Conditional Knockout of Crhr2 in Serotonergic Neurons Decreases the Expression of *slc6a4* mRNA in the Ventral Dorsal Raphe Nucleus

By Joseph H. Malzbender

Department of Molecular, Cellular & Developmental Biology

University of Colorado Boulder

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Thesis Advisor:

Dr. Christopher A. Lowry, Department of Integrative Physiology and the Center for Neuroscience

Defense Committee:

Dr. Kevin Jones, Department of Molecular, Cellular & Developmental Biology and the Center for Neuroscience

Dr. Kenneth Krauter, Department of Molecular, Cellular & Developmental Biology and the Institute for Behavioral Genetics

Abstract

Exposure to stressors triggers the release of corticotropin-releasing hormone (Crh), which mediates the physiological and behavioral stress response through activation of corticotropin-releasing hormone receptor type-1 and 2 (CRHR2). Crhr2 is a G protein-coupled-receptor found on the surface of cells across the brain, including serotonergic neurons in the dorsal raphe nucleus (DRN). Studies performing global knockout of Crhr2 show anxiolytic effects, but cannot attribute these effects to changes in Crh signaling in specific neural systems. This study utilized a Pet1-driven Cre-mediated knockout of Crhr2 from serotonergic neurons to elucidate the effects of Crh signaling on serotonergic neurons in the DRN. To measure the impact of deletion of Crhr2 in serotonergic neurons on genes that are important modulators of serotonergic signaling, we measured mRNA expression levels of serotonin transporter gene *slc6a4*. Using *in situ* hybridization histochemistry we analyzed *slc6a4* mRNA expression across subregions of the DRN and median raphe nucleus. We found a localized decrease in the expression levels of *slc6a4* mRNA in the mid-rostrocaudal region of the ventral dorsal raphe nucleus (DRV). A decrease in *slc6a4* mRNA, if representative of levels of serotonin transporter protein expression and trafficking to the plasma membrane, would suggest an increase in the serotonergic signaling of brain regions innervated by this population of neurons. Crhr2 primarily has an excitatory role in neuronal signaling and deletion of Crhr2 would remove an important excitatory input to these neurons. Decreased *slc6a4* mRNA expression could implicate a possible compensatory role for the loss of excitatory Crhr2 in the mid-rostrocaudal DRV.

Keywords(5): coritcotropin-releasing hormone receptor type 2 (Crhr2), dorsal raphe nucleus (DRN), *in-situ* hybridization histochemistry, slc6a4, stress

Introduction

Corticotropin-releasing hormone (CRH) is a neuropeptide that is released in response to exposure of an organism to environmental stressors, and initiates the hypothalamic-pituitary adrenal (HPA) axis response by stimulation of secretion of adrenocorticotropic hormone from the anterior pituitary. CRH is also able to act as a neurotransmitter in the brain, and acts on two G protein-coupled-receptors: corticotropin-releasing hormone receptor-1 and 2 (CRHR1 and CRHR2). CRH and the related hormone urocortin (Ucn) are able to coordinate the stress response via the Crhr1 and Crhr2 receptors.

Experiments have shown that Crhr2-deficient mice have enhanced anxiety (Kishimoto et al, 2000) and a hyperactive HPA axis response to stress (Bale et al, 2000), suggesting that Crhr2 has an anxiolytic role. However, these findings were obtained using global knockout studies of Crhr2, and could not implicate a specific neural system in being responsible for these effects. It is also possible that this anxiolytic-like behavior in the global knockouts could be due to a compensatory mechanism changing the behavioral phenotype in these mice (Hammack et al, 2003). Other experiments contradict these findings, and have shown that when Crhr2 receptors were inactivated by antagonists, anxiety-like behavior was repressed (Takahashi et al, 2001; Bakshi et al, 2002), implicating Crhr2 to have an anxiogenic role.

Projections of serotonergic (5-hydroxytrptamine; 5-HT) neurons from the brainstem dorsal raphe nucleus (DRN) may have a role in producing anxiety (Graeff et al, 1996). In mice with central CRH over-expression it has been shown that levels of Crhr2 mRNA were significantly elevated in the DRN (Korosi et al, 2006), suggesting that Crhr2 in the DRN may mediate stress-induced release of serotonin. Using dual *in situ* hybridization histochemistry studies, it has been found that Crhr2 mRNA is exclusively expressed in serotonergic neurons of the mid-rostrocaudal levels of the DRN; in contrast, approximately half of Crhr2 mRNA is expressed in GABAergic neurons in caudal levels (Day et al, 2004). These results suggest that there is a link between serotonergic neuron activation and Crhr2. They also suggest that subregions of the DRN may have different roles in the behavioral effects of Crhr2 expression. Based on this it seems that conditional knockouts of Crhr2 are needed to be able to implicate a specific neural system and understand behavioral phenotypes.

To do this, a conditional knockout of Crhr2 specific to serotonergic neurons in mice was created. This was done using a Pet-1 Cre-lox system by breeding mice that had loxP sites flanking exon 4 of the Crhr2 gene, with mice that had Cre driven under an enhancer of Pet1, which is specific only to serotonergic neurons (Scott et al, 2005). We evaluated expression levels of genes that control serotonergic neurotransmission such as *hrt1a, slc6a4, and tph2*. This paper will focus only on the expression levels of *slc6a4 (solute carrier family 6, member 4).* Through this model we were able to gain insight into the sub-regional effects of serotonergic transmission within the DRN, and able to look at potential compensatory changes in response to Crhr2 knockout.

Slc6a4 gene encodes for an integral membrane transporter that transports serotonin from the synaptic cleft back into the presynaptic neuron in a sodium dependent manner, and terminates the action of serotonin. Polymorphisms of the *slc6a4* promoter have been linked to the anxiety-related trait of neuroticism in humans (Mazzanti et al, 1998). Analysis of *slc6a4* mRNA

expression using *in situ* hybridization histochemistry revealed a localized decrease in the mid-rostrocaudal ventral dorsal raphe (DRV) in mice with selective deletion of Crhr2 in serotonergic neurons.

Materials and Methods

Animals

All animals were housed in standard polycarbonate breeding cages (Makrolon Type II; 265 mm L x 205 mm W x 140 mm H); all water and food were available *ad libitum* under constant laboratory conditions (light cycle 12 hr : dark cycle 12 hr ; room temperature 22 °C; air humidity 55%). All experiments were performed in accordance with the guidelines of the government of Upper Bavaria, Germany for the care and use of laboratory animals.

Conditional Crhr2 knockout mice (Crhr2CKO mice)

Mice harboring a floxed *crhr2* allele were generated using an embryonic stem cell clone with conditional potential, which was obtained from the EUCOMM/KOMP consortium (http://www.mousephenotypeorg/martsearch_ikmc_project/about/eucomm.). In these mice exon 4 of the *crhr2* gene is flanked by two loxP sites and can be removed by Cre recombinase, causing disruption of the receptor expression in Cre-positive cells. Based on these floxed Crhr2 mice a set of conditional Crhr2 knockout mice was established using the Cre/loxP system. This approach allowed us to spatially control the inactivation of Crhr2 in those neurons that share similar neurochemical identity, while sparing its expression in others. Initially the conditional Crhr2 allele contained a LacZ reporter and selection cassettes which caused disruption of Crhr2 expression throughout all tissues. To restore the expression of Crhr2, these Crhr2 reporter mice (lacZ/LacZ) were bred with Flp-deleter mice (Rodriguez et al, 2000). From the F1 generation animals heterozygous for the conditional *crhr2* allele (+/lox) and carrying gene for Flp were selected and bred to wild-type mice in order to obtain mice heterozygous for the conditional *crhr2* allele and devoid of Flp recombinase. Homozygous offspring for the conditional *crhr2* allele (lox/lox) were selected and used for breeding to mice transgenic for neurotransmitter-specific Cre-recombinase (ePet-Cre mice for serotonergic systems). In the following generation we obtained offspring that were heterozygous for the floxed *crhr2* allele (+/lox) and carried Cre-recombinase. These mice were bred to double floxed *crhr2* mice (lox/lox). In subsequent experiments mice homozygous for *crhr2* (lox/lox) with (CKO) or without (Ctrl) the Cre-recombinase were used for experiments.

Crhr2$^{CKO\text{-}5HT}$ mice

To establish conditional Crhr2 KO mice with deletion of Crhr2 selectively in serotonergic neurons (Crhr2$^{CKO\text{-}5HT}$ mice), homozygous floxed control mice (Crhr2^{Ctrl}) were bred with mice expressing Cre recombinase under the control of the Pet1 enhancer, the expression of which is exclusive to serotonergic neurons (Scott et al., 2005). Once expressed, the Cre recombinase targets the floxed exon 4 of Crhr2, thereby silencing the expression of functional Crhr2, selectively in serotonergic neurons.

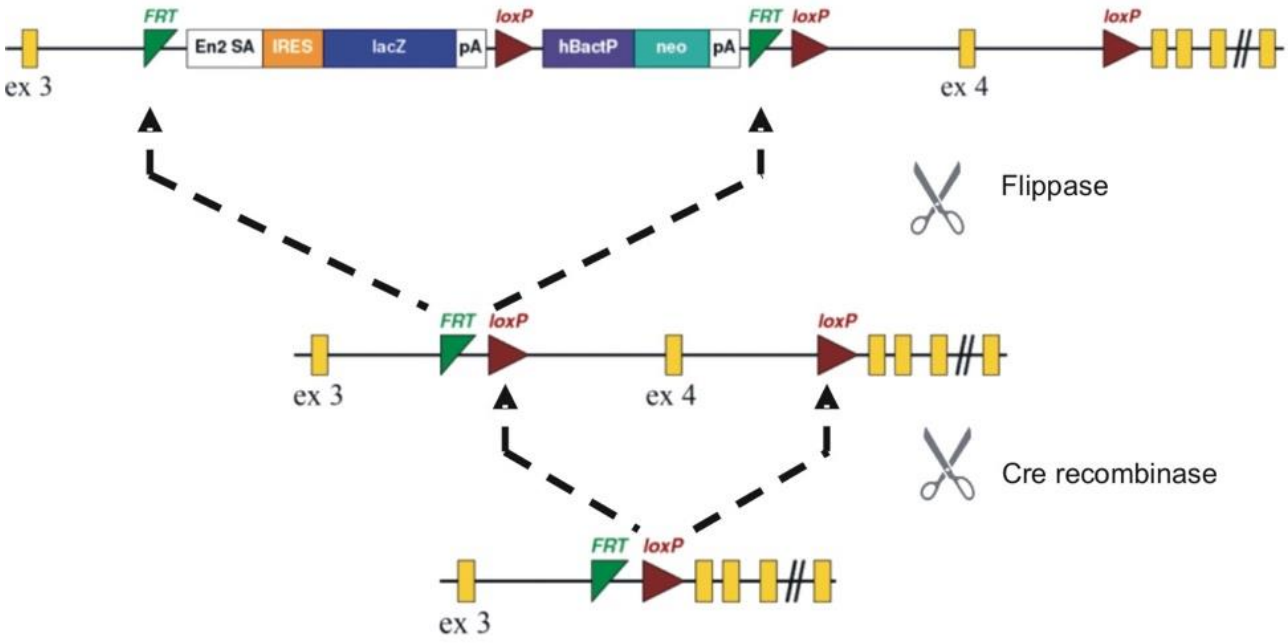

Figure 1. Simplified scheme for generation of conditional Crhr2 knockout mice (Crhr2CKO). The originally targeted *crhr2* locus contains a LacZ reporter and a Neo selection cassette. Upon Flp-mediated recombination both cassettes are deleted and the expression of Crhr2 is restored. Cre-mediated recombination leads to deletion of exon 4 with a downstream premature stop codon, which results in the functional inactivation of Crhr2.

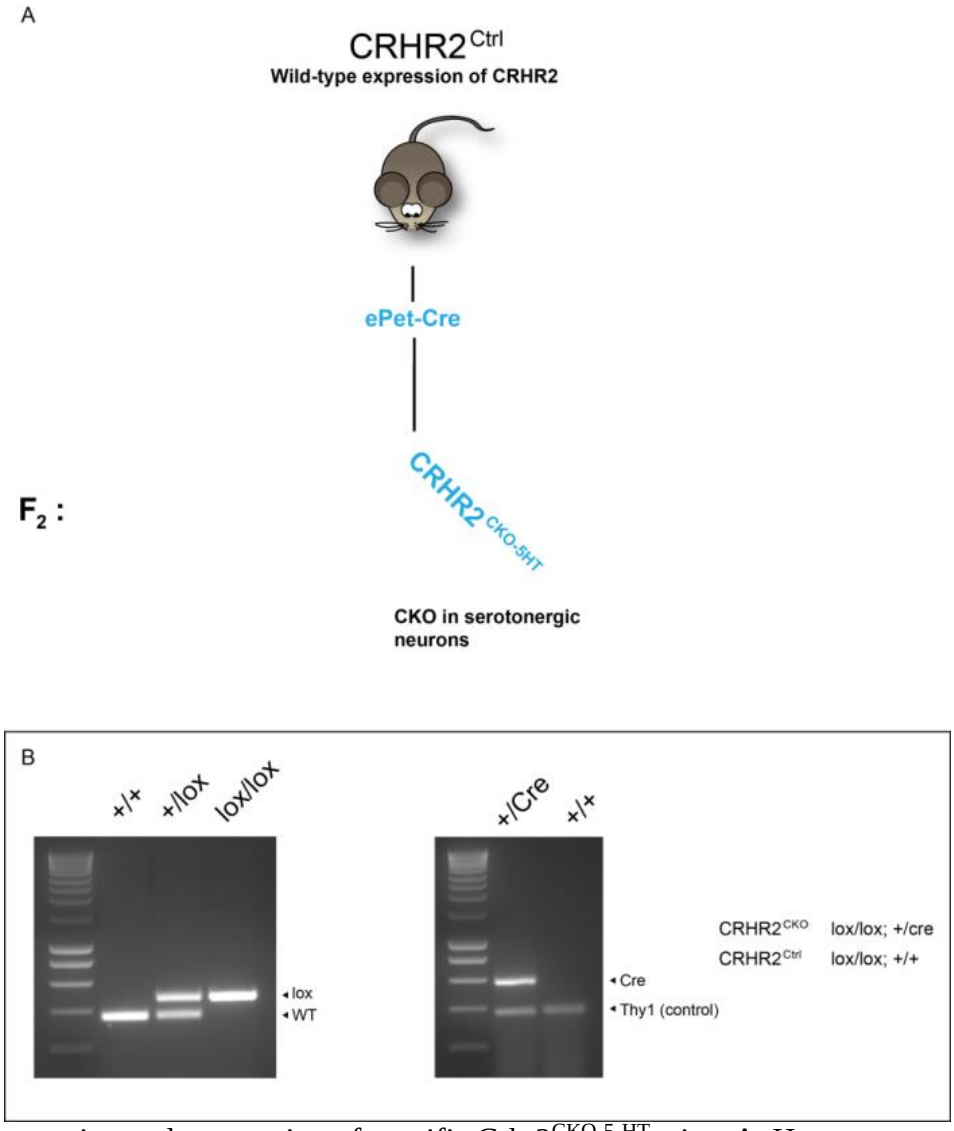

Figure 2. Generation and genotyping of specific Crhr2$^{CKO\text{-}5\text{-}HT}$ mice. A, Homozygous floxed mice were bred to a Cre-specific mouse line in which expression of Cre-recombinase is driven by the ePet promoter. B, Genotyping results of Crhr2 transgene and WT alleles and presence of Cre recombinase in Crhr2^{CKO} mice.

Tissue collection and processing

All animals were sacrificed via rapid decapitation. After decapitation, brains were removed, snap-frozen in ice-cold isopentane, and stored at -80 °C. Brains were placed on dry ice and shipped from the Max Planck Institute of Psychiatry to the University of Colorado Boulder. Brains were blocked into forebrain and hindbrain pieces with a cut in the coronal plane at the caudal border of the mammillary bodies (approximately -3.40 mm bregma) using a mouse brain matrix (RBM-2000C, ASI Instruments, Warren, MI, USA), to ensure a consistent coronal plane of sectioning. Brains were sectioned throughout the midbrain and pons using a Leica 1900 cryostat (North Central Instruments, Plymouth, MN, USA; 5 alternate sets of 12 µm sections). Brain tissue sections were mounted onto 75x25x1 mm, positively charged VWR Vistavision™ Histobond® microscope slides (16004-406, VWR International, Radnor, PA, USA) and stored at –80 °C.

Slc6a4 in situ hybridization histochemistry

For *in situ* hybridization histochemical analysis of *slc6a4* mRNA expression in the dorsal and median raphe nuclei, one oligonucleotide probe complementary and specific to *slc6a4* mRNA was synthesized. The complimentary base sequence of the synthetic oligonucleotide probe was (5'-ACTGCAGAGTACCCATTGGATATTTGGCTAGGCTCTGCCCTGTCCGCTGT-3') (Integrated DNA Technologies). Following transcription, probe was radiolabeled at the 3' end with [35-S]-deoxyadenosine-5'-(alpha-thio)-triphosphate (dATP, Cat. No. NEG034H001MC, PerkinElmer) using the enzyme terminal transferase (Promega) for 1 hr at 37 °C in a water bath to create a DNA oligonucleotide probe complementary to bases 207-256 of murine *slc6a4* mRNA (Hansen and Mikkelson, 1998).

The oligonucleotide probes were then cleaned (QIAquick Nucleotide Removal® kit, Cat. No. 28304, Qiagen, Valencia, CA, USA), and *in situ* hybridization histochemistry was performed (Gardner et al, 2009). Briefly, slides were equilibrated to room temperature and then immersed in 4% paraformaldehyde in 0.05 M phosphate buffered saline (PBS) for 10 min. Following two washes in 0.05 M PBS, slides were placed into freshly prepared 0.25% acetic anhydride in 0.9% NaCl containing 0.1 M triethanolamine (TEA) for 10 min. Sections were then dehydrated through a graded series of alcohol washes, delipidated in chloroform, rehydrated through a second series of alcohol washes, and then allowed to air dry.

Oligonucleotide probe hybridization solution (50% formamide, 20 × standard saline citrate (SSC), 25 mg/ml yeast tRNA, 10 mg/ml sheared salmon sperm DNA, 50X Denhardt's solution, 50% dextran sulphate, 10 mM dithiothreitol (DTT) and 1×10^6 cpm total radiolabeled probe) was placed on each slide (90 µl), covered with Parafilm M® coverslips, and incubated overnight in a humidified 37 °C chamber. The next day coverslips were removed in 1X SSC and each slide was washed with agitation 4 × 15 sec in 1X SSC. Slides were then put through 4 × 15 min washes in 1X SSC at 55 °C in a shaking water bath, 1X SSC at room temperature for 2 × 30 min and then briefly (1–2 sec) in distilled water at room temperature. Slides were then air-dried, and apposed to a BioMax MR autoradiography film (Cat. No. 871 5187, Carestream Health, Rochester, NY, USA) along with ^{14}C standards (Cat. No. ARC0146C, American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) for a period of 36 days.

Semi-quantitative analysis of slc6a4 mRNA expression

For semi-quantitative analysis of *slc6a4* mRNA expression, digital autoradiography images were analyzed with ImageJ (NIH, Bethesda, MD, USA), while the researcher was blinded to the treatment groups, to measure gray value x area (as a measure of mRNA expression) using matrices in the shape of each subdivision of the brainstem DRN or median raphe nucleus (MnR). Area (mm^2) was defined as the area (within each matrix) that fell above a gray value threshold that was kept consistent throughout analysis. Based on Gardner et al 2009, a total of 12 rostrocaudal sections, designated levels +6 to −5, containing 7 major subdivisions of the DR, were analyzed (dorsal raphe nucleus, dorsal part, DRD, −4.244 to −4.644 mm bregma; dorsal raphe nucleus, ventral part, DRV, −4.244 to −4.724 mm bregma; dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region, DRVL/VLPAG, −4.544 to −4.724 mm bregma; dorsal raphe nucleus, caudal part, DRC, −4.724 to −4.904 mm bregma; dorsal raphe nucleus, interfascicular part, DRI, −4.784 to −4.904 mm bregma) (Paxinos & Franklin, 2003). An average value was computed for the DRVL/VLPAG using values from both the left and right hemisphere. The MnR was examined from −4.544 mm −4.644 mm bregma. Background measurements were taken in the lateral periaqueductal gray. The average background gray value for each section was subtracted from the mean gray value for the above threshold pixels for each subregion; the resulting number was then multiplied by the area of the subregion (in mm) to generate a semi-quantitative measurement of gene expression within that region. Values for each of the 7 DR subdivisions were averaged at each rostrocaudal level, and average *slc6a4* mRNA expression levels in the entire DR were also calculated for each mouse. An atlas of *slc6a4* mRNA expression was used to aid in keeping the analysis consistent (Figure 3).

Statistical analysis

For statistical comparisons, the software package SPSS (version 22.0, SPSS Inc., Chicago, IL, USA) was used. Average levels of gene expression for each DRN subdivision at each rostrocaudal level of the DRN in each treatment group were generated. Outliers were identified using the Grubbs' test (Grubbs, 1969) and removed. Following outlier removal, an overall linear mixed model (LMM) with repeated measures (subregion and rostrocaudal level, with rostrocaudal level nested within subregion) was used to detect overall fixed effects of serotonin neurons with knocked out Crhr2 receptor ($KO^{R2\text{-}5HT}$), raphe subregion, rostrocaudal level and interactions among these factors (Table 1). Following this, additional LMMs were conducted on subsets of the data containing only data from individual DRN subdivisions to determine the locations of effects identified in the overall model. An autoregressive heterogeneous covariance structure was specified for all LMMs. Individual pairwise comparisons between $KO^{R2\text{-}5HT}$ and control mice were made using independent samples *t*-tests, the significance was set at $p < 0.05$ (Figure 4).

Results

The initial Linear Mixed Model (LMM) analysis showed a main effect in $KO^{R2\text{-}5HT}$, *Raphe subregion*, *Rostrocaudal level* as well as a $KO^{R2\text{-}5HT}$**Rostrocaudal level* interaction. The LMM analysis found a main effect of $KO^{R2\text{-}5HT}$ across all rostrocaudal levels ($F_{(1, 77.3)} = 6.710$, $p < 0.01$; Table 1). Using a LMM, analysis of *slc6a4* mRNA expression revealed an interaction between $KO^{R2\text{-}5HT}$ and *Rostrocaudal level(Raphe subregion)* ($F_{(74, 36.3)} = 3.5$, $p < 0.01$; Table 1). Follow up analyses of *slc6a4* mRNA expression in individual subregions revealed a main effect of $KO^{R2\text{-}5HT}$ only in the DRV ($F_{(1,125.3)} = 8.4$, $p < 0.01$; Table 1, Figure 4). Post hoc analyses revealed a localized decrease in *slc6a4* mRNA expression only in the DRV at −4.424 mm from bregma (Level 3), a more rostral region of the DR. At −4.544 mm from bregma (Level 1) of the DRV, the comparison approached statistical significance ($p = 0.074$). It should also be noted that mean *slc6a4* mRNA expression in entire DR did not show any significance ($F_{(1,26.0)} = 2.1$, $p < 0.2$; Table 1).

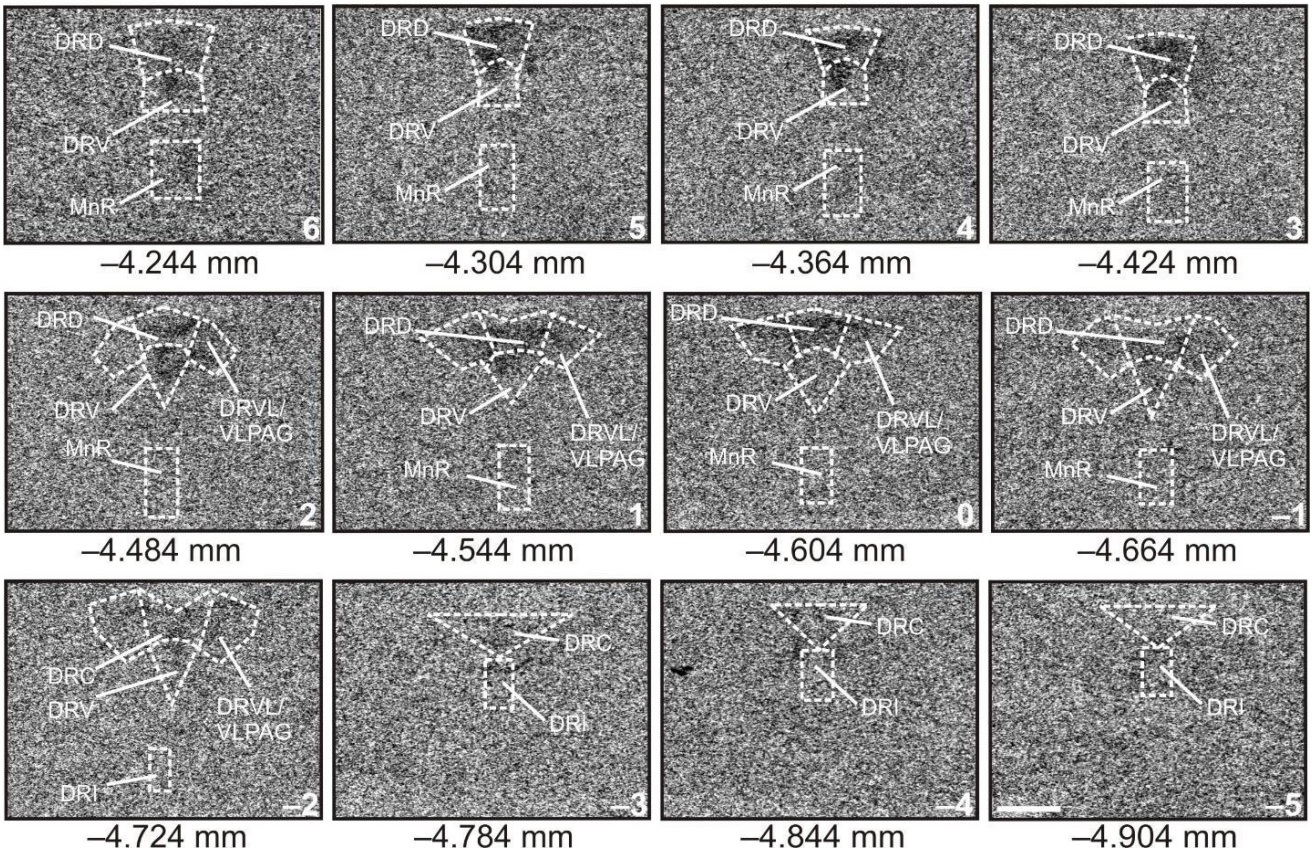

Figure 3. Atlas used to analyze *slc6a4* (solute carrier family 6, member 4) mRNA expression in subregions of the murine dorsal raphe nucleus and median raphe nucleus. Borders of each subregion are denoted by white dashed lines. Rostrocaudal location of each section is shown below each photomicrograph in millimeters from bregma. The white numbers in the bottom right of each panel indicate experimenter designated rostrocaudal level used in analysis. Scale bar, 500 µm. Abbreviations: dorsal raphe nucleus, caudal part (DRC); dorsal raphe nucleus, dorsal part (DRD); dorsal raphe nucleus interfascicular part (DRI); dorsal raphe nucleus, ventral part (DRV); dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region (DRVL/VLPAG); median raphe nucleus (MnR).

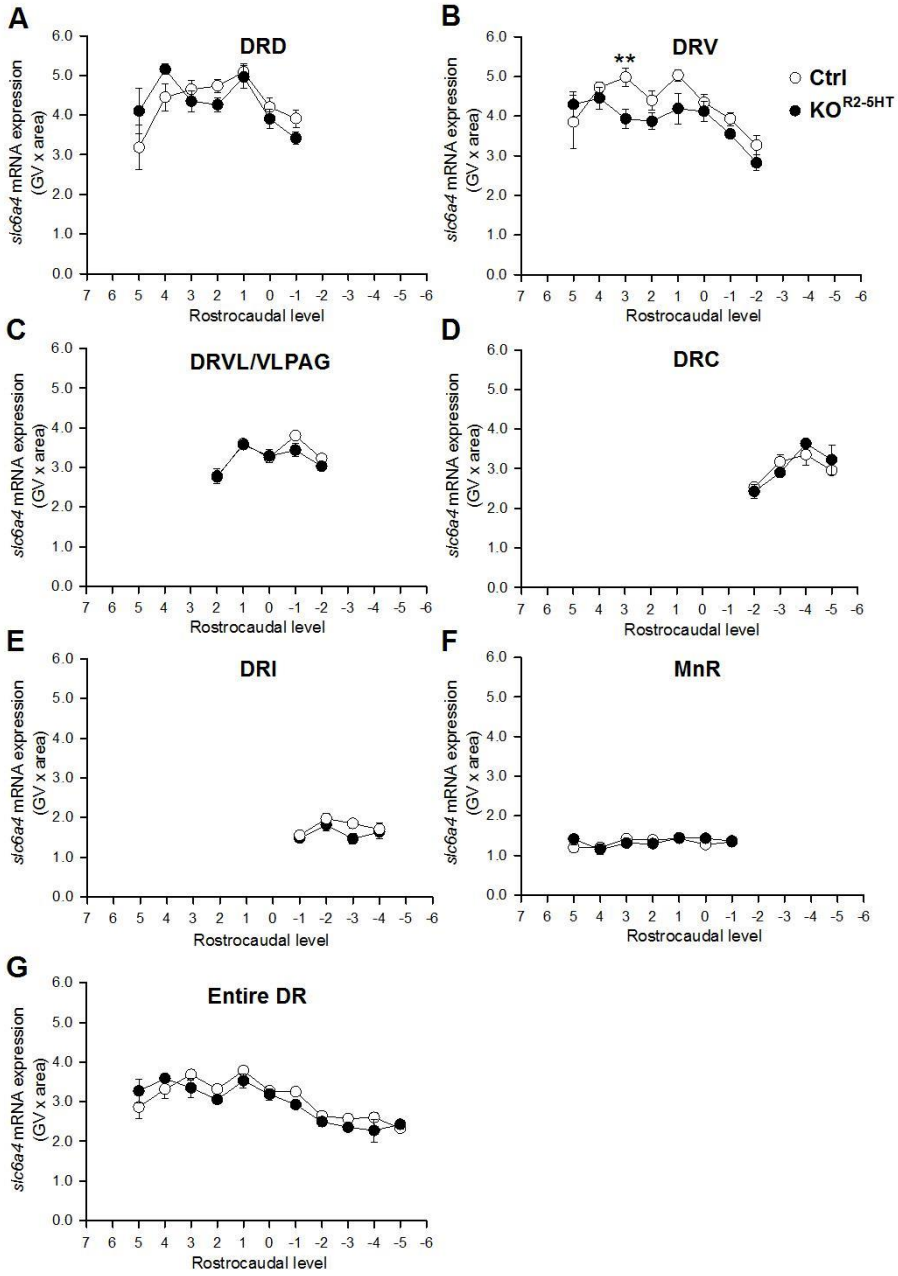

Figure 4. Expression of *slc6a4* (solute carrier family 6, member 4) mRNA across the rostrocaudal extent of the dorsal raphe nucleus and median raphe nucleus of fCrhr2xePet mice (KO $^{R25\text{-}HT}$) mice (black) and Crhr2 (lox/lox) control mice (white). Expression levels are indicated as average gray value multiplied by area ± SEM. Bregma levels are 0.60 μm apart, with Level 0 defined as –4.604 mm bregma. **p < 0.01 Post hoc independent samples *t*-test. Abbreviations: dorsal raphe nucleus, caudal part (DRC); dorsal raphe nucleus, dorsal part (DRD); dorsal raphe nucleus interfascicular part (DRI); dorsal raphe nucleus, ventral part (DRV); dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region (DRVL/VLPAG); median raphe nucleus (MnR); gray value (GV).

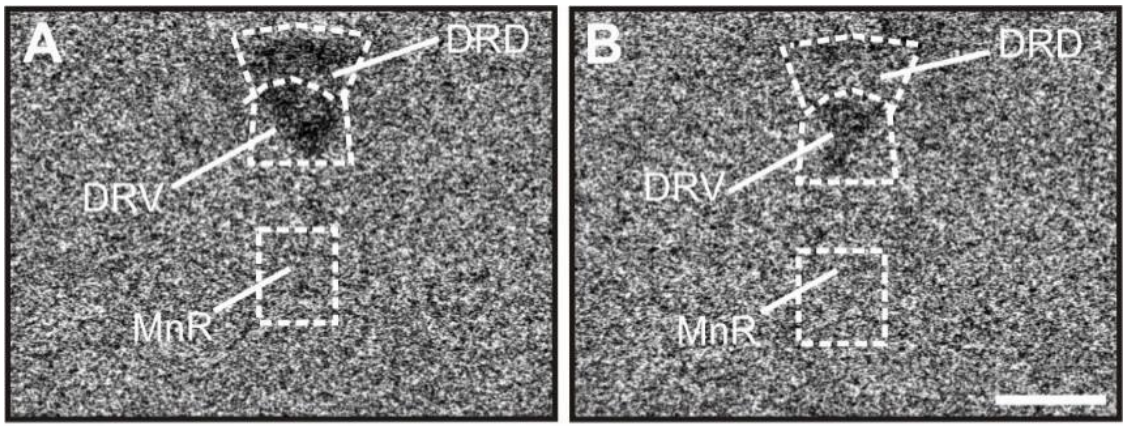

Figure 5: Photomicrographs illustrating *slc6a4* (solute carrier family 6, member 4) expression in Crhr2 (lox/lox) control mice (A) and fCrhr2xePet mice ($KO^{R2\text{-}5HT}$) (B) at –4.484 mm bregma. Scale bar, 500 µm. Borders of each subregion are denoted by white dashed lines. Abbreviations: dorsal raphe nucleus, dorsal part (DRD); dorsal raphe nucleus, ventral part (DRV); median raphe nucleus (MnR).

Table 1: Fixed effects of the Linear Mixed Models used to analyze *slc6a4* mRNA expression.

Model	Source	Test statistic	*p*-value
Overall Analysis			
Entire dataset			
	$KO^{R2\text{-}5HT}$	$F_{(1, 77.3)} = 6.710$	0.01
	Raphe subregion	$F_{(6, 59.2)} = 514.1$	0.001
	Rostrocaudal level	$F_{(10,49.5)} = 27.2$	0.001
	$KO^{R2\text{-}5HT}$*Rostrocaudal level(Raphe subregion)	$F_{(74,36.3)} = 3.548$	0.001
Subregional Analyses			
Mean *slc6a4* mRNA expression in entire DR			
	$KO^{R2\text{-}5HT}$	$F_{(1,26.0)} = 2.1$	0.2
	Rostrocaudal level	$F_{(10,19.4)} = 22.8$	0.001
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(10,19.4)} = 1.6$	0.2
DRD			
	$KO^{R2\text{-}5HT}$	$F_{(1,15.0)} = 0.001$	1.0
	Rostrocaudal level	$F_{(6,19.6)} = 8.3$	0.001
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(6,19.6)} = 1.3$	0.3
DRV			
	$KO^{R2\text{-}5HT}$	$F_{(1,25.3)} = 8.4$	0.008
	Rostrocaudal level	$F_{(7,15.7)} = 10.3$	0.001
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(7,15.7)} = 1.0$	0.4
DRVL/VLPAG			
	$KO^{R2\text{-}5HT}$	$F_{(1,24.8)} = 2.1$	0.2
	Rostrocaudal level	$F_{(4,22.2)} = 15.1$	0.001
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(4,22.2)} = 1.0$	0.5
DRC			
	$KO^{R2\text{-}5HT}$	$F_{(1,12.8)} = 0.2$	0.7
	Rostrocaudal level	$F_{(3,11.9)} = 10.7$	0.001
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(3,11.9)} = 1.3$	0.3
DRI			
	$KO^{R2\text{-}5HT}$	$F_{(1,14.2)} = 2.6$	0.1
	Rostrocaudal level	$F_{(3,26.8)} = 4.9$	0.008
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(3,26.8)} = 0.7$	0.6
MnR			
	$KO^{R2\text{-}5HT}$	$F_{(1,32.8)} = 0.2$	0.7
	Rostrocaudal level	$F_{(6, 60.6)} = 1.4$	0.03
	$KO^{R2\text{-}5HT}$*Rostrocaudal Level	$F_{(6,60.6)} = 0.8$	0.8

Discussion

The conditional knockout of Crhr2 in serotonergic neurons in the DRN showed decreased *slc6a4* mRNA expression in the mid-rostrocaudal region of the DRV (Figure 4), and expression levels were not changed in any other subregions of the DRN or the median raphe nucleus (MnR). The DRV is the main source of efferent projections to the sensory motor cortex, as well as projecting to the frontal cortex, motor cortex, visual cortex, and barrel field cortex. The DRV contains a high density of small round serotonergic neurons, and has a high density of tryptophan hydroxylase 2, serotonin transporter, and 5-HT receptors. Projections from the DRV to the caudate putamen and cortical targets suggests these neurons may be involved in cognitive and motor function. The DRV has also been associated with diseases such as anorexia, obsessive compulsive disorder, and Tourette's syndrome (Lowry et al, 2008).

Decreases in 5-HT transporter mRNA expression in the DRN and MnR have been found following 3 to 6 weeks of voluntary wheel running, and this may contribute to the anxiolytic properties of physical activity. More specifically they found that 6 weeks of voluntary wheel running decreased serotonin transporter mRNA levels in the dorsal and mid-rostrocaudal regions of the DRV, and not in caudal regions of the DRV (Greenwood et al, 2005). Similarly, our study found a decrease of *slc6a4* mRNA in a rostral level but not in the caudal levels of the DRV. The diminished *slc6a4* mRNA expression levels in the DRV could suggest a compensatory excitation for the serotonergic neurons that are lacking Crhr2. This could serve as a possible source for anxiolytic-like behavior observed in other Crhr2 knockout studies. These results can not implicate the DRN as the source for anxiolytic-like behavior observed, but suggests that a decrease of serotonin in the mid-rostrocaudal DRV may contribute to these behavioral phenotypes.

Using fMRI on humans performing a monetary decision-making task it was found that the lateral orbitofrontal cortex was activated by negative reward anticipation, losing outcomes, and evaluating the wrong choice (Liu et al, 2007). The lateral orbitofrontal cortex is known to project selectively to the DRV compared to other regions of the DRN and has been shown to have a role in processing unpleasant stimuli (Raison et al, 2015). If in fact a decrease of *slc6a4* mRNA is representative of a decrease in 5-HT transporters on serotonergic neurons in the mid-rostrocaudal DRV it would suggest increased activation of serotonin signaling in this subregion. If you also account for the fact that the DRV seems to play an important role in processing unpleasant stimuli, then it might suggest that this decrease of *slc6a4* mRNA could play a compensatory role to the loss of Crhr2 receptor. Activation of serotonergic neurons in the mid-rostrocaudal levels may be a way of processing a stressor. However, the mechanism between $KO^{R2\text{-}5HT}$ mice and serotonin transporter is unknown, and changes in *slc6a4* expression could be due to other neural systems' effects on the mid-rostrocaudal region.

From the results of *slc6a4* expression in the dorsal raphe nucleus and median raphe nucleus it is not possible to implicate these regions as the reason for the observed anxiolytic-like effects of Crhr2 knockout mice. One reason the DRN could not be implicated based on these results is because Pet1 is a driver for serotonergic neurons, but is not exclusive to the DRN, and is expressed in serotonergic neurons across the brain. Pet1, the driver for Cre expression is also not found in all serotonergic neurons in the dorsal raphe nucleus (Gaspar et al, 2012), suggesting that

there are serotonergic neurons in the DRN that were not deficient for Crhr2. Another reason is that the mechanisms behind the interaction of Crhr2 on *slc6a4* mRNA in the mid-rostrocaudal DRV are not known. However, based on the above arguments, the decrease in *slc6a4* mRNA expression in the mid-rostrocaudal DRV points to a possible compensatory change to potentiate signaling of serotonergic neurons in the DRV to help account for the decreased signaling that comes with Crhr2 knockout mice.

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