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Genome-wide DNA hydroxymethylation identifies potassium channels in the nucleus accumbens as discriminators of methamphetamine addiction and abstinence

Jean Lud Cadet[1], **Christie Brannock**[1], **Irina N. Krasnova**[1], **Subramaniam Jayanthi**[1], **Bruce Ladenheim**[1], **Michael T. McCoy**[1], **Donna Walther**[1], **Arthur Godino**[2], **Mehdi Pirooznia**[3], **Richard S. Lee**[3]

[1]Molecular Neuropsychiatry Research Branch, NIDA Intramural Research Program Baltimore, MD, USA

[2]Département de Biologie, École Normale Supérieure de Lyon, Lyon, France

[3]Department of Psychiatry and Behavioral Sciences, Johns Hopkins School of Medicine, Baltimore, MD, USA

Abstract

Epigenetic consequences of exposure to psychostimulants are substantial but the relationship of these changes to compulsive drug taking and abstinence is not clear. Here, we used a paradigm that helped to segregate rats that reduce or stop their methamphetamine (METH) intake (non-addicted) from those that continue to take the drug compulsively (addicted) in the presence of footshocks. We used that model to investigate potential alterations in global DNA hydroxymethylation in the nucleus accumbens (NAc) because neuroplastic changes in the NAc may participate in the development and maintenance of drug-taking behaviors. We found that METH-addicted rats did indeed show differential DNA hydroxymethylation in comparison to both control and non-addicted rats. Non-addicted rats also showed differences from control rats. Differential DNA hydroxymethylation observed in addicted rats occurred mostly at intergenic sites located on long and short interspersed elements (LINEs and SINEs, respectively). Interestingly, differentially hydroxymethylated regions (DHMRs) in genes encoding voltage (Kv1. 1, Kv1. 2, Kvb1, and Kv2. 2)- and calcium (KCNMA1, KCNN1 and KCNN2)-gated potassium channels observed in the NAc of non-addicted rats were accompanied by increased mRNA levels of these potassium channels when compared to mRNA expression in METH-addicted rats. These observations indicate that changes in DHMRs and increased expression of specific potassium

Address correspondence to: Jean Lud Cadet, M. D., Molecular Neuropsychiatry Research Branch, DHHS/NIH/NIDA IRP, 251 Bayview Boulevard, Baltimore, MD 21224, (443) 740-2656 (Phone), (443) 740-2856 (Fax), jcadet@intra.nida.nih.gov.

Author contributions

JLC, the corresponding author, supervised all aspects of the experiment, performed IPA analysis, and wrote the manuscript. CB performed IPA and PANTHER analyses of the data. INK wrote the animal protocol and performed the self-administration experiment. SJ performed the hMeDIP assay and contributed to the writing of the paper. BL, MTM, and DW performed quantitative PCR analyses. BL and MTM also dissected rat brains and provided technical assistance with the self-administration experiment. AG assisted with data analysis. MP performed the bioinformatics analyses on the hMeDIP sequencing data. RSL performed the hMeDIP sequencing experiments.

Conflict of interest

The authors declare no conflict of interest.

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channels in the NAc may promote abstinence from drug taking behaviors. Thus, activation of specific subclasses of voltage- and/or calcium-gated potassium channels may provide an important approach to the beneficial treatment for METH addiction.

Keywords

DNA hydroxymethylation; addiction; epigenetics; self-administration

INTRODUCTION

Drug self-administration (SA) offers an important model to investigate transcriptional and epigenetic programs that may drive the transition to addicted states after initial drug exposure [1–3]. Behavioral phenomena observed during drug SA are regulated by a diversity of interconnected but distinct brain regions that include the nucleus accumbens (NAc) and dorsal striatum, among others [4–6]. The NAc is an important link in the circuit of addiction because it connects to brain regions that subsume positive memory [7] and decision making [8], two cognitive processes that regulate drug taking behaviors in rodents and humans [9, 10]. Indeed, pharmacological manipulations that affect some of these structures have been shown to increase, attenuate, or stop animals from self-administering various drugs of abuse [11, 12]. Among these brain regions, the NAc appears to play critical roles in the initiation and maintenance of drug self-administration [13]. Importantly, dopaminergic neurons from the ventral tegmental area (VTA) [14] and glutamatergic neurons from the prefrontal cortex [15] send projections to the NAc and can regulate plastic mechanisms that form the substrates of addiction [1, 6]. These neuroplastic changes appear to occur via alterations of transcripts and protein levels in intrinsic NAc neurons. [16–19].

Similar to other psychostimulants, methamphetamine (METH) addiction is highly prevalent throughout the world and is associated with a diversity of neuropsychiatric signs and symptoms [20]. Some of the long-term effects of this drug may be the results of molecular events that occur in the rodent striatum and NAc after either contingent or noncontigent administration of the drug [20–23]. In addition to changes in the expression of immediate early genes (IEGs) [4, 22], METH can also alter the expression of several neuropeptides including prodynorphin in the METH SA model [23]. Nevertheless, experimental approaches that use all rats that self-administer a drug to measure biochemical or physiological consequences of drugs do not strictly model human conditions [24–26] because not all humans who use rewarding drugs become addicted to them. Importantly, criteria for the addiction diagnosis include the persistence of drug taking in the presence of adverse consequences (APA, DSM V) by definition. It is also clear that a substantial number of individuals who have experimented with drugs can self-impose abstinence because of psychosocial complications associated with drug use [27].

In order to investigate epigenetic dysfunctions that may subsume METH addiction, we used a model of compulsive drug taking that was recently shown to produce a subgroup of rats that continue to lever press compulsively for cocaine despite adverse consequences [28–30]. Here, we show that there also exists a subpopulation of rats that continue to lever press for

METH even in the presence of footshocks whereas other rats became progressively more abstinent with increasing shock intensity. Moreover, we discovered that rats that differ phenotypically also exhibited substantial differences in DNA hydroxymethylation in peaks located near or within genes that are relevant to synaptic plasticity and cognition.

MATERIALS AND METHODS

Subjects

Male Sprague–Dawley rats, 14-16 weeks old (Charles River, Raleigh, NC), weighing 350–400 g before surgery, were used in our experiments. Rats were housed in the animal facility under a reversed 12: 12 h light/dark cycle with food and water available ad libitum except during the first 1–2 days of self-administration training when regular home-cage food was limited to about 20 g/day. All procedures followed the guidelines outlined in the *NIH Guide for the Care and Use of Laboratory Animals* (eighth edition; http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory_Animals.pdf) and were approved by the local NIDA animal care committee.

Intravenous surgery

Surgery for the implantation of intravenous catheter was done essentially as described before [26, 31]. Rats were anesthetized with ketamine (50 mg/kg, intraperitoneal) and xylazine (5 mg/kg, intraperitoneal) and inserted with silastic catheters into the jugular vein. The catheters were attached to a modified 22-gauge cannula that was mounted to the rats' skulls with dental cement. We injected buprenorphine (0.1 mg/kg, subcutaneous) after surgery to relieve pain and allowed the rats to recover for 7 days before METH SA training. During the recovery, training and punishment phases, we flushed the catheters every 24–48 h with gentamicin (Henry Schein; 5 mg/ml) and sterile saline.

Apparatus

Rats were trained in SA chambers located inside sound-attenuating cabinets and controlled by a Med Associates system (Med Associates, St. Albans, VT). Each chamber was equipped with two levers located 8.5 cm above the grid floor and the grid floors were connected to electric shock generators. Presses on the retractable active lever activated the infusion pump. Presses on the inactive lever had no reinforced consequences. The catheters of rats were connected to an i.v. line (Plastics One, Roanoke, VA) attached to a liquid swivel (Instech, Plymouth, PA) via polyethylene-50 tubing that was protected by a metal spring.

Training phase

The training procedure for METH SA was similar to that used in previous studies [26]. On the first day of training, the rats were brought to the SA room where they were chronically housed in SA chambers. Rats were randomly assigned to either METH SA or control groups. Rats (n=24) were trained to self-administer dl-METH HCl (NIDA) during three 3-h sessions/day (the sessions were separated by 30 min) over 20 days under a fixed-ratio-1 (FR-1) with a 20-s timeout reinforcement schedule (Figure 1a). Lever presses were accompanied by a 5-s compound tone-light cue. We trained the rats in cycles of 2 days of drug SA and one day off in order to control for weight loss which is a common side effect of

METH in laboratory animals [32]. METH was dissolved in sterile saline and the rats self-administered the drug at a dose of 0.1 mg/kg/infusion over 3.5 s (0.1 ml/infusion) [26]. Control rats (n=10) self-administered saline (0.1 ml/infusion). The number of infusions per 3-h session was limited to 35 in order to control for potential overdosing. At the end of each 3-h session, the housing light was turned off, and the active lever was retracted.

Footshock phase

During the shock phase, the rats continued to self-administer METH every day (9-h sessions) under the same FR-1 20-s timeout reinforcement schedule used during training. In addition, 50% of the reinforced active lever-presses resulted in the concurrent delivery of a 0.5-s footshock through the grid floor [26]. The initial foot-shock was set at 0.18 mA and was increased by 0.06 mA daily up to 0.30 mA. Thereafter, the rats received footshocks of 0.30 mA for 4 days followed by 0.36 mA for another 4 days. We chose to increase the shock progressively because adverse conditions in humans progress over time. Punished responses continued to produce the tone-light cue and 0.1 mg/kg/infusions of METH.

Tissue collection

We euthanized the rats at 2 hours after the last shock session. Brains were removed from the skull and the NAc was collected to be used in hydroxymethylated DNA immunoprecipitation (hMeDIP) sequencing (Seq) (hmC-Seq) and quantitative PCR analysis that are described in detail in the Supplemental Methods.

Analysis of hmC-Seq data

A combinatorial approach was used for high confidence peak calling. Statistically significant peaks of mapped reads were detected by using the model-based analysis of ChIP-Seq (MACS) program [33] on each hmC-Seq file against the matching input file under the stringent condition that satisfies fold enrichment ≥10 and the false discovery rate (FDR) ≤0.05%. Replicates were combined by retaining only those peaks that meet the majority rule condition (>50% of samples identify a peak) as described previously [34]. To measure the reproducibility at the level of peak calling, IDR (irreproducible discovery rate) analysis [35] was applied to the sets of peaks identified from replicates. Next, peaks were identified using PePr [36] (peak-calling prioritization pipeline) that uses a negative binomial distribution to identify consistent or differential binding sites in ChIP-Seq experiments with biological replicates. Peaks called in both MACS2-IDR and PePr pipelines were considered as high confidence calls.

Peak annotations were performed using HOMER (hypergeometric optimization of motif enrichment) [37]. The annotation includes peaks in the TSS (transcription start site), TTS (transcription termination site), Exon (Coding), 5′ untranslated region (5′ UTR) Exon, 3′ UTR Exon, or Intron. The locations outside these were defined as intergenic regions and include the nearest promoter, nearest gene, distance to TSS, and motif occurrences in peaks. In addition to associating peaks with nearby genes, the annotation includes the Gene Ontology Analysis and the Genomic Feature association analysis that look for enrichment of various gene function and genomic annotations in the list of peaks/regions, respectively.

Pathway Analysis

We used Ingenuity Pathway Analysis (IPA) software (Qiagen, Valencia, CA) to identify molecular functions, canonical pathways, and biological networks. We also used the PANTHER (protein annotation through evolutionary relationships) classification system to analyze genes that showed differential DNA hydroxymethylation (http://www.pantherdb.org/) [38].

Statistical analyses

We analyzed the behavioral data with the statistical program SPSS and followed significant effects ($p<0.05$) with SPSS *post hoc* contrasts within the repeated measures ANOVA module. For the training and footshock phases, the dependent variables were the number of METH infusions during the training days and the punishment sessions. The dependent variables for the extinction tests were total (non-reinforced) active lever-presses and inactive lever-presses.

RESULTS

Training phase

Figure 1a shows the timeline for the drug self-administration experiments. All METH-trained rats significantly escalated their intake of the drug per session during the first 16 days and then maintained their intake for the remainder of the training session (Figure 1b). The repeated measures mixed ANOVA for rewards earned included the between-subject factor group [control, SS (shock-sensitive), SR (shock-resistant)] and the within-subject factor of day (training days 1-20). This analysis showed a significant effect of day x group ($F_{(38,608)}= 18.4$, $p<0.001$). The significant interaction indicates that METH intake continued to increase for the first 16 training days for METH-trained rats whereas saline intake decreased and stabilized after 7 days for control rats. There were no significant differences in reward acquisition during training between METH-trained SS and SR groups ($p > 0.05$) (Figure 1b).

Footshock phase

During the footshock phase, we increased shock intensity over several days (Figure 1c). The shocks led to the segregation of the METH-trained animals into two phenotypes. One group continued to compulsively press the lever for METH while the other group progressively decreased their intake (Figure 1c). As the intensity of footshocks increased over days, METH-reinforced responding decreased in SS (non-addicted), but not in SR (METH-addicted) group. The statistical analysis of METH infusions earned by SS and SR rats included the between-subjects factor of group (SS, SR) and within-subjects factor of shock day (shock days 1-10). There was a significant effect of shock day x group ($F_{(9,198)}= 5.09$, $p<0.001$), indicating that addicted rats self-administered more METH infusions than non-addicted rats, an effect that was most pronounced at the higher shock dose (0.36 mA) (Figure 1c). Statistical analysis showed no significant differences in the number of METH infusions between SS and SR groups at the end of SA training (Figure 1d). However, in the end of footshock phase, SS rats dramatically reduced the number of METH infusions in

comparison to their pre-shock intake and in comparison to SR rats. In contrast, SR rats did not reduce the number of METH infusions versus their pre-shock level (Figure 1d).

DNA hydroxymethylation

Two hours following the SA and footshock regimen, the animals were euthanized and genomic DNA was extracted from the NAc. Isolated DNA was immunoprecipitated using an antibody against hydroxymethylated cytosine. Immunoprecipitated DNA was made into sequencing libraries and subjected to next-generation sequencing. Reads generated from sequencing underwent quality control measures (see supplementary methods) and were aligned to the rat genome (UCSC Genome Browser rn5). The MACS2 peak-calling program was used to convert loci with enriched reads as peaks. We considered peaks that satisfied fold enrichment ≥10 and the false discovery rate (FDR) ≤0.05. In addition, we chose peaks that exist for more than three of the six animals per group. Using the criteria described above, comparisons were performed between SR and CT (SRvsCT), SS and CT (SSvsCT), and SR and SS (SRvsSS) groups. The hMeDIP Seq dataset has been deposited and is freely available at http://psychiatry.som.jhmi.edu/pirooznia/RLee_ChIP-seq/.

Figure 2 and Supplementary Table S1 show the results of the overall DNA hydroxymethylation experiments. We found significant differentially hydroxymethylated (DHM) peaks between SRvsCT, SSvsCT, and SRvsSS comparisons. There were 475 (427 hyper- and 48 hypo-hydroxymethylated) peaks that showed differential DNA hydroxymethylation regions in the SRvsCT comparison, 808 (797 hyper and 11 hypo) peaks in the SSvsCT comparison, and 992 (598 hyper and 394 hypo) peaks in the SRvsSS comparison (Supplementary Figures S1, S2 and Figure 3). The Venn diagrams also show the overlaps among the 3 comparisons for the number of total genes (Figure 2a) and intragenic genes (Figure 2b). Figure 2a shows that there were 211 genes with differentially hydroxymethylated peaks unique to the SRvsCT comparison, 479 genes restricted to the SSvsCT comparison, and 547 genes in the SRvsSS comparison, indicating that METH-addicted and non-addicted rats could also be segregated based on their genome-wide DNA hydroxymethylation profiles.

Figure 2c shows the distribution of differentially hydroxymethylated peaks in the 3 pairwise comparisons. The majority of peaks were found in intergenic sites and in repeat elements. There were very few changes located in transcription start sites (TSS) or in exons. Interestingly, many changes occurred in long interspersed elements (LINEs) [39], with the smaller number of changes occurring in the SSvsCT comparison (27.9% against 56.2% and 55.6% for SRvsCT and SRvsSS, respectively) (Figure 2c). Changes in DNA hydroxymethylation also occurred in short interspersed elements (SINEs), with peak counts being similar across the 3 comparisons.

We also analyzed the DNA hydroxymethylation data by IPA and PANTHER programs in order to put the genes within biological contexts of potential relevance to neuronal plasticity, learning and memory, and/or diseased states (Supplementary Figures S1 and S2). Biological processes with hyper-hydroxymethylated peaks in the SRvsCT comparison included biological adhesion, developmental process, localization, and rhythmic process (Supplementary Figure S1). Molecular functions involved in addicted rats included

transcription factor activity, receptor activity, and transporter activity. Protein classes include receptors, cell adhesion molecules, and calcium binding proteins. Several biological processes were also affected in the NAc of the non-addicted rats (SSvsCT comparison) (Supplementary Figure S2). These include biological adhesion and immune system process. Peaks with increased DNA hydroxymethylation when comparing addicted to non-addicted rats (SRvsSS) are located within or near genes that are transcription factors, receptors, and transporters (Figure 3a). Peaks with decreased DNA hydroxymethylation in the addicted/non-addicted comparison were also located in genes that encode for proteins that are transcription factors, receptors, and transporters (Figure 3b).

Figure 4a shows the IPA results that confirm the role for some of these genes in potassium transport, cognition, and synaptic plasticity. Indeed, several genes that code for potassium channels were found in the SSvsCT comparison. These include KCNAJ2 (intergenic L1), KCNMA1 (intron 4), and KCNN2 (intergenic L1) that show increased DNA hydroxymethylation in the non-addicted rats (Table 1). Similarly, the SRvsSS comparison also contained peaks in and around genes that code for potassium channels, including KCNA4 (intergenic), KCNB2 (intergenic L1) and KCNH1 (intron) that also show increased DNA hydroxymethylation, whereas KCND3 (intergenic L1) and KCNT2 (intergenic L1) showed decreased hydroxymethylation (Table 1). Figures 4b and 4c show example of peaks located in intragenic regions of two genes coding for potassium channels.

Quantitative PCR of potassium channels

In order to investigate the impact of differential DNA hydroxymethylation on gene expression, we used quantitative PCR to measure the expression of several genes that code for potassium channels in the NAc. Figures 4d–4k illustrate the results of METH SA on gene expression in the footshock-induced divergent phenotypes. Non-addicted rats show increased expression of KCNA1 (Kv1.1) [$F_{(2,29)} = 5.05$, $p<0.013$] and KCNA2 (Kv1.2) [$F_{(2,25)} = 8.81$, $p<0.001$] expression in comparison to METH-addicted and control groups (Figures 4d and 4e, respectively). In addition, there were significant increases in the expression of KCNAB1 (also called KCNA1B, Kvb1) [$F(_{2,27}) = 5.51$, $p <0.01$] in the non-addicted group in comparison to the addicted rats (Figure 4f). KCNB2 (Kv2.2) [$F(_{2,27})= 7.5$, $p<0.0025$] expression was also increased in the non-addicted rats in comparison to control and compulsive METH takers (Figure 4g).

The non-addicted rats also showed increased expression of other subtypes of potassium channels (Figures 4h–4k). KCNMA1 (BK, SLO), coding for the large conductance calcium-activated K+ channel subfamily M alpha 1 showed significant increased expression [$F(_{2,26}) = 5.21$, $p<0.013$] in the non-addicted rats in comparison to the control and the addicted rats (Figure 4h). Both KCNN1 (SK1, KCa2.1) [$F(_{2,27}) = 5.70$, $p<0.0086$], encoding for the small conductance Ca++ activated subfamily N alpha, member 1, and KCNN2 (SK2, KCa2.2) [$F(_{2,26}) = 7.44$, $p<0.0028$] showed increased mRNA levels in the non-addicted rats in comparison to the control and METH-addicted rats (Figures 4i and 4j, respectively). KCNN3 mRNA expression was not affected in (Figure 4k).

In order to test for the specificity of the changes in gene expression, we also measured mRNA levels of potassium channels in the prefrontal cortex (PFC) and the dorsal striatum

(Supplementary figures S3 and S4). In the PFC, only KCNN2 showed significant changes [$F_{(2,25)} = 3.43$, $p=0.0481$] in mRNA levels, with the non-addicted group showing about 27% increases in comparison to the control group (Figure S3). In the dorsal striatum, KCNA1 [$F_{(2,28)} = 8.82$, $p<0.0011$] and KCNA2 [$F_{(2,29)} = 8.60$, $p<0.0012$] showed significant decreases in both non-addicted and addicted rats in comparison to the control group (Figure S4), with there being no differences between the two METH-exposed rats. There were no significant changes in mRNA expression of any of the other potassium channels (Figure S4). In general, these results indicate that the expression of potassium channels in the PFC and dorsal striatum did not help to differentiate compulsive METH takers from non-addicted rats.

DISCUSSION

METH addiction is a complex neuropsychiatric syndrome that is accompanied by pervasive changes in behaviors [40] and a high rate of recidivism [41]. The recent development of animal models that better mimic drug addiction in rodents [29, 30, 42] should help to improve the characterization of cellular and molecular substrates of this complex behavioral syndrome and to usher the development of better pharmacological therapies. Here we show that compulsive METH taking is associated with large scale changes in DNA hydroxymethylation in the rat NAc, consistent with a potential role for DNA hydroxymethylation processes in addiction [43]. Genes that were affected in the METH-addicted rats included those that participate in calcium signaling, among others. In contrast, non-addicted rats exhibited increased expression of mRNAs that code for several K+ channels in NAc, but not in the PFC and dorsal striatum.

K+ channels are a very large family of transmembrane proteins [44–46] that regulate biological processes by controlling K+ flow across membrane pores [47] and the firing properties of neurons [48, 49]. Indeed, they play important roles in controlling the length of action potentials and stabilizing membrane potentials [48, 49]. They are also involved in the regulation of diverse cellular processes including neurotransmitter release [50, 51]. There are more than 100 mammalian genes that code for several subfamilies of K+ channels that are distributed throughout the brain and influence regional functions based on their co-distribution with various neurotransmitter systems. Functional K+ channels consist of homo- and heterotetrameric structures composed of four alpha subunits with a central core and auxiliary beta subunits [44, 46]. K+ channels are located in somas, dendrites, spines, and axon terminals where they can impact neuronal signaling based on their structural characteristics.

Voltage-gated potassium channels are differentially expressed in addicted and non-addicted rats.

In the present study, we have discovered that non-addicted rats showed differential DNA hydroxymethylation and increased expression of two shaker genes, KCNA1 and KCNA2, that code for voltage-gated K+ (Kv) channels Kv1.1 and Kv1.2 [44], respectively, in comparison to compulsive METH takers. Importantly, there were also increases in the expression of the accessory protein, KCNAB1, which codes for the potassium channel subunit beta-1 [52, 53] that forms heteromultimeric complexes with alpha subunits such as

Kv1.1 and Kv1.1 [54]. Association of alpha and beta subunits in the brain increases the flexibility and diversity of potassium channels [54]. Voltage-gated potassium channels are located on axons and axon terminals [55] and serve to dampen neuronal excitability through the regulation of action potentials and the firing potentials of neurons [56]. Interestingly, the shaker locus in drosophila that exhibit motoric behaviors was found to encode a potassium channel [57]. Engineered Kcna1-null mice suffer from episodic eye blinking, increased startle response, hippocampal hyper-excitability, and seizures [58]. Deletion of Kcna2 also produces seizure activities in mice [59]. Interestingly, Kcna1b-deficient mice showed normal synaptic plasticity in the hippocampus but impaired learning in the Morris water maze test [60]. These data implicate a role of these K+ channels in the manifestation of complex behaviors. Thus, when taken together with the increased excitability reported in mice with Kv deletion, the increased expression of KCNA1 and KCNA2 mRNAs observed in the non-addicted rats suggests the possibility that activation of these channels in the NAc might have served to suppress compulsive METH taking in the presence of adverse consequences. In addition to the increased expression of KCNA1 and KCNA2 in the non-addicted rats, there were also significant increases in the mRNA levels of the shab-related delayed rectifier K+ channel, KCNB2 (Kv2.2) [61] in the non-addicted rats in comparison to controls rats. Kv2.2 is highly expressed in the brain and is important in the regulation of somatodendritic excitability [49, 62]. When taken together, the increased expression of voltage-gated potassium channels suggests that these channels might be co-regulated to promote abstinence. Our data also hint to the potential use of activators of Kv channels [63] in therapeutic interventions against METH addiction.

Increased expression of calcium-gated potassium channels in non-addicted rats.

We also discovered that the mRNA expression of 3 Ca++-activated K+ channels (KCNMA1, KCNN1, and KCNN2) was upregulated in the non-addicted rats, suggesting that diverse potassium channels might work in concert to control or attenuate neuroadaptive mechanisms involved in the regulation of drug taking. Indeed, these calcium-activated channels are also found in subcellular locations such as soma, dendrites, and axon terminals where they are known to regulate neuronal firing properties, neurotransmitter release, synaptic plasticity, as well as learning and memory [46, 50, 64]. It is thus possible to suggest that the increased expression in the non-addicted animals may help to enhance mnemonic processes related to remembering adverse consequences associated with METH taking. The suggestion that these channels might be important to METH addiction is consistent with recent reports that have documented a role for these potassium channels in alcohol addiction [65–67]. For example, chronic alcohol produced downregulation of surface SK2 (KCNN2) expression and reduced apamin-sensitive SK currents while activation of SK2 channels by 1-ethyl-2-benzimidazolinone (1-EBIO) reduced alcohol intake [66]. Interestingly, it has been reported that genetic datasets related to addiction contain families of potassium channels including KCNN1 for METH [67]. Importantly, the authors also found that alcohol dependence was associated with decreased KCNN3 expression and blocking of KCNN3 activity increased alcohol intake. These reports are consistent with the fact that the SK channel activator, chlorzoxazone, can reduce excessive alcohol intake [68]. Taken together with observations concerning alcohol addiction, our findings suggest that activators of SK channels [69] might be important therapeutic targets for the treatment of substance use disorders.

Conclusion

In conclusion, quantitative analyses of DNA hydroxymethylation in METH-addicted and non-addicted rats documented the presence of several differentially hydroxymethylated peaks in the NAc of these two phenotypes. Prominent among those are members of the classes of potassium channels including voltage- and calcium-activated channels that showed increased mRNA expression in the NAc of non-addicted rats. These observations are consistent with data that had proposed a role of small-conductance calcium-gated potassium channels in alcohol addiction [66] and voltage-gated channels in the effects of cocaine [70, 71]. Thus, our experiments identify, for the first time, a potential role for different classes of voltage-activated K+ channels in regulating abstinence and compulsive drug taking in a rat model of METH addiction. It is also important to note that Kv1 and Kv2 channels are differentially distributed on somatodendritic and axonal sites, suggesting the possibility that co-regulation of these channels may, in part, serve to promote plastic changes that are responsible for the two dichotomous phenotypes. In any case, our large-scale quantitative analyses provide a rich source of information that can be mined for further assessments of specific roles that individual K+ channels might play in various aspects of rodent models of addiction. Finally, the present discoveries should help to usher novel drug development programs in the search for powerful medications in the therapeutic war against METH addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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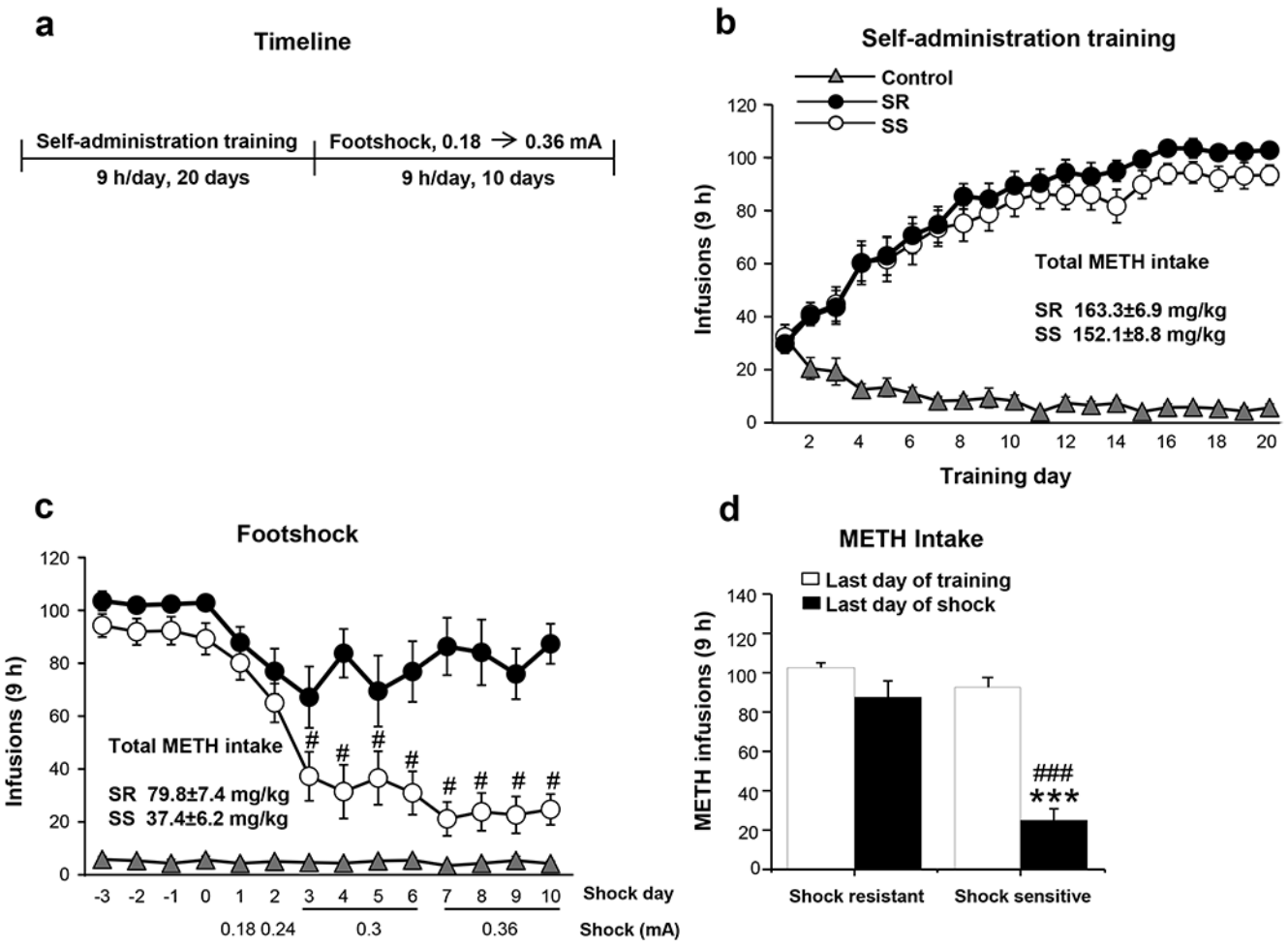
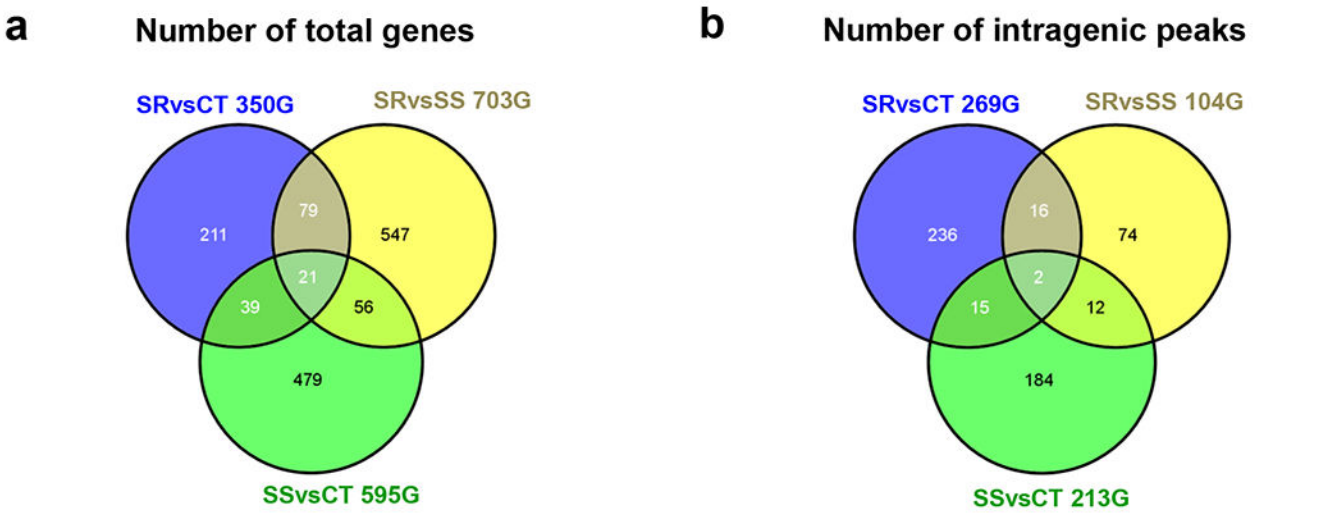


Figure 1.

Prolonged methamphetamine self-administration and contingent footshock produce rats with non-addicted and compulsive drug taking phenotypes. (**a**) Timeline of the experiment. (**b**) All rats escalate their intake of METH during the training phase of the experiment. The inset shows that SR and SS rats took similar amount of METH before the shock phase. (**c**) Footshocks suppress lever pressing in shock-sensitive (SS) rats but not in shock-resistant (SR) compulsive METH takers. The inset illustrates the fact that SS rats took substantially less METH than SR rats during that phase. (**d**) Lever pressing on the last day of training and last day of footshocks. Key to statistics: # $P < 0.05$, ### $P < 0.001$, in comparison to SR rats; *** $P < 0.001$ in comparison to last day of training.



c **Distribution of hydroxymethylated peaks in METH-addicted and non-addicted rats**

			SRvsCT		SSvsCT		SRvsSS	
			Count	%	Count	%	Count	%
Intragenic	TSS / Promoter		0	0.0	3	0.5	3	0.4
	Exon		0	0.0	1	0.2	0	0.0
	Intron		13	3.4	45	7.0	64	7.9
	TTS		1	0.3	3	0.5	4	0.5
Repeated elements	LINE	Intragenic	46	12.1	38	5.9	130	16.1
		Intergenic	167	44.1	141	22.0	318	39.5
	SINE	Intragenic	25	6.6	47	7.3	47	5.8
		Intergenic	23	6.1	52	8.1	48	6.0
	Satellite repeats	Intragenic	1	0.3	5	0.8	16	2.0
		Intergenic	36	9.5	20	3.1	53	6.6
	Simple repeats	Intragenic	14	3.7	72	11.2	14	1.7
		Intergenic	9	2.4	115	17.9	20	2.5
	LTR		10	2.6	10	1.6	24	3.0
	Others		1	0.3	6	0.9	3	0.4
scRNA			10	2.6	12	1.9	9	1.1
Other intergenic			23	6.1	71	11.1	53	6.6
Total			379	100	641	100	806	100

Figure 2.

Whole genome profiling in METH-addicted (shock-resistant, SR) and non-addicted (shock-sensitive, SS) rats. The Venn diagrams show the number of (**a**) all hydroxymethylated peaks and (**b**) only intragenic peaks in the different METH and footshock-induced phenotypes. (**c**) Distribution of hydroxymethylated peaks in the 3 pairwise comparisons.

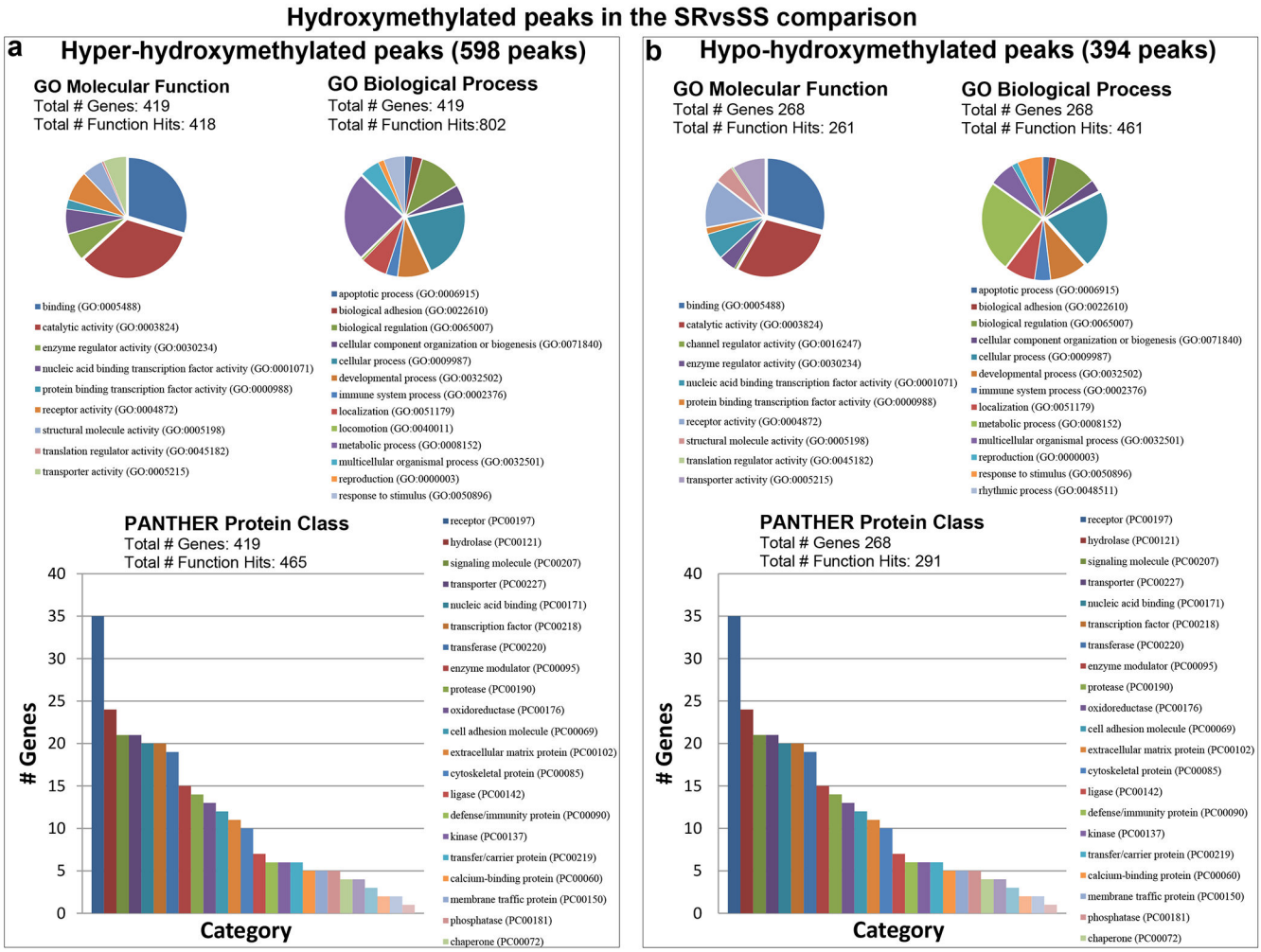
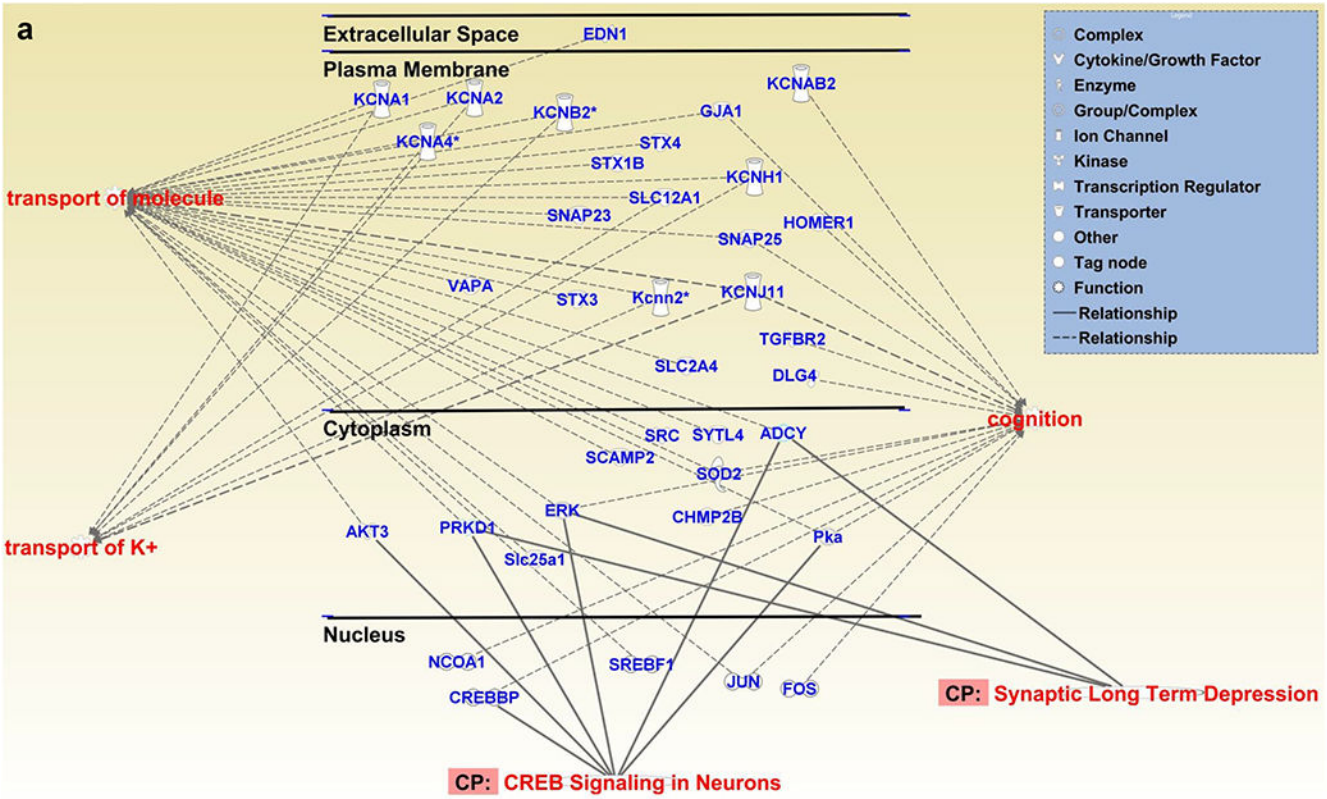


Figure 3.

Functional annotation of differentially hydroxymethylated peaks between METH-addicted (shock-resistant, SR) and non-addicted (shock-sensitive, SS) rats. The data sets were grouped according to (**a**) hyper-hydroxymethylated and (**b**) hypo-hydroxymethylated peaks in the SRvsSS comparison. Gene families were grouped based on their molecular functions, biological processes, and protein classes according to the PANTHER analysis. Differences in hydroxymethylated peaks were considered significant as described in the text. The genes with differentially hydroxymethylated peaks are listed in supplementary Table S1.



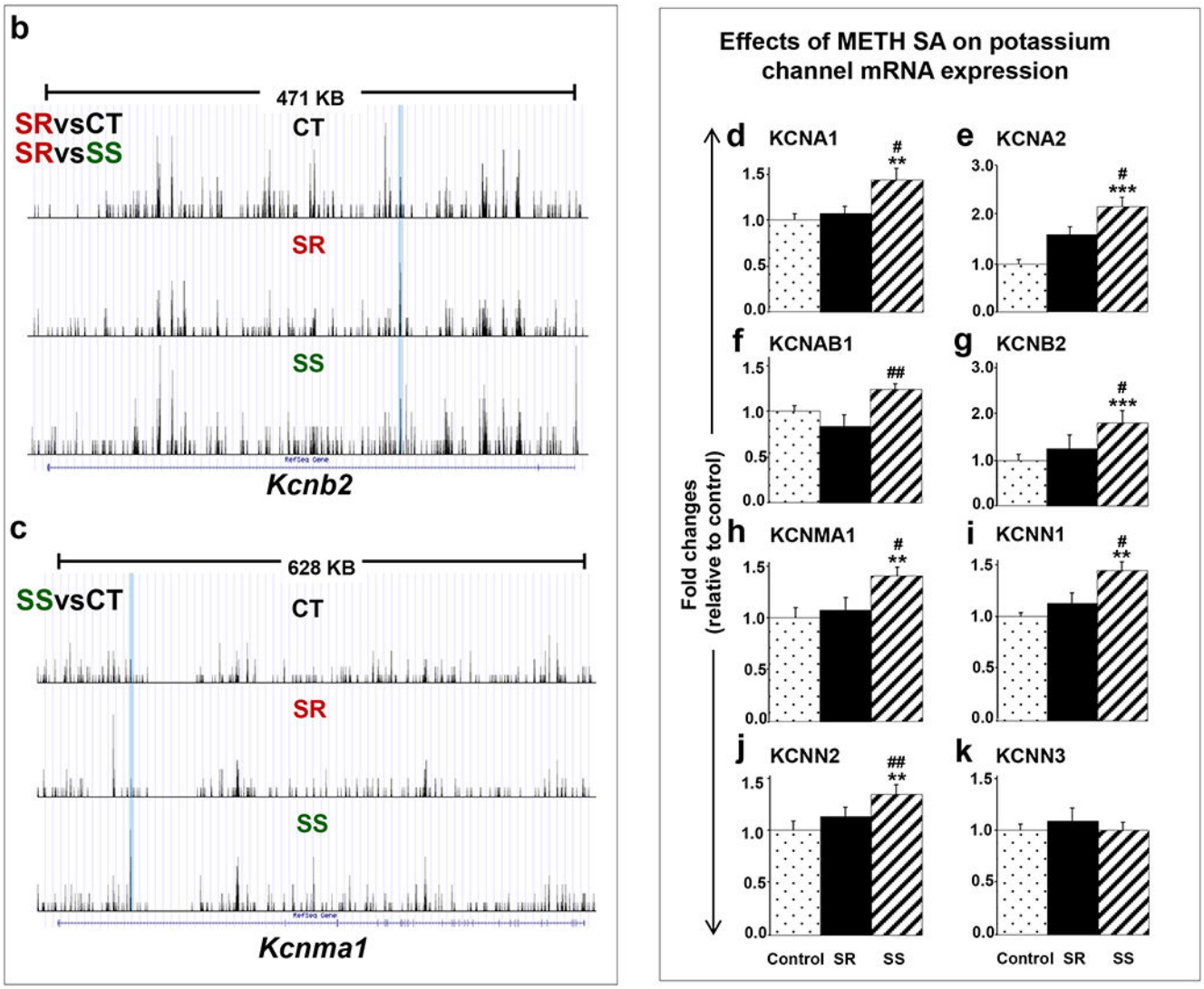


Figure 4.

Effects of METH SA on DNA hydroxymethylation and expression of potassium channels in the NAc. (**a**) IPA shows that genes with differentially methylated peaks participate in transport of molecular, potassium transport, and cognition. (**b, c**) UCSC Genome Browser images of hydroxymethylated peaks in Kcnb2 and Kcnma1 intragenic sites, respectively. (**d-k**) mRNA expression of potassium channels in non-addicted (SS) and compulsive (SR) METH takers. Key to statistics: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ in comparison to control; # $P < 0.05$, ## $P < 0.01$, in comparison to SR.

Table 1.

List of potassium channel genes

Gene	Comparisons	Up/Down	Chromosome	Peak Location
	SR v CT			
Kcnb2		up	chr5	Intron
Kcnn2		up	chr18	Intergenic
	SS v CT			
Kcnip2		up	chr1	Intergenic
Kcnj2		up	chr10	Intergenic
Kcnj2		up	chr10	Intergenic
Kcnj3		up	chr3	Intergenic
Kcnk12		up	chr6	Intergenic
Kcnma1		up	chr15	Intron
Kcnn2		up	chr18	Intergenic
	SR v SS			
Kcna4		up	chr3	Intergenic
Kcnb2		up	chr5	Intron
Kcnb2		up	chr5	Intergenic
Kcnb2		down	chr5	Intergenic
Kcnd3		up	chr2	Intergenic
Kcnh1		up	chr13	Intron
Kcnk1		up	chr19	Intergenic
Kcnk1		up	chr19	Intergenic
Kcnn2		up	chr18	Intergenic
Kcnn2		down	chr18	Intergenic
Kcnt2		down	chr13	Intergenic
Kctd13		up	chr1	Intron