Published in final edited form as:

Genesis. 2016 November ; 54(11): 593–601. doi:10.1002/dvg.22985.

Generation of a *NK1R-CreER* Knockin Mouse Strain to Study Cells Involved in Neurokinin 1 Receptor Signaling

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Abstract

The Neurokinin 1 Receptor (NK1R), which binds Substance P, is expressed in discrete populations of neurons throughout the nervous system, where it has numerous roles including the modulation of pain and affective behaviors. Here, we report the generation of a *NK1R-CreER* knockin allele, in which CreER^{T2} replaces the coding sequence of the *TACR1* gene (encoding NK1R) in order to gain genetic access to these cells. We find that the *NK1R-CreER* allele mediates recombination in many regions of the nervous system that are important in pain and anxiety including the amygdala, hypothalamus, frontal cortex, raphe nucleus, and dorsal horn of the spinal cord. Other cell types that are labeled by this allele include amacrine cells in the retina and fibroblasts in the skin. Thus, the *NK1R-CreER* mouse line is a valuable new tool for conditional gene manipulation enabling the visualization and manipulation of cells that express NK1R.

Keywords

genetics; Neurokinin 1 receptor; substance P receptor; NK1R; TACR1; Cre- loxP system; CreER; tamoxifen; Substance P

INTRODUCTION

The Neurokinin 1 Receptor (NK1R), encoded by the gene *TACR1*, is a Gq-coupled seven transmembrane receptor (Kwatra *et al.*, 1993). Its functions include the modulation of nociception, drug reward, stress/anxiety, emesis, vasodilation, salivation, and inflammation (Garcia-Recio and Gascon, 2015). The ligand for NK1R, Substance P (encoded by *TAC1*), localizes to synaptic vesicles and is released in a calcium dependent manner (Iversen *et al.*, 1976; Pickel *et al.*, 1979). Substance P binding to NK1R leads to activation of phospholipase C and adenylate cyclase (Grady *et al.*, 2000). The precise downstream pathways activated upon SP-NK1R signaling are dependent on the cell type and cellular environment, and can

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include mitogen-activated protein kinases, nuclear factor-kappa B, and protein kinase C. Prior to activation by Substance P, NK1Rs are localized on the plasma membrane, but upon activation, they are rapidly internalized into the cytoplasm via endosomes (Mantyh *et al.*, 1995a; Mantyh *et al.*, 1995b). NK1R internalization is proportional to the intensity and frequency of the stimulus (Mantyh *et al.*, 1995a) and is reversible (Mantyh, 2002).

Recently, NK1R signaling has generated significant interest as a potential therapeutic target, and several groups have generated specific antagonists. The NK1R antagonist Aprepitant is clinically approved as an antiemetic to prevent post-operative and chemotherapy-induced nausea and vomiting (Kramer *et al.*, 1998). A second NK1R antagonist, VPD-737, showed safety and efficacy in a phase 2 clinical trial for the treatment of chronic itch. Given the diverse nature of NK1R functions and its clinical utility, we thought that a genetic tool to study NK1R-expressing cells would be of broad interest. Thus, we developed a *NK1R-CreER* knockin mouse with the goal of having inducible control of neurons that express NK1R. Here, we describe the generation of this mouse line and we characterize the recombination that is observed using a Cre-responsive reporter allele.

RESULTS AND DISCUSSION

To gain temporal and spatial control of cells that express NK1R, we targeted CreER^{T2} to the endogenous *TACR1* locus, which encodes NK1R (Figure 1A). CreER^{T2} is a fusion of Cre Recombinase to the ligand-binding domain of the estrogen receptor that has been mutated such that it binds 4-hydroxytamoxifen. This fusion enables tamoxifen-dependent nuclear translocation of CreER^{T2} for site-specific recombination across loxP sites (Feil *et al.*, 1997). To generate a knockin to the endogenous locus, the coding region of *TACR1* in exon 2 was replaced by that of *CreER^{T2}* followed by a strong polyadenylation sequence (CreER^{T2}pA; (Taniguchi *et al.*, 2011). The targeting vector featured a loxP-flanked PGK-neomycin positive selection cassette (Neo) downstream to the 3'UTR (Figure 1A). Linearized targeting vectors were electroporated into Sv129 (J1) mouse ES cells (Li *et al.*, 1992) and two G418-resistant clones showed homologous recombination at the *TACR1* locus by PCR of the 5' and 3' arms (Figures 1B and 1C). Southern blotting of each arm was performed to confirm correct targeting (Figures 1D and 1E). Routine genotyping by PCR gave rise to products of the anticipated size in wild type, heterozygous, and *NK1R^{CreER/CreER}* mutant mice (Figure 1F). Since *CreER^{T2}* replaced the endogenous *TACR1* gene, we expected homozygous mice to be knockouts for NK1R protein. Consistent with this idea, immunostaining of brain sections from wild type and *NK1R^{CreER/CreER}* littermates revealed the loss of NK1R protein in homozygous *NK1R-CreER* mice, as illustrated in the striatum (Figure 1G).

To analyze the recombination mediated by the *NK1R-CreER* allele, we used the Cre-dependent *Rosa^{ls}tdtomato* reporter (also known as *Ai14*; Jax Labs). Double transgenic mice were injected with tamoxifen (IP, 0.4mg/g) as young adults (between P25 and P29) and analyzed approximately one week later. We observed sparse tdtomato labeling in many regions of the nervous system. To analyze whether tamoxifen was required for recombination, we also analyzed reporter mice that had not received tamoxifen treatment. In the absence of tamoxifen, the *NK1R-CreER* allele showed no recombination in most tissues and brain areas (Figure 1H). However, in the striatum a couple of recombined cells were

occasionally observed, presumably due to CreER translocation to the nucleus independent of tamoxifen (data not shown). Thus, the vast majority (>99%) of recombination mediated by the *NK1R-CreER* allele requires tamoxifen treatment.

Next, to investigate whether the recombination mediated by the *NK1R-CreER* allele was occurring in neurons that express NK1R, we co-stained sections from these mice with NK1R-directed antibodies. We found that almost all recombined cells showed NK1R immunoreactivity (NK1R-IR). However, with a single dose of tamoxifen, only a subset of NK1R expressing cells showed recombination, as assessed by tdtomato expression. Notably, within the CNS, all cells that showed tdtomato expression colocalized with NeuN, suggesting that *NK1R-CreER* causes recombination in neurons (Figure 1H). Together, these findings suggest that a subset of NK1R-expressing neurons is genetically marked in tamoxifen-treated *NK1R-CreER* mice.

Although a large number of areas and cell types in the nervous system showed recombination with the *NK1R-CreER* allele, here we highlight brain regions with prominent tdtomato expression. These regions include the striatum, hypothalamus, cortex, amygdala, periaqueductal gray (PAG), and dorsal raphe nucleus (Figure 2). Dense recombination was observed throughout the striatum, including the caudate putamen (Figure 2Ai-ii), and most recombined cells in this region showed clear NK1R-IR localized to the somatic and dendritic membranes (Figure 2Aii). These neurons are thought to be D1 medium spiny neurons (Blomeley and Bracci, 2008), which project to the basal ganglia (Gerfen, 1992), and whose activation results in reward reinforcement (Kravitz *et al.*, 2012).

Although the vast majority of tdtomato-expressing cells showed obvious NK1R expression, we occasionally observed a tdtomato positive cell that lacked visible NK1R immunostaining (Figure 2Aii). This mismatch in reporter and NK1R protein may reflect a developmental change of NK1R expression. In this regard, it is noteworthy that NK1R expression is patchy in the striatum during development, but this pattern is lost in adult mice, when NK1R expression is more uniformly distributed, suggesting the cells that express NK1R may change over time (Tokuno *et al.*, 1996).

Other regions in which dense recombination was observed include broad regions of the hypothalamus, including the lateral hypothalamus and the anterior hypothalamic nucleus (Figure 2Aiii), and the amygdala (Figure 2Biii). Intriguingly, the pattern of endogenous NK1R expression in these regions is characterized by NK1R puncta that are predominantly restricted to dendrites rather than the soma (Figure 2Aiii), occasionally making NK1R-expressing cells difficult to identify. Nevertheless, most recombined cells in these regions showed clear NK1R expression.

In the cortex, NK1R-expressing neurons are broadly distributed, but sparse. *NK1R-CreER* allele causes recombination in a large proportion of these neurons, which show strong NK1R expression throughout the soma and dendrites (Figure 2Bii). Based on morphology, we hypothesized that these are interneurons.

In the midbrain, the *NK1R-CreER* allele causes recombination in a subset of NK1R-expressing neurons in the periaqueductal grey (PAG) (Figure 2Cii) and in the dorsal raphe

nucleus (Figure 2Ciii). Expression of NK1R in this region is strong along dendrites and faint on somatic membranes.

Moving to the most anterior region of the brain, there is strong *NK1R-CreER* mediated expression in the granule layer of the olfactory bulb (Figure 3A). Endogenous expression of NK1R is very punctate and is strongest in the external plexiform layer (Figure 3Aii). This recombination is consistent with the Allen Brain Atlas *in situ* data that shows *NK1R* mRNA in the granule layer of the main olfactory bulb (Lein *et al.*, 2007).

In the brainstem, *tdtomato* is expressed in many cells of the inferior colliculus (Figure 3B). Here, endogenous NK1R expression is localized in a sparse punctate fashion along dendrites (Figure 3Bii). In more posterior regions of the brainstem, NK1R is expressed in the pontine central grey as well as the lateral parabrachial nucleus, as illustrated (Figure 3Cii).

We also observe *NK1R-CreER* mediated recombination in cells in NK1R immuno-positive neurons in lamina I as well as deep layers of the spinal cord dorsal horn (Figure 3D). NK1R is expressed in the majority of spinal projection neurons (Todd *et al.*, 2000), which are important for pain (Khasabov and Simone, 2013; Mantyh *et al.*, 1997; Nichols *et al.*, 1999) and itch (Carstens *et al.*, 2010). Since many of the spinal projection neurons target the lateral parabrachial nucleus, labeled processes observed in this regions may either arise locally from neurons within this nucleus or from the spinal projection neurons that terminate therein (Todd, 2010).

Within the retina, NK1R is expressed in subsets of bipolar and amacrine cells (Catalani *et al.*, 2004). When tamoxifen is delivered approximately 3 weeks after eye opening, the *NK1R-CreER* allele appears to capture amacrine cells across the retina that express endogenous NK1R (Figure 4A–C). To characterize these amacrine cell subtypes, retinas from NK1R-CreER mice were costained with tyrosine hydroxylase (TH, Figure 4D), the pan-amacrine cell marker Pax6 (Figure 4E), and choline-acetyl-transferase (ChAT, Figure 4F). *NK1R-CreER* cells colocalize with a subset of TH-expressing amacrine cells as well as non-TH-expressing amacrine cells, but not with ChAT-expressing amacrine cells (Figure 4). These findings are consistent with previous studies suggesting that NK1R is expressed by a variety of amacrine cell subtypes (Catalani *et al.*, 2006; Catalani *et al.*, 2004).

During early postnatal development, NK1R is expressed in the brain and the retina (Ardelt *et al.*, 1996; Catalani *et al.*, 2006). To assess *NK1R-CreER* mediated recombination during development, we injected tamoxifen at P2 and assayed expression at P6. At this age, we see low levels of recombination throughout the brain, with the most recombination in the lateral parabrachial nucleus (Figure 5A) and striatum (Figure 5B). Interestingly, the expression pattern of endogenous NK1R changes during development in the striatum (Ardelt *et al.*, 1996). Between P2 and P7 NK1R expression is patchy and mosaic-like, whereas in adult mice NK1R is expressed in neurons that are uniformly distributed throughout the striatum (Ardelt *et al.*, 1996; Tokuno *et al.*, 1996). Thus, use of the *NK1R-CreER* allele and tamoxifen administration at specific time points may help distinguish whether the change in expression pattern from patchy to uniform reflects a change in NK1R expression or the migration of NK1R-expressing cells.

Previous reports suggest that the neurokinin receptor family is expressed on mast cells, keratinocytes, merkel cells, fibroblasts, endothelial cells, and Langerhans cells (Fantini and Johansson, 1995; Grady *et al.*, 1996; Ho *et al.*, 1997; Song *et al.*, 2000; Staniek *et al.*, 1997). However, the precise expression profile and function of NK1R in the skin is not well understood. Using our *NK1R-CreER* mouse, we see Cre-mediated recombination in the dermis, but not the epidermis (Figure 6A). *NK1R-CreER* mediated recombination is evident in Thy1.2-IR cells, which labels T-cells, fibroblasts, and keratinocytes (Figure 6Bi), but not in MHCII-labeled cells, which label dendritic cells and macrophages (Figure 6Bii). These findings suggested that NK1R-CreER cells in the dermis are fibroblasts. To test investigate whether these cells are fibroblasts but not T cells, we used vimentin, which is expressed in most fibroblast subtypes, as well as CD3, which is a marker for T cells. We observed that many NK1R-CreER cells colocalize with vimentin (Figure 6C) but not CD3 (Figure 6D). Thus, based on the morphology, location in the dermis, and immunoreactivity of tdTomato cells with Thy1.2 and vimentin, *NK1R-CreER* likely mediates recombination in a subset of fibroblasts.

MATERIALS AND METHODS

Animal Husbandry

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 *NK1R-CreER* knockin mouse

The targeting vectors were constructed from 129s6/SvEvTAC mouse genomic fragments, which were amplified by PCR and sequenced. The coding region of the *TACR1* gene exon 2 was replaced with the coding region for *CreERT²* followed by a strong polyadenylation signal (CreERT²-pA) by fusion PCR (Taniguchi *et al.*, 2011). The fusion product was then introduced into a targeting vector that contained 5' and 3' homologous arms. The 5' and 3' homologous arms were PCR amplified from genomic DNA using the following primers: 5' arm (F: GTGGAATGGGAGCAGAATGA and R: CGTAATCTGGCATTCTGGG) and the 3' arm (F: TGCACGAGACTAGTGAGACG and R: AAGATAAGATGCCTCGCAGG). The targeting vector contained a loxP-flanked neomycin selection cassette. This manipulation was confirmed by restriction digest and sequencing. 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 *TACR1* locus by PCR. For Southern blot analysis, genomic DNA was digested with EcoRV and blots were hybridized with an ~500 bp probe that was external to the gene targeting construct. Confirmed ES cells were injected into C57BL/6 blastocysts and implanted into pseudopregnant females to generate chimeric offspring. Clones 143 and 189 demonstrated successful homologous recombination and mice derived from each clone showed indistinguishable patterns of recombination. The experiments presented here used mice derived from clone 189. Mice are available upon request.

Genotyping PCR

For routine genotyping, the primers were as follows: Forward Primer (common): ACCTCCTGAGTAGCAGGACT; WT reverse primer: AGGTCCAGGGGTTCTAGCAT, *NK1R-CreER* reverse primer: CATGTTTAGCTGGCCCAAAT. These primers give PCR products of 671 bp for the WT allele and 430 bp for the *NK1R-CreER* knockin allele.

Tamoxifen Administration

Tamoxifen (Sigma) was dissolved in corn oil at 37°C for 3 hours, shaking and stored in a light-blocking container at 4°C for up to 2 weeks (10 mg/ml). Tamoxifen was administered at 3 weeks or P2 by intraperitoneal injection (0.4 mg/g body weight). Tissue was harvested 1 week after tamoxifen administration.

Immunohistochemistry

NK1R-CreER mice were bred to a Cre-dependent reporter line, *Rosa^{lsl}tdtomato* (also known as Ai14; Jax Labs) that expresses tdTomato following Cre-induced recombination. For immunohistochemistry, mice were fixed with 4% paraformaldehyde in PBS by intracardial perfusion. Tissues were post-fixed for 30 min to overnight at 4°C. After post-fix, all tissue was washed extensively with PBS, cryopreserved in 30% sucrose in PBS overnight, embedded in OCT (Sakura Finetek, Torrance, CA), and frozen. Depending on the tissue, sections were cut at 20µm on a cryostat (Leica CM 1950) and placed on slides or cut at 40 µm on a vibratome (Leica VT 1200S). For immunostaining sections cut on the cryostat, sections were blocked in 10% goat serum and 0.25% triton-X in PBS for 1h at RT. Sections were incubated with primary antibodies in block overnight at 4°C. Slides were washed 4 x 5 min in PBS containing 0.1% triton-X. Free-floating sections were cut on the vibratome then washed with 50% ethanol for 30 minutes to aid in antibody penetration. Sections were immunostained using 0.3M PBST (0.3M NaCl, 0.3% Triton X-100 in PBS) with 5% goat serum containing primary antibodies rocking for 2 – 3 nights at 4°C.

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-NK1R (1:10,000; Sigma S8305), mouse anti-NeuN (1:500; Millipore MAB377), mouse anti-PAX6 (1:200, Developmental Studies Hybridoma Bank), goat anti-ChAT (1:500, Millipore), rabbit anti-TH (1:1000, Millipore), anti-MHCII conjugated to Alexa647 (1:400, Biolegend), anti-CD3 conjugated to FITC (1:400, Biolegend), and anti-Thy1.2 conjugated to FITC (1:400, eBiosciences), anti-vimentin (1:400; Biolegend). 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; 20x or 60x oil-immersion objective) or a Nikon 90i fluorescence microscope with an automated stage (10x or 20x objective). Whole brain sections were stitched together using the 10x objective, automated stage, and Nikon stitching algorithm.

Acknowledgments

This research was supported by NIH grants R01 AR063772 and R21 AR064445 to SER and R01 AR060744 and R01 AR067187 to DHK. LMS and MSK are supported by a T32 NS 73548-3. A portion of this work was supported by a grant from the Rita Allen Foundation to SER, who is a Rita Allen Foundation Pain Scholar. The *NK1R-CreER* line was generated with help from the Mouse Gene Manipulation Facility of the Boston Children's Hospital Intellectual and Developmental Disabilities Research Center (IDDRC), which is supported by NIH P30-HD 18655.

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Figure 1. Generation of a *NK1R-CreER* knockin mouse

A. Targeting of *TACR1*. The *NK1R-CreER* KI targeting vector was constructed by fusion PCR, replacing the coding region of *NK1R* in exon 2 with *CreER*^{T2p} followed by a loxP-flanked neomycin (Neo) cassette. Open reading frames are indicated with arrows. LoxP sites are indicated with black triangles. PCR products and Southern probes are marked with black bars. EcoRV (E) and CsiI (C) are indicated with letters. **B – C.** PCR-based screening for homologous recombination at the 5' (B) and 3' arms (C). PCR products of appropriate size are observed in two (of two hundred) clones, 143 and 189. **D – E.** Southern blot analysis of EcoRV-digested (D) or CsiI-digested (E) genomic DNA from clones 143 and 189 using the 5' or 3' probes, respectively, results in fragments of the predicted sizes, indicating correct targeting. **F.** Genotyping PCR of tail lysates from mice that are homozygous for the *CreER* (Cre/Cre, 430bp), heterozygous (het; 430bp and 671bp), or wild type (wt, 671bp) at the *TACR1* locus. **G.** Immunohistochemistry for *NK1R* (green; *NK1R*-immunoreactivity (*NK1R-IR*)) or NeuN (red) in coronal sections of the striatum of adult mice (wild type or homozygous *NK1R^{CreER/CreER}* mice, derived from clone 189) demonstrates that the homozygous mice have no detectable *NK1R* protein. Scale bar = 10 μ m. **H.** Striatum from

NK1R^{CreER}; Rosa^{Isl1tdtomato} mice that did not receive tamoxifen (left) and did receive tamoxifen (right). Sections are stained with NeuN (blue) and NK1R antibody (green). Scale bar = 50 μ m.

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Figure 2. Tamoxifen-mediated recombination in the brain of *NK1R-CreER* mice

Coronal sections through the brain moving anterior (A) to posterior (C) in *NK1R-CreER*; *Rosa^{lsl}tdtomato* mice that were treated with tamoxifen at 3 weeks and analyzed one week later. **Ai, Bi, Ci.** Tiled 10x wide-field images of the entire coronal section (top: tdtomato; bottom: merge tdtomato and NK1R-IR; scale bar = 1 mm). **Aii - Cii and Aiii - Ciii.** Further magnification (60x, maximum intensity projections) of the region indicated by the white box in Ai - Ci. Scale bars = 50 μ m. Tdtomato (red), NK1R-immunoreactivity (NK1R-IR; green), and merge are shown for each image. Caudate putamen (Aii), hypothalamus (Aiii), amygdala (Bii), cortex (Biii), periaqueductal grey (Cii), and dorsal raphe nucleus (Ciii).

Figure 3. Tamoxifen-mediated recombination in the CNS of *NK1R-CreER* mice

Coronal sections through the olfactory bulb (**A**), midbrain (**B**, **C**), and lumbar spinal cord (**D**) in an adult *NK1R-CreER; Rosa^{lsl}tdtomato* mouse showing tdtomato (red) and NK1R-immunoreactivity (green). Tiled 10x wide-field images of the entire coronal section; scale bar = 1 mm. **Aii** – **Diii**. Further magnification (60x confocal, maximum intensity projection) of the olfactory bulb (**Aii**), inferior colliculus (**Bii**), and lateral parabrachial nucleus (**Cii**), lamina I of the dorsal horn (**Dii**), and deep dorsal horn (**Diii**), as indicated by the white boxes. Scale bars = 50 μ m.

Figure 4. *NK1R-CreER* mediated recombination in amacrine cells of the retina

A. Transverse section (20 μ m, 60x confocal) through the retina of a *NK1R-CreER*; *Rosa^{lsl} tdtomato* mouse. **B – E.** Transverse sections of the mouse retina showing tdtomato, NK1R-IR (B) TH-IR (C) Pax6-IR (D) or ChAT-IR (E), as indicated. Scale bars = 20 μ m. Colocalization of *NK1R-CreER* with amacrine cell markers is indicated by asterisks. Abbreviations: inner nuclear layer (INL), inner plexiform layer (IPL).

Figure 5. *NK1R-CreER* mediates recombination in the brain of neonatal mice
 20x single wide-field images from *NK1R-CreER; Rosa^{sl}tdtomato* mouse at P6 that was treated with tamoxifen at P2. **A.** Lateral parabrachial nucleus and **B.** striatum. Hoechst (blue) and tdTomato (red) in cells with *NK1R-CreER* mediated recombination. Scale bars = 100 μ m.

Figure 6. *NK1R-CreER* mediated recombination in fibroblasts in the skin

A. Tiled 10x wide-field image of skin section from an adult *NK1R-CreER; Rosa^{lsI tdTomato}* mouse immunostained with antibodies against MHCII (MHCII-IR; labels dendritic cells and macrophages), and Thy1.2 (Thy1.2-IR; labels T-cells, fibroblasts, and keratinocytes). Nuclei stained with DAPI. Scale Bar = 100 μm. Asterisks = colocalization **B – D.** 40x wide-field images of the skin from *NK1R-CreER* mouse immunostained with antibodies to Thy1.2 (Bi), MHCII (Bii), vimentin (C) and CD3 (D). Scale bars= 50 μm.