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# Elimination of *Kalrn* expression in POMC cells reduces anxiety-like behavior and contextual fear learning☆

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ABSTRACT

Kalirin, a Rho GDP/GTP exchange factor for Rac1 and RhoG, is known to play an essential role in the formation and maintenance of excitatory synapses and in the secretion of neuropeptides.
Mice unable to express any of the isoforms of *Kalrn* in cells that produce POMC at any time during development (POMC cells) exhibited reduced anxiety-like behavior and reduced acquisition of passive avoidance behavior, along with sex-specific alteration in the corticosterone response to restraint stress. Strikingly, lack of *Kalrn* expression in POMC cells closely mimicked the effects of global *Kalrn* knockout on anxiety-like behavior and passive avoidance conditioning without causing the other deficits noted in *Kalrn* knockout mice. Our data suggest that deficits in excitatory inputs onto POMC neurons are responsible for the behavioral phenotypes observed.


## Introduction

The human *KALRN* gene encompasses several functional domains and generates multiple isoforms (Fig. 1A); *KALRN* has been implicated in cardiovascular disease, ischemic stroke, schizophrenia, Alzheimer's disease and attention deficit hyperactivity disorder (Beresewicz et al., 2008; Krug et al., 2010; Kushima et al., 2012; Wang et al., 2007; Wu et al., 2012; Youn et al., 2007a, 2007b). The rodent *Kalrn* gene gives rise to similar developmentally regulated and functionally distinct isoforms that were named based on the lengths of their mRNAs (Johnson et al., 2000; McPherson et al., 2004; Penzes et al., 2011). Kalirin-7 (Kal7) expression is limited to neurons, whereas Kal9 and Kal12 are broadly expressed (Ma et al., 2008a, 2008b; Mandela et al., 2012; Penzes et al., 2001a, 2001b; Wu et al., 2012). Kal12, the largest isoform, includes a lipid binding Sec14 domain, nine spectrin repeats, two guanine nucleotide exchange factor (GEF) domains, two SH3 domains, an Ig/FnIII domain and a putative kinase domain (Mandela and Ma, 2012; Miller et al., 2013; Rabiner et al., 2005); Kal7 includes a PDZ binding motif and is localized to the post-synaptic density.

Kalirin was first identified as an interactor with the cytosolic domain of peptidylglycine α-amidating monooxygenase (PAM), an enzyme essential for the synthesis of many bioactive peptides (Alam et al., 1996). The spectrin repeats common to the full-length isoforms of Kalirin are responsible for its ability to interact with PAM and for the formation of Kalirin/iNOS heterodimers (Ratovitski et al., 1999). In extracts of hippocampi from patients with Alzheimer's disease, there is a paucity of Kal7 and an excess of iNOS (Nathan et al., 2005; Youn et al., 2007a, 2007b). Although early studies demonstrated a role for Kalirin in controlling peptide hormone secretion, subsequent investigations focused on the role of Kal7 in dendritic spine formation and function. Roles for the larger isoforms of Kalirin in the cardiovascular, skeletal and neuromuscular systems have subsequently been demonstrated (Huang et al., 2013; Mandela et al., 2012; Wu et al., 2012).

Mice globally lacking the exon encoding the PDZ binding motif unique to Kal7 ($Kal7^{KO}$ mice) exhibit decreased anxiety-like behavior and decreased acquisition of passive avoidance behavior (Ma et al., 2008a, 2008b). In order to ablate all of the major isoforms of Kalirin, exon 13, which encodes part of the spectrin repeat region, was flanked by *lox-p* sites, generating $KalSR^{KO}$ mice (Mandela et al., 2012; Wu et al., 2012). In addition to the deficits observed in $Kal7^{KO}$ mice, the growth curves for male and female global Kalirin knockout mice ($KalSR^{KO}$) are delayed, and female $KalSR^{KO}$ mice have difficulty with parturition and lactation. Primary pituitary cultures prepared from $KalSR^{KO}$ mice exhibit increased basal secretion of growth hormone and prolactin (Mandela et al., 2012).

Given the crucial role of the hypothalamic–pituitary–adrenal axis in the response to stress (Bhargava et al., 2000; Young et al., 1990), and the deficiencies in the stress responses seen with global *Kalrn* knockout mice (Mandela et al., 2012), we wanted to test the consequences of eliminating Kalirin expression only in POMC cells ($Kal^{POMC\text{-}KO}$). Studies on the role of individual neuropeptides in *Caenorhabditis elegans* have revealed their ability to modulate the circuitry involved in adjusting

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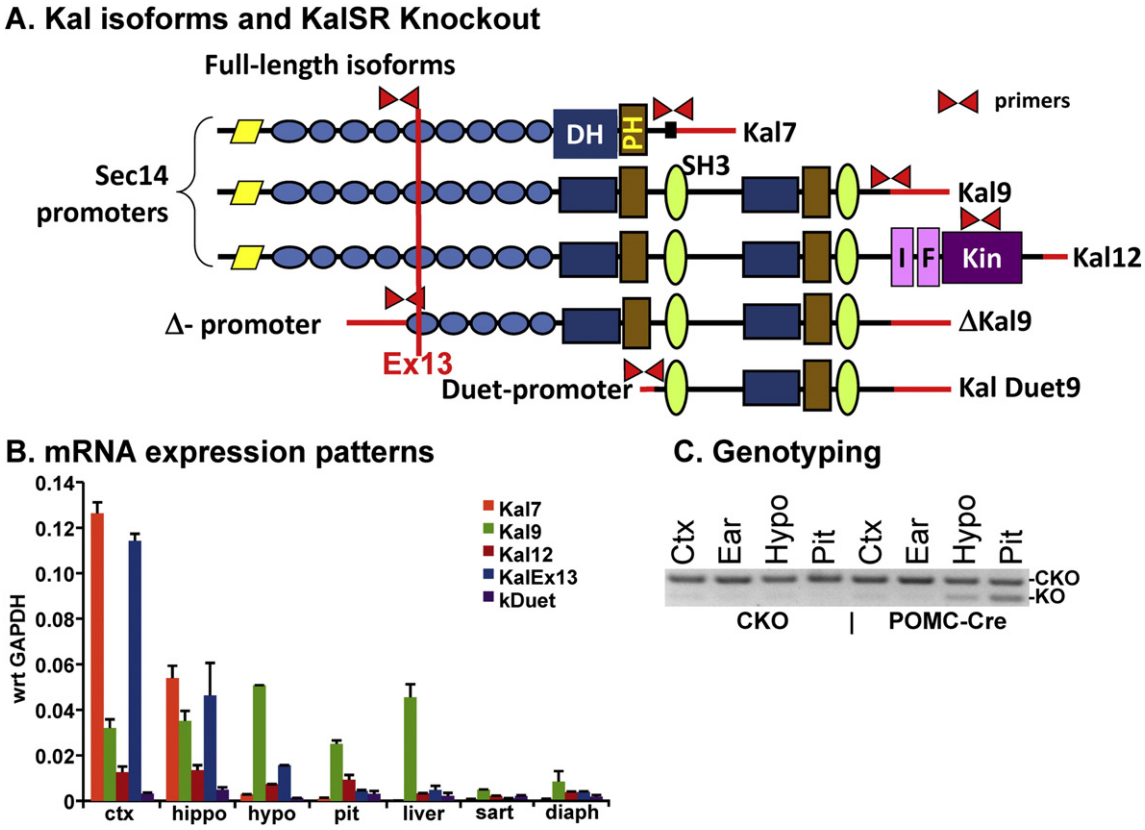


**Fig. 1.** Kalirin isoforms and the Kalirin POMC-Cre mouse. A) The major isoforms of Kalirin are indicated, along with the position of the Exon 13 ablation. The black box at the COOH-terminus of Kal7 is a PDZ-binding motif. B) Real time polymerase chain reaction (qPCR) was performed on RNA from the indicated tissues as described (Mandela et al., 2012). Results are expressed with respect to the GAPDH signal in the same samples on the same 96-well plate. C) Genotyping from the tissues indicated was performed as described; the products expected from KalSR$^{CKO}$ and KalSR$^{KO}$ mice are indicated (Mandela et al., 2012).

behavioral responses to environmental inputs (Bargmann, 2012). Mice of the desired genotype were generated by breeding global Kalirin conditional knockout mice (KalSR$^{CKO/CKO}$) to mice expressing Cre-recombinase under the control of the POMC promoter (Balthasar et al., 2004). Cells that express POMC at any time during development are referred to as POMC cells; although they may not produce POMC in the adult, expression of POMC and Cre-recombinase during development means that Kalirin expression will have been eliminated in these cells. POMC cells that actually express POMC in the adult are referred to as POMC-producing cells. The major sites of POMC production in the adult include anterior pituitary corticotropes, intermediate pituitary melanotropes and POMC neurons in the arcuate nucleus of the hypothalamus and nucleus of the tractus solitarius (NTS) (Eipper and Mains, 1980; Khachaturian et al., 1985). The anterior pituitary POMC product, adrenocorticotropic hormone (ACTH), stimulates glucocorticoid production and secretion by the adrenal cortex; glucocorticoid receptors are found in many neurons and mediate the multiple effects of stress on nervous system function (Bhargava et al., 2000; Dallman et al., 1987; Young et al., 1990). POMC-producing neurons synthesize non-acetylated ACTH(1–13)$NH_2$ (Emeson and Eipper, 1986), which signals satiety (Ellacott and Cone, 2006), and β-endorphin, a potent endogenous opiate (Khachaturian et al., 1985). Behavioral and biochemical approaches were used to test the hypothesis that a lack of Kalirin expression in POMC cells contributes to a subset of the behavioral alterations observed in KalSR$^{KO}$ mice.

## Methods

In order to eliminate both the full-length and Δ-isoforms of Kalirin, *lox-p* sites were inserted both before and after Kalirin Exon 13 (Fig. 1A), which is 3′ to the Δ-isoform initiation site in the intron preceding Exon 11 (Mandela et al., 2012). The KalSR$^{CKO/CKO}$ mice thus created were of normal weight, reproduced well and had an unaltered distribution level of Kalirin isoforms. The floxed allele has been bred more than 15 generations into the C57Bl/6 background. Knockout of Kalirin in POMC neurons and endocrine cells was accomplished by mating KalSR$^{CKO/CKO}$ mice to POMC-Cre mouse lines that target expression of Cre-recombinase using the POMC promoter (JAX #5965). The pups were genotyped for the presence of both *lox-p* and Cre (Mandela et al., 2012). Mice were designated as Kalirin knockouts in POMC cells (KalSR$^{POMC-KO}$) when they expressed Cre-recombinase and were homozygous for the floxed Kalirin allele (KalSR$^{CKO/CKO}$). KalSR$^{CKO/CKO}$ mice (KalSR$^{CKO}$) were used as controls for KalSR$^{POMC-KO}$ mice; these mice were identical to wild type mice in all behavioral and biochemical tests done so far (Mandela et al., 2012).

### *POMC Cre-recombinase specificity*

Homozygous Rosa26-LacZ mice (R26R; JAX# 3474; B6.129S4-*Gt(ROSA)26Sor*$^{tm1Sor}$/J) were bred with the POMC-Cre mice from JAX. The offspring whose genomes contained both the Cre and β-galactosidase coding sequences were used to localize the sites of Cre-recombinase expression with X-gal staining. Adult mice were sacrificed, pituitaries and brains were collected, and frozen sections were prepared and stained with X-gal for *lacZ* (β-galactosidase) activity. Because the pattern of expression of Cre in the POMC-Cre mice has been a matter of controversy (Balthasar et al., 2004; King and Hentges, 2011; Morrison and Munzberg, 2012; Padilla et al., 2010, 2012), the POMC-Cre mice were also bred with homozygous Rosa26-TdTomato mice (JAX# 7905; B6.129S6-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J) and the pattern of TdTomato fluorescence was examined.