). *KOR-Cre* causes recombination in bipolar cells in the inner nuclear layer (INL) and in amacrine cells the ganglion cell layer (GCL). (C–E) Transverse sections of mouse retina showing ChR2-eYFP (C) Pax6 (D) or Merge (E). Examples of *KOR-cre* recombined amacrine cells are marked by the arrowheads. B, C, scale bar = 10 μm.

Millipore), mouse anti-PAX6 (1:200, Developmental Studies Hybridoma Bank). Secondary antibodies: Alexa Fluor-488 donkey anti-rabbit (1:500, Life Technologies), Alexa Fluor-555 donkey anti-goat (1:500, Life Technologies), Alexa-Fluor-555 donkey anti-mouse (1:500, Life Technologies).

Imaging

All sections were imaged with either a Nikon A1R confocal microscope (Nikon, Tokyo Japan; 20× or 60× oil-immersion objective) or an Olympus BX53 florescence microscope (Olympus, Tokyo Japan, 4× or 10× objective).

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