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Epigenetic regulation of the kappa opioid receptor gene by an insertion-deletion in the promoter region

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1 Supplementary Material (containing 1 Supplementary Table and 1 supplementary Figure)

28 references

Abstract

Preclinical and clinical studies have demonstrated that the kappa opioid receptor (KOR) regulates reward, hedonic tone and emotions. At therapeutic level, on-going clinical trials are assessing the potential of targeting the KOR for the management of depression, anxiety disorders and substance use disorders. However, genetic polymorphisms in the KOR gene that potentially contribute to its implication in these phenotypes have been poorly studied. Here we investigated an insertion-deletion in the promoter region of KOR (rs35566036), recently associated with alcohol addiction, in a cohort of depressed subjects who died by suicide, as well as psychiatrically healthy individuals. Focusing on 3 brain regions (anterior insula, anterior cingulate cortex, and mediodorsal thalamus), we characterized the functional impact of this structural variant on the expression and patterns of DNA methylation of the KOR gene, using qPCR and targeted Bisulfite-Sequencing, respectively.

While there was no significant change in the expression of KOR as a function of the insertion-deletion, or as a function of disease status in any brain region, we found that this variant strongly determines DNA methylation in KOR promoter, leading to a significant decrease in methylation levels of 8 nearby CpG dinucleotides located approximately 500 base pairs upstream the transcription start site. In addition, our results suggest a possible association between the insertion-deletion and depression; however, this result should be tested in larger populations. In sum, in this study we uncovered an epigenetic mechanism potentially contributing to KOR dysfunction in carriers of the insertion-deletion.

Keywords

Kappa opioid receptor, insertion-deletion, DNA methylation, gene expression, genotype, depression

Introduction

The kappa opioid receptor (KOR) belongs to the endogenous opioid system, and is known to tightly regulate essential physiological functions, including reward (Wee and Koob, 2010), mood (Lutz and Kieffer, 2013), and stress homeostasis (Bruchas et al., 2010). Over the last two decades, it has been demonstrated that dysregulation of KOR function is implicated in the pathophysiology of numerous psychiatric disorders such as anxiety, depression and addiction (Lalanne et al., 2014). Pharmacological research also suggests that KOR antagonists might have beneficial properties in the treatment of depressed mood (Falcon et al., 2016) and anxiety-related symptoms (Carlezon and Krystal, 2016), as well as in the prevention of stress-induced relapse in drug addicts (Lalanne et al., 2017; Lalanne et al., 2014), among others.

Genetic polymorphisms in the opioid system, including the KOR gene, have been largely investigated for their potential association with addiction to alcohol, heroin and cocaine (Butelman et al., 2012). Beyond substance use disorders, it is possible that KOR genetic variants may also impact on the risk of major depressive disorder. The later aspect, however, has been relatively poorly documented, with only limited evidence for an association between KOR polymorphisms and stress sensitivity (Xu et al., 2013).

An insertion-deletion was identified in 2008 by Edenberg et al. (2008) in the KOR promoter region (INDEL, rs35566036). This variant corresponds to the deletion of 11 base pairs (bp) from -1975 to -1985 upstream the transcription start site, and to the insertion of 841 bp at -1986, resulting in a net insertion of 830 bp. Luciferase assays provided *in vitro* evidence suggesting that the INDEL might lower the transcriptional activity of the KOR promoter. Surprisingly, to our knowledge, the *in vivo* relevance and potential implication in depression of this variant remains currently unexplored. Therefore, this study was designed to characterize how the expression and epigenetic landscape of the KOR locus might be affected in brain tissue by the INDEL. Focusing on a cohort of depressed subjects who died by suicide, and psychiatrically healthy individuals as control group, we genotyped the INDEL and quantified KOR expression in 3 brain regions implicated in mood control: the anterior insula (AI), the dorsal part of the anterior cingulate cortex (ACC), and the mediodorsal thalamus (MDT). We chose to focus our analyses on these three brain regions, due to their well-known involvement in emotional regulation and to remain consistent with a former study of ours (Lutz et al., 2015b). Furthermore, we characterized

the impact of the INDEL on DNA methylation, a form of epigenetic plasticity that modulates gene activity, brain function and behaviour (Lutz et al., 2015a; Lutz et al., 2017a).

Our results indicate that the INDEL does not directly affect KOR expression *in vivo*, at least in the brain regions examined, while it strikingly predicts DNA methylation within the KOR promoter. We propose that such major DNA methylation differences occurring as a function of the INDEL may contribute to epigenetic plasticity at the KOR locus, and help explain how this variant might be implicated in addictive or depressive conditions.

Experimental procedures

Cohort (Table 1)**.** Our cohort consisted of both males and females who were depressed and died by suicide, as well as psychiatrically healthy controls who died by accidental causes. Brain tissue was obtained from the Douglas-Bell Canada Brain Bank. Psychological autopsies were conducted for each subject as described elsewhere (Lutz et al., 2017b). Tissue samples from three brain regions (AI, ACC, MDT) were dissected as described in Supplementary Material (see also (Lutz et al., 2015b)).

Quantitative polymerase chain reaction (qPCR). qPCR was performed as described previously (Lutz et al., 2015b) to quantify the expression of the *OPRK1* gene encoding the KOR, in quintuplicates, using 2 housekeeping genes (GAPDH, β-actin; Table 2). Only samples with a RNA integrity number (RIN) >5 were used (5 out of 82 subjects discarded).

***Genotyping*.** Allele frequencies for the INDEL were determined by PCR amplification of brain DNA (Table 1), using a Platinum *Taq* DNA Polymerase (Invitrogen®) and parameters and primers described by Edenberg *et al* ((2008), and see supplementary material).

DNA Methylation. DNA methylation was analysed as described by Masser et al (see (2013) and supplementary material). Briefly, genomic DNA was bisulfite converted, and then amplified by PCR (see primers in Supplementary Table 1). Libraries were prepared using the NEXTERA XT kit, indexed by PCR, and sequenced on an Illumina MiSeq.

***Bioinformatic analysis*.** As described previously (Chen et al., 2017), adaptors were trimmed from sequencing reads, which were further trimmed when Phred quality scores dropped below 20. Bismark v0.14.4 was run with Bowtie 2 to align reads to the hg19 human genome. The extraction of the number of unconverted and converted cytosine for each CG was done with the script bismark_methylation_extractor provided with Bismark. All cytosines had coverages above 10, and therefore all data were included into statistical analyses. The DNA methylation percentage was calculated for each cytosine as the unconverted (cytosine) read counts divided by total read counts (cytosine and thymine).

Statistical analysis. Statistical analyses were carried out by the IBM SPSS package v23. Frequencies of the INDEL were analysed using χ^2 test. For KOR expression, a general linear model was used to analyse group differences. Potential correlations between KOR expression and variables that commonly act as confounders (brain pH, *post-mortem* interval PMI, RIN, and age) were assessed separately for each brain region of interest, and only those variables showing a significant Pearson correlation were included in each model. Averaged DNA methylation levels were analysed similarly using a GLM. DNA methylation values at individual CG sites in KOR promoter were analysed using a repeated-measures ANOVA, with CG sites as the within-subject factor. Statistical significance was set at $p<0.05$.

Results

Frequency of the INDEL. A total of 82 subjects (Table 1) were genotyped for the KOR INDEL (Fig.1A). Observed and expected frequencies by genotype are presented in Fig.1B. The distribution of the INDEL in control subjects was in Hardy-Weinberg equilibrium (HWE), as assessed using a χ^2 test (df=1, N=30): $\chi^2=0.52$, $p=0.47$. Genotypic association analysis, using a dominant model, demonstrated a significant association between presence of the INDEL and clinical group (df=1, N=82): $\chi^2=6.41$, $p=0.01$. A binary logistic regression analysis revealed that the presence of the INDEL significantly associated with depression $p=0.014$; OR=3.55; 95% CI 1.30 - 9.70.

Effects of the INDEL on KOR expression. In AI, KOR mRNA levels are positively correlated with age (r=0.359, N=77, p=0.001) and RIN (r=0.226, N=77, p=0.048). While controlling for the effects of age and RIN, we found no main effect of genotype, N: SS=44, SL=33 (F(1,73)=0.005, p=0.946) (Fig.2), sex (F(1,73)=0.244, p=0.623), substance use disorder (F(1,73)=0.003, p=0.958), or depression (F(1,73)=0.143, p=0.706) on KOR expression.

In the ACC, brain pH showed a significant negative correlation with KOR expression (r=-0.311, N=46, p=0.035). While controlling for the effect of brain pH, we found no main effect of genotype, N: SS=28, SL=18 (F(1,43)=0.766, p=0.386) (Fig.2), sex (F(1,43)=0.042, p=0.838), substance use disorder (F(1,43)=2.671, p=0.116), or depression (F(1,43)=1.688, p=0.201) on KOR expression.

In the MDT, PMI positively correlated with KOR expression (r=0.294, N=45, p=0.050). While controlling for the effect of PMI, we found no main effect of genotype, N:SS=27, SL=17 (F(1,42)=0.190, p=0.665) (Fig.2), sex (F(1,42)=0.052, p=0.821), substance use disorder (F(1,42)=1.776, p=0.190), or depression (F(1,42)=2.717, p=0.107) on KOR expression.

Effects of the INDEL on DNA methylation. We generated base-resolution DNA methylation data in 5 genomic regions of *OPRK1* (Fig.3A). Because the INDEL had no effect on KOR expression in any brain region investigated, we arbitrarily focused on DNA extracted from anterior insula tissue. As expected, levels of DNA methylation were very low (below 5%, Fig.3B) in the CG island covering exons 1 and 2 (Weber et al., 2007). While brain pH, PMI and gender had no significant effects on DNA methylation in any genomic region, age significantly positively correlated with DNA methylation in exon1 (r=0.28, N=77, p=0.015) and exon2 (r=0.40, N=77, p<0.001). The latter effects of age which, although statistically significant, were subtle, are consistent with previous genome-wide data showing that increasing DNA methylation levels during aging occurs in restricted CG sites, notably within CG islands (Horvath, 2013; Jones et al., 2015).

There was no significant effect of depression and suicide, or substance use disorder, on DNA methylation in any genomic region (data not shown). In contrast, we found that the INDEL very strongly correlated with average DNA methylation levels in the Promoter region (as assessed with a 470-bp amplicon interrogating 10 CG sites, see Fig.3A), where heterozygotes (SL) showed decreased DNA methylation levels (F(1,75)=28.1, p<0.0001). Looking at individual

CG sites within this region, a repeated-measures ANOVA (Fig.3C) confirmed the significant effect of the INDEL ($F(1,75)=28.1$, $p<0.0001$), found significant DNA methylation differences among CG sites ($F(9,675)=870$, $p<0.0001$) and a significant interaction between the 2 factors ($F(9,675)=2.29$, $p=0.015$). Post-hoc comparisons with Bonferroni corrections showed that DNA methylation was lower in SL carriers for 8 CGs: CG1 ($p<0.0001$), CG2 ($p=0.014$), CG5 ($p=0.0004$), CG6 ($p<0.0001$), CG7 ($p=0.034$), CG8 ($p=0.022$), CG9 ($p=0.0003$), and CG10 ($p<0.0001$), while there was no difference for CG3 ($p=0.76$) and CG4 ($p=0.10$). In contrast, there was no effect of the INDEL in any other genomic region: exon 1 ($F(1,74)=3.41$, $p=0.069$), controlling for age; exon 2 ($F(1,74)=0.44$, $p=0.51$), controlling for age; exon 3 ($F(1,75)=2.76$, $p=0.10$); exon 4 ($F(1,75)=0.009$, $p=0.92$). Finally, because DNA methylation has been shown to affect mRNA splicing, we conducted an isoform-specific expression analysis, which failed to detect any effect of genotype on 2 KOR transcripts (data not shown): NM_000912.4 ($F(1,73)=0.504$, $p=0.48$) or NM_001282904.1 ($F(1,74)=0.013$, $p=0.91$), while controlling for age and RIN, respectively.

Discussion

The present study was designed to explore the potential implication of the KOR INDEL in KOR regulation and depression pathophysiology. Our results show that the INDEL was more frequently detected in depressed suicide completers than controls (Fig.1B), suggesting a possible association. Because it is now widely acknowledged that individual genetic variants only account for a very small fraction of the phenotypic variability associated with depression, the present result obtained in a small cohort may represent a false positive that may not stand replication in larger cohorts of depressed subjects. It appears nevertheless consistent with the association previously reported between alcoholism and the KOR INDEL, and with the rodent literature documenting the role of KOR in both addiction and depression models (Lalanne et al., 2014).

Preclinical data indicate that the mechanisms through which KOR interacts with reward processes and mood likely involve the regulation of multiple brainstem monoaminergic nuclei and cortical structures (Lalanne et al., 2014). In human, a recent PET-Scan study suggested that trauma-related psychopathology associates with changes in KOR availability in the ACC and AI (Pietrzak et al., 2014). We therefore hypothesized that the regulation of KOR expression by the

INDEL might account for its relationship with depression in key brain structures. We however, found no differential KOR expression in the AI, ACC, or MDT of depressed suicide completers compared with psychiatrically healthy individuals. These findings are consistent with Peckys and Hurd (2001) whose data did not provide evidence of impaired KOR expression in the ACC or dorsolateral prefrontal cortex of depressed subjects. A potential explanation for these negative findings is that depression-associated dysregulation of KOR signalling may occur in other brain structures, and may result from altered expression of dynorphins, its endogenous ligands, as opposed to receptor expression. Accordingly, downregulation of prodynorphin mRNA has been reported in amygdala nuclei, in the periamygdaloid cortex, and in the caudate nucleus of depressed subjects (Anderson et al., 2013; Hurd, 2002; Hurd et al., 1997).

Our results further indicate that presence of the INDEL did not result in any change in KOR expression in any brain region investigated, nor did it seem to affect any gene located nearby the OPRK1 locus (see Supplementary Figure 1). These findings contrast with results obtained by Edenberg et al. (2008) in HepG2 cells. The authors reported that the transcriptional activity of a Luciferase reporter gene, when driven by a construct containing the minor INDEL allele of the KOR promoter region, was decreased in comparison with the unmodified KOR promoter. Since our sample did not contain subjects who were homozygous for the INDEL, this discrepancy may result from a dosage effect, whereby a single wild-type allele may be sufficient in brain tissue to mask a potentially lower expression by the allele carrying the INDEL. KOR expression and DNA methylation was measured herein at the levels of both alleles combined, and future studies will be necessary to clarify this point. Finally, it is possible that the impact of the INDEL variant on the genomic architecture and transcriptional regulation of the KOR native locus may not be completely recapitulated by an artificial genomic construct expressed in non-neuronal cells.

Considering that the length and base composition of DNA are major determinants of epigenetic regulatory processes, we next hypothesized that the INDEL, which represents a net insertion of 830bp in KOR promoter region, might impact on DNA methylation. Indeed, our results demonstrate that the presence of the INDEL leads to an average 3.9% reduction in levels of DNA methylation over 10 consecutive CG sites. This promoter region located immediately downstream from the INDEL was the only site affected, as there was no effect of genotype on other genomic regions more distantly located along the KOR gene. Notably, in a study by Ji et al

(2015) methylation levels at 3 CG sites located in the KOR promoter region were increased in peripheral blood samples from Alzheimer's patients versus healthy controls. The authors determined using a luciferase reporter assay that the promoter fragment containing these CG's up-regulated gene expression. Accordingly, the significant DNA methylation differences that we found associated with KOR INDEL at the level of the promoter region may have an impact on this locus activity, one that could involve alterations in the recruitment of regulatory factors. Interestingly, the ENCODE consortium documents a putative binding site for the EZH2 transcription factor (a member of the Polycomb repressive complex and an essential regulator of epigenetic gene silencing (Vire et al., 2006)) within the Promoter region where we observed differential methylation as a function of the INDEL. Therefore, differential DNA methylation as a function of the INDEL might possibly interfere with EZH2 binding in KOR promoter.

Our data do not support a direct relationship between KOR expression and the INDEL, at least in the 3 brain regions examined, or in the sub-genual ACC (Lutz et al., 2017b) (see Supplementary Figure 1). Such a relationship remains possible in other brain regions. Therefore, additional investigations will be crucial to completely understand whether KOR expression is dysregulated in the depressed brain or in relation to other conditions involving KOR signalling (e.g. addiction, pain), and the contribution of the INDEL and DNA methylation changes to these processes. In addition, we speculate that decreased DNA methylation in the KOR promoter of subjects carrying the INDEL might be responsible for a differential molecular reactivity of this genomic locus, which may only unveil under certain circumstances (eg, stress), and may contribute to psychopathology. Of note, such "meta-plasticity" has been suggested in animal models for other genomic loci (Baker-Andresen et al., 2013), and shown to contribute to behavioural adaptation. For instance, maternal deprivation in male rats has been shown to induce DNA demethylation of the corticotropin-release hormone (CRH) promoter in the hypothalamus of adult rats (Chen et al., 2012). Despite such differential methylation, basal CRH expression was not affected. However, following an acute restraint stress in adulthood, maternally deprived rats had significantly higher CRH overexpression in comparison to controls, unveiling a latent enhanced vulnerability to stress. In summation, our study unravels how the INDEL in the Promoter region of the KOR gene impacts on its epigenetic landscape. Future studies will be necessary to confirm whether this genetic variant truly represents a risk factor for depression, and how the associated epigenetic modifications affect KOR-dependent processes in the living brain.

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Figure legends

Table1. Sample Demographics. The study population consisted of 52 MDD+suicide (mean age ± SD=46.2±15.4 years) and 30 matched controls (46.8±21.1 years). For MDD+suicide and control groups, 35% and 13%, respectively, had a substance use disorder. No statistically significant differences were observed between the MDD+suicide and control groups for gender, age, brain pH, PMI, and RIN values.

Figure1. A) The Kappa opioid receptor insertion-deletion (INDEL) was genotyped using the primers and methods described previously by Edenberg et al. (Baker-Andresen et al., 2013), yielding a 336 bp band without the INDEL, in homozygous SS carriers, or a 1166 bp band with the INDEL, in heterozygous SL carriers. **B)** 2x2 contingency table of observed and (expected) genotype frequencies for each group.

Figure2. Effect of the INDEL on KOR expression in the anterior insula (AI, N: SS=44, SL=33), anterior cingulate cortex (ACC, N: SS=28, SL=18), and mediodorsal thalamus (MDT, N: SS=27, SL=17) of all subjects, independent of clinical grouping. There was no significant effect of the INDEL on expression levels of KOR mRNA in any of the brain areas investigated. Values are mean ± sem.

Figure3. Effects of the INDEL on *OPRK1* DNA methylation in the anterior insula (AI) of all subjects, independent of clinical grouping (N: SS=44, SL=33). **A)** Diagram of the *OPRK1* locus and of the 5 targeted genomic regions. **B)** Presence of the INDEL had a significant effect on average DNA methylation across the promoter but not exon 1 – 4. **C)** Base-resolution DNA methylation across the promoter revealed significant CpG site specific differences by presence or absence of the INDEL. Values are mean ± sem. * $p<0.05$, ***$p<0.001$, ****$p<0.0001$.

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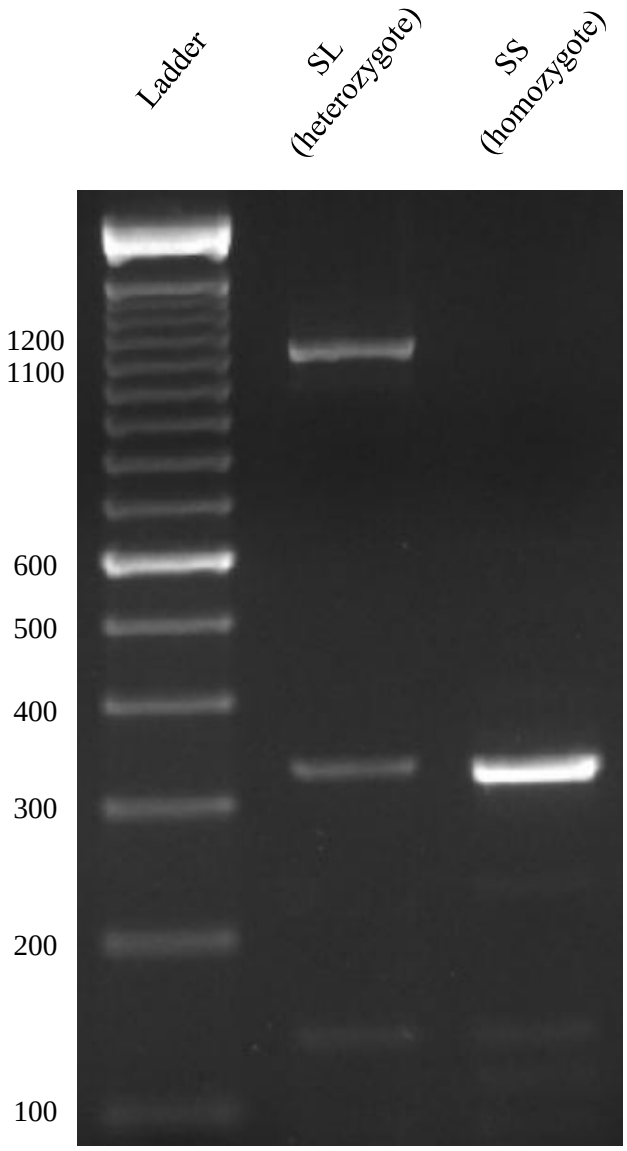
Table 1

	Depressed Suicides	Controls
N	**52**	**30**
Male/Female	42/10	24/6
Age (years)	46.2 ± 15.4	46.8 ± 21.1
Mood disorder	52	0
Alcohol/Drug Abuse	18	4
Brain pH	6.58 ± 0.36	6.45 ± 0.30
PMI (h)	24.4 ± 18.6	24.1 ± 19.7

Figure 1

A)



B)

	Depressed Suicides	Controls	
Genotype	Counts		*Row Totals*
SS	25 (30.4)	23 (17.6)	48
SL	27 (21.6)	7 (12.4)	34
Column Totals	52	30	82

Odds Ratio:
P = 0.014; OR = 3.55; 95% C.I 1.30 - 9.70

Figure 2

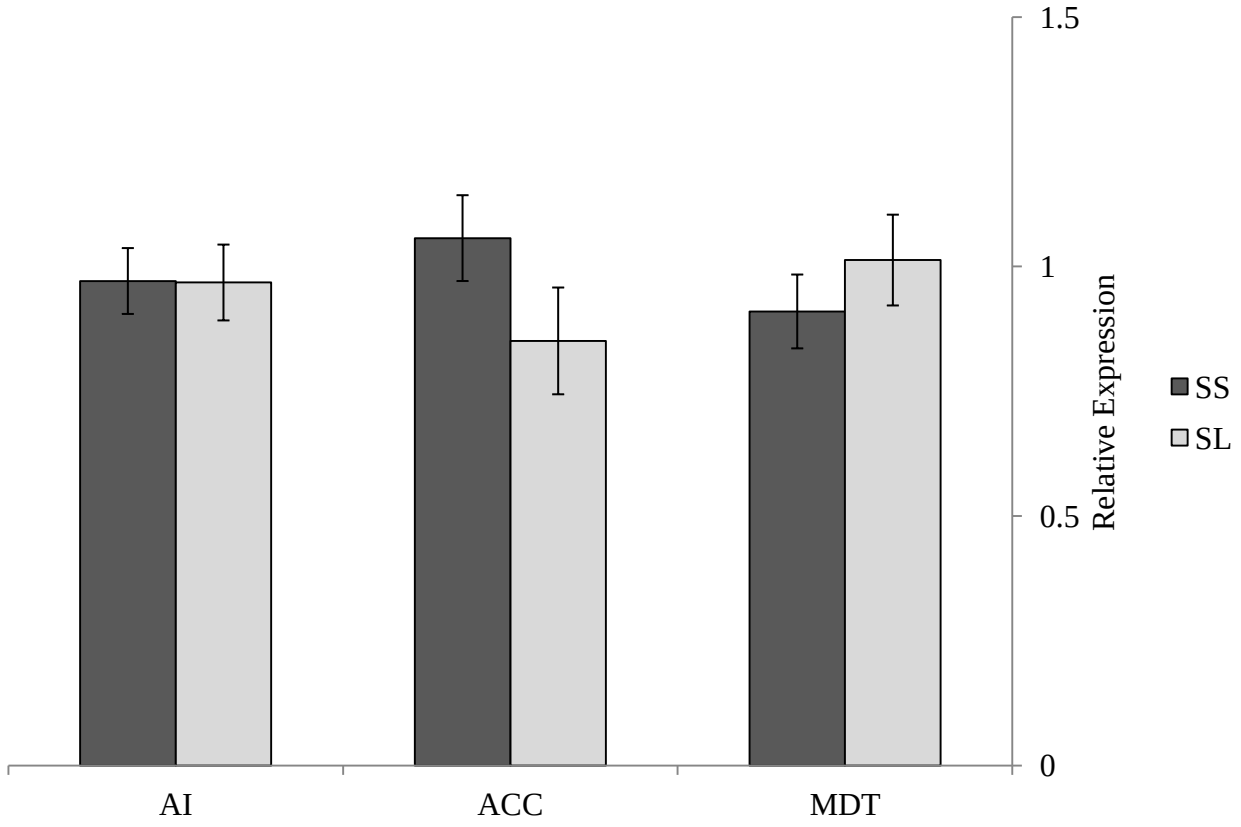
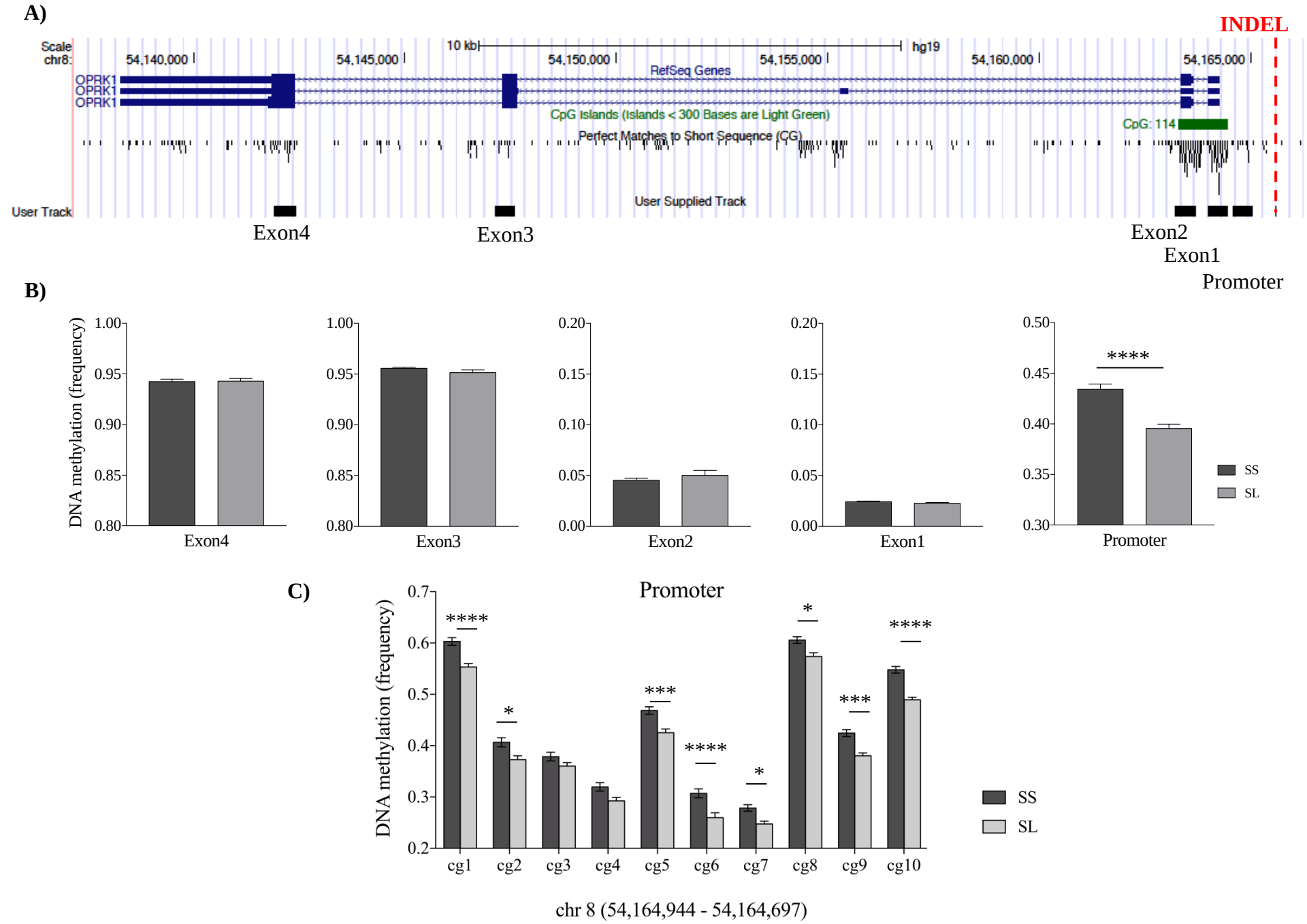


Figure 3


A)
INDEL
Scale
chr8:
54,140,000
54,145,000
10 kb
54,150,000
54,155,000
hg19
54,160,000
54,165,000
RefSeq Genes
OPRK1
OPRK1
OPRK1
CpG Islands (Islands < 300 Bases are Light Green)
CpG: 114
Perfect Matches to Short Sequence (CG)
User Track
User Supplied Track
Exon4
Exon3
Exon2
Exon1
Promoter
B)
DNA methylation (frequency)
1.00
0.95
0.90
0.85
0.80
0.20
0.15
0.10
0.05
0.00
0.50
0.45
0.40
0.35
0.30
****
Exon4
Exon3
Exon2
Exon1
Promoter
SS
SL
C)
Promoter
DNA methylation (frequency)
0.7
0.6
0.5
0.4
0.3
0.2
****
*
***
****
*
*
***
****
cg1
cg2
cg3
cg4
cg5
cg6
cg7
cg8
cg9
cg10
SS
SL
chr 8 (54,164,944 - 54,164,697)

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Contributors

PEL designed the study. PEL and DA conducted all experiments. PEL, DA and RB undertook the statistical analysis. PEL and DA wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to declare.