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EFFECT OF KEPI (*Ppp1r14c*) DELETION ON MORPHINE ANALGESIA AND TOLERANCE IN MICE OF DIFFERENT GENETIC BACKGROUNDS: WHEN A KNOCKOUT IS NEAR A RELEVANT QUANTITATIVE TRAIT LOCUS

J. DRGONOVA,[a] D. B. ZIMONJIC,[b] F. S. HALL[a] AND G. R. UHL[a]*

[a]*Molecular Neurobiology Branch, NIDA- IRP/NIH/DHHS, 333 Cassell Drive, Baltimore, MD 21224, USA*

[b]*Laboratory of Experimental Carcinogenesis, NCI/NIH/DHHS, 31 Center Drive, Bethesda, MD 20892, USA*

Abstract—We previously identified KEPI as a morphine-regulated gene using subtractive hybridization and differential display PCR. Upon phosphorylation by protein kinase C, KEPI becomes a powerful inhibitor of protein phosphatase 1. To gain insights into KEPI functions, we created KEPI knockout (KO) mice on mixed 129S6×C57BL/6 genetic backgrounds. KEPI maps onto mouse chromosome 10 close to the locus that contains the μ-opioid receptor (Oprm1) and provides a major quantitative trait locus for morphine effects. Analysis of single nucleotide polymorphisms in and near the Oprm1 locus identified a doubly-recombinant mouse with C57BL/6 markers within 1 Mb on either side of the KEPI deletion. This strategy minimized the amount of 129S6 DNA surrounding the transgene and documented the C57BL/6 origin of the Oprm1 gene in this founder and its offspring. Recombinant KEPIKO mice displayed (a) normal analgesic responses and normal locomotion after initial morphine treatments, (b) accelerated development of tolerance to analgesic effects of morphine, (c) elevated activity of protein phosphatase 1 in thalamus, (d) attenuated morphine reward as assessed by conditioned place preference. These data support roles for KEPI action in adaptive responses to repeated administration of morphine that include analgesic tolerance and drug reward. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: quantitative trait locus, morphine analgesia, morphine reward, morphine tolerance, protein phosphatase 1, conditioned place preference.

Protein phosphorylation is implicated in many aspects of brain function. Phosphorylation of serine and threonine residues depends on the balanced activities of more than three hundred serine/threonine protein kinases (Manning et al., 2002; Caenepeel et al., 2004) and a smaller number of serine/threonine protein phosphatases. The regulation of these phosphatases is complex, however. For example, protein phosphatase 1 (PP1), a principal serine/threonine phosphatase, is regulated through its association with over 50 proteins that modulate its activity temporally and spatially. These PP1 subunits include inhibitor-1 (Ppp1r1a), DARPP-32 (Ppp1r1b), Cpi17 (Ppp1r14a), PHI-1 (Ppp1r14b), and KEPI (Ppp1r14c) (Perez and Lewis, 1992; Eto et al., 1999; McLaren et al., 2000; Yamawaki et al., 2001; Liu et al., 2002). These inhibitors are virtually all phosphoproteins themselves; their activities are regulated by kinases and phosphatases.

We identified KEPI as a morphine-regulated gene using subtractive hybridization and differential display PCR (Liu et al., 2002). After phosphorylation by protein kinase C (PKC), KEPI inhibits PP1 with an IC_{50} of about 10^{-9} M (Liu et al., 2002). KEPI maps to mouse chromosome 10 near the gene for the μ-opioid receptor (Oprm1), a principal site for morphine actions (Sora et al., 1997, 2001). KEPI is expressed in heart, muscle, and the CNS. Its multifocal, largely neuronal expression patterns in the CNS include regions associated with reward, locomotor control and nociception, such as striatum, nucleus accumbens, amygdala, thalamus, periaqueductal grey, and spinal cord (Liu et al., 2002; Gong et al., 2005).

To gain insights into KEPI functions at biochemical and behavioral levels, we created defined and characterized KEPI knockout (KO) mice. While the initial animals were developed on a mixed 129S6×C57BL/6J background, the proximity of the KEPI gene to Oprm1 lead us to consider possible effects of "hitch-hiking" genes from the 129S6 genetic background, including Oprm1 (Gerlai, 1996). Oprm1 variants provide a major contribution to a quantitative trait locus (QTL) that influences morphine antinociception and self-administration (Berrettini et al., 1994; Belknap et al., 1995; Bergeson et al., 2001). We thus identified recombinant KO animals with C57BL/6 markers in the appropriate genomic region by analyzing single nucleotide polymorphisms (SNPs) in and near the Oprm1 locus. This strategy allowed us to evaluate the contribution of KEPI to morphine-related traits without confounding influences from Oprm1 variants, and provides a generally useful approach for evaluating mice in which engineered gene variants lie near QTLs for phenotypes in which the gene of interest may be involved.

EXPERIMENTAL PROCEDURES

Generation of KEPI KO mice

A 129/SvEvTac genomic library in λ fixII vector (Stratagene, Cedar Creek, TX, USA) was screened with a 32P-labeled probe amplified from the mouse 129S6 (129S6/SvEvTac) genomic DNA by PCR. The primers used for the probe amplification (J24 and

*Corresponding author. Tel: +1-443-740-2799; fax: +1-443-740-2122.
E-mail address: guhl@intra.nida.nih.gov (G. R. Uhl).

Abbreviations: CPP, conditioned place preference; ES, embryonic stem; KO, knockout; MPE, maximal possible effect; PAG, periaqueductal grey; PCR, polymerase chain reaction; PKC, protein kinase C; PP1, protein phosphatase 1; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; WT, wild type.

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Table 1. DNA primers used in the study

Primer name	Primer sequence	Primer name	Primer sequence
J24	CACTCCCAGCTGTCCTAGGAG	rs29358253f	CTCTAATGCCAGTGGAGCGT
J25	CTAGGAAGTCGTCCGAGGTGG	rs29358253r	CACCGCAATCGGATAGCACT
J30	GGAGTTCTGGCTTCTAGAAGAAC	rs29358253ex	CTCTGTACTTCTCCCTTCTC
J31	CATCATGTGGTCACCACATGCC	rs29382724f	CCAGAGCAAGCGTCTCTGG
J32	TCTGGCACACTGTCTTCCCTGA	rs29382724r	GAGTGGGATGCCAAATTACCT
J33	CATGGCTAGCATGCCCTGGCA	rs29382724ex	TGCAATCGGGTTCTGCTAAC
J37	ACTGACACTACGTGAGAAGTTTCTCCCACCTTCA	rs29382421f	CCATTGGAAATACGGTGACTC
J38	TCAGAGGTAGAGTGCACTTGCC	rs29382421r	CAAAGCACATTGTACACATGAG
J39	CTTCACTGGCAACAGTTATGGAG	rs29382421ex	CACACGTCTTCTAAAACGAAC
J40	AAGGGAGGCAATGATTGCTGCC	rs4228112f	GATCCACAGAACGGAGAGGA
vNEOf	CCGCTATCAGGACATAGCGTTG	rs4228112r	GCGCATTTCGAGTCTGCTTTT
KEPIf	GAGTCTGCCTATGTGTATTTCCAT	rs4228112ex	CTCTATTCTTTGGTATGGATTG
KEPIr	CTCATAGACTATCTTGTTCCTCTC	rs29361179f	TCGTCCACCTTCAGCATCCA
J85	CTCCGCAAGGATCCAGCGTCTAGG	rs29361179r	GCCCTTGACCAACTCAACAC
J86	CGAAGCTCTTTCCTCCTCGCT	rs29361179ex	GTTTGTCATTACAATGAGCCA
rs8244279f	TTCAGCAGTAATGTAGATAAGGT	qGAPDHf	GCATGGCCTTCCGTGTTC
rs8244279r	GGAGACCGATGAATGTGAGC	qGAPDHr	CACCACCTTCTTGATGTCATC
rs8244279ex	AGTTCATATGGCTGAGCTGAA	qPKCCf	GCACCTCCTTTCAGACCACG
rs16821161f	TGTGGAAGGCATGGTGGAGA	qPKCCr	GGTCAGTGCTGGCGCTGCC
rs16821161r	ATGGATTTGAGGCACACAAACT	qPKCEf	ACTGGGTACTGCTGGAGCAG
rs16821161ex	GGAACCGACTTGACGTAGC	qPKCEr	GTAAGTATTGGCTCTTCCCGC
rs29360540f	CAGAGCCAAACATGGTAGCTC	qPP1CAf	GTGCCATGATGAGTGTGGATG
rs29360540r	GAGGCAGTGTCTCACGCTG	qPP1CAr	CAGGCCGCTGAACTGCCCAT
rs29360540ex	TGAGGCTGATATAGGATGAAG	qPP1CBf	GGCGAGTTTGACAATGCTGGT
rs29334299f	CATCCCATGCTCTTATTTGGG	qPP1CBr	CCCACCATACTGGTACTTAGC
rs29334299r	ATGTAGGCAGCCTGCCTGG	qPP1CCf	CTCCATAAGCATGATTTGGATCT
rs29334299ex	AGCAGAAACTCGATGGGCC	qPP1CCr	CAGAGTGACTAACTGCCTCTTT

J25, Table 1) targeted the first exon. Two positive clones, λ13 and λ23 were further examined by PCR with primer pairs annealing 5′ (J30–J31, Table 1) and 3′ (J32–J33, Table 1) from the first exon and by restriction analysis to confirm that they contained the KEPI gene. λ13 was digested with *Xba* I (this and all other restriction enzymes were from NEB, Beverly, MA, USA) and the 3.5 kb fragment was cloned into the pBSIIKS+vector (Stratagene). Digestion of λ23 with *BamH* I produced a 3.9 kb piece downstream of exon 1 which was inserted 3′ from the *Xba* I insert. A Spe I fragment of the Neo cassette was inserted between the two arms and the *Sal* I fragment of the TK gene was then inserted in the opposite orientation downstream of the *BamH* I arm (Fig. 1a). The final construct, pJD9, was linearized with *Not* I and electroporated into 129.3 mouse MC1 (126S6/SvEvTac) embryonic stem (ES) cells (JHU Transgenic Core Laboratory, Baltimore, MD, USA). Primers vNEOf (in the Neo cassette, Table 1) and J39 (outside the construct, Table 1) were used for a PCR screen of 376 colonies resistant to G418 (Invitrogen, Carlsbad, CA, USA) and Gancyclovir. This and all other reagents were purchase from Sigma, St. Louis, MO, USA (unless stated otherwise). Plasmid pJD10 derived from pJD9 by inserting a PCR amplicon (J37–J38, Table 1) into a *Dra* III site was used as a positive control. Embryonic stem (ES) cell clones positive by PCR were further confirmed by Southern blot after DNA digestion with *Bgl* II (Fig. 1b). The 640-bp probe spanning the 5′ *Xba* I site was generated by PCR with primers J40 and J31 (Table 1). Eight of the correctly targeted ES cell clones were karyotyped and two with the correct number of chromosomes were microinjected into C57BL/6J E3.5 blastocysts at the Johns Hopkins University transgenic core facility. Heterozygous KEPI KO offspring of the resulting male chimeras were mated with each other and their progeny genotyped by PCR with primers KEPIf, KEPIr, and vNEOf (Table 1) to amplify wild type (WT) (527 bp) and KO (922 bp) alleles simultaneously (Fig. 1c). To prepare DNA for genotyping, ear punches (approximately 1.5 mm diameter) were digested for 6 h at 55 °C in 50 μl of a buffer containing 10 mM Tris–HCl pH 8.5, 5 mM EDTA, 0.1% SDS, 200 mM NaCl, and 10 mg/ml proteinase K (Invitrogen). The resulting hydrolyzate was diluted 4× with H_2O and directly used for PCR reaction.

SNP genotyping

SNPs around the KEPI locus were selected from the Mouse Genome Informatics Database (http://www.informatics.jax.org) and genotyped by primer extension followed by MALDI-TOF-based allele detection (Sequenom, San Diego, CA, USA) according to the manufacturer's protocol. DNA was prepared as described above and further diluted threefold. Primers for primary PCRs and extension primer sequences are listed in Table 1. One male with cross-overs closest to KEPI (Fig. 2) was mated with a C57BL/6J female and the resulting heterozygote offspring were mated to produce the "Recombinant KEPI KO Line."

RNA extraction and reverse transcription-PCR

Total brain RNA was prepared with RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from oligo dT primers using Superscript III (Invitrogen, Carlsbad, CA, USA). Quantitative real time PCR was performed on ABI, Prism 7900HT Sequence Detection System with primers as listed in Table 1 and SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. Expression levels were normalized against GAPDH.

Preparation of brain extracts for phosphatase assay

Unless otherwise stated, all the steps were performed at 0–4 °C. Mice were euthanized by spinal cord dislocation followed by decapitation, brains rapidly dissected, frozen, and stored at −80 °C