Inhibition of histone deacetylation rescues phenotype in a mouse model of Birk-Barel intellectual disability syndrome

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Mutations in the actively expressed, maternal allele of the imprinted KCNK9 gene cause Birk-Barel intellectual disability syndrome (BBIDS). Using a BBIDS mouse model, we identify here a partial rescue of the BBIDS-like behavioral and neuronal phenotypes mediated via residual expression from the paternal Kcnk9 (Kcnk9pat) allele. We further demonstrate that the second-generation HDAC inhibitor CI-994 induces enhanced expression from the paternally silenced Kcnk9 allele and leads to a full rescue of the behavioral phenotype suggesting CI-994 as a promising molecule for BBIDS therapy. Thus, these findings suggest a potential approach to improve cognitive dysfunction in a mouse model of an imprinting disorder.
Birk-Barel intellectual disability (ID) dysmorphism syndrome (BBIDS or KCNK9 imprinting syndrome, OMIM 612292) is typically associated with congenital central hypotonia, developmental delay, intellectual disability, severe feeding problems, and hyperactivity. The disease is inherited autosomally dominantly with maternal-only transmission, as the KCNK9 gene is embryonically paternally silenced (imprinted) in man and mouse. It encodes the potassium channel subunit TASK3, which dimerizes to form two-pore domain potassium (K2P) leak channels. The mouse Kcnk9 gene maps to an imprinted cluster on mouse chromosome 15 together with further imprinted genes, i.e., the brain-specific maternally expressed genes Ago2, Chrac1, and Trappc9 and the paternally expressed Peg13 genes.

Kcnk9 mRNA expression is widespread in the central nervous system (CNS), in rodents with notably high levels in cerebellar granule neurons, the locus coeruleus (LC), the dorsal raphe nuclei, hippocampal CA1 and CA3 pyramidal neurons, and several hypothalamic nuclei. Homozygous deletion of Kcnk9 in the mouse (Kcnk9KOmat) reduces the resting potassium conductance and enhances spike firing accommodation in adult cerebellar granule neurons. Kcnk9KOhom mice further display increased nocturnal motor activity, cognitive deficits, as well as a reduced sensitivity to inhalation anesthetics and the cannabinoid receptor agonist WIN55212-2 mesylate. More recently, RNAi-based knockdown of Kcnk9 and expression of a dominant-negative mutant KCNK9, which had been associated with the human disease phenotype were shown to impair neuronal migration during mouse cortical development. However, the phenotype of mice with heterozygous deletion of the active maternal Kcnk9 allele (Kcnk9KOmat) thus mimicking BBIDS has not yet been characterized.

DNA methylation is well established as a key player in imprinting regulation by multiple studies, which characterized the kinetics and mechanisms of methylation reprogramming at imprinting control regions (ICRs). The critical involvement of post-translational histone modifications in transcriptional regulation, in general, is also well accepted, but much less explored for ICRs. It is assumed that an interplay between DNA methylation and histone acetylation is essential for proper erasure and resetting of imprints in the germline as well as selective imprint maintenance during postzygotic reprogramming. A differentially methylated region (DMR) which is methylated on the maternal allele and assumed to be involved in Kcnk9 gene regulation has been only identified in the promoter region of Peg13. Promoter CpG islands of Kcnk9 are unmethylated, but display high levels of active histone H3 lysine 4 monomethylation (H3K4me1) and histone H3 lysine 27 acetylation (H3K27ac) chromatin marks in brain tissues.

Here, we characterize the behavioral and neuronal phenotype of mice with heterozygous deletion of the active maternal Kcnk9 allele (Kcnk9KOmat). We demonstrate partial behavioral rescue in these animals compared with full knockout animals and show that epigenetic manipulation stimulates Kcnk9KOmat expression sufficiently, to rescue the behavioral phenotypes, thereby opening new avenues for treatment of cognitive dysfunctions in BBIDS.

Results
Deletion of Kcnk9 leads to impaired behavior. To assess behavioral deficits along the BBIDS phenotype in Kcnk9KO mice, we performed in vivo experiments in adult wild type (WT) as well as Kcnk9KOmat and Kcnk9KOhom mice with a deletion of Kcnk9 exon 2 as previously described (Fig. 1a). Following the strictly monoallelic expression pattern of Kcnk9 in mouse brain (<1% paternal expression), we expected largely concordant phenotypes in mice carrying a deletion of both Kcnk9 alleles (Kcnk9KOmat) or only the maternal Kcnk9 allele (Kcnk9KOmat), respectively.

No differences in behavior between Kcnk9KOhom, Kcnk9KOmat, and WT littermates were seen in elevated plus maze and open field testing for anxiety (Supplementary Fig. 1a, b) and in the rotarod-test for motor coordination (Supplementary Fig. 1c). Given the described intellectual disability in BBIDS patients, we then examined whether working memory was affected in Kcnk9KO mice. Spontaneous alternation relies on the natural tendency of rodents to explore a novel environment and can be quantified using a Y-maze task. The ability to remember the immediately preceding choice is considered an indicator for active working memory. In the Y-maze, spontaneous alternation was defined as consecutive entries into all three arms without revisiting an arm. The average percentage of spontaneous alternation made by Kcnk9KOhom, Kcnk9KOmat, and WT mice across a 10-min trial was analyzed and revealed a significant reduction of spontaneous alternation by about 10% in both Kcnk9KOhom and Kcnk9KOmat mice compared with WT littermates (Fig. 1b). There was no significant difference in the extent of the working memory impairment between Kcnk9KOhom and Kcnk9KOmat mice (Fig. 1b). Our findings indicate an impaired working memory in Kcnk9KOhom and Kcnk9KOmat mice, which is consistent with previously reported observations in Kcnk9KOhom mice.

Circadian rhythms in mammals are endogenously coordinated oscillations of biological parameters such as the sleep/wake cycles with an overall period length of about 24 h. KCNK9 channels are expected to contribute to the in vivo electrical activity of neurons in those brain regions associated with the regulation of circadian rhythms and arousal. Spontaneous motor activity analysis during light (resting) and dark (active) phase was performed, resembling day and night in diurnal humans, respectively. A significantly increased overall locomotor activity during the dark phase was found in Kcnk9KOhom and Kcnk9KOmat mice compared with WT controls, describing an exaggerated nocturnal activity in Kcnk9KO animals (Fig. 1c), which is in consensus with previous observations. Surprisingly, an intermediate phenotype for dark phase activity was observed in the Kcnk9KOmat animals with statistically significant differences to both WT and Kcnk9KOhom animals. No significant differences between genotypes were observed in the overall locomotor activity during the light phase (Fig. 1c, Supplementary Table 1).

Together, these data demonstrate that the loss of Kcnk9 in mice results in the impairment of behavioral parameters resembling components of the BBIDS phenotype. Interestingly, we found an intermediate phenotype for nocturnal locomotor activity in animals with a loss of only the actively expressed maternal allele (Kcnk9KOmat) suggesting the involvement of the silenced paternal allele.

Non-canonical imprinting of Kcnk9 in the mouse brain. In human and mouse brain KCNK9 was reported to be monoallelically expressed from the maternal allele while the paternal allele is silenced. To elucidate if the intermediate phenotype of Kcnk9KOmat animals was due to residual expression from the paternal allele, we analyzed the parent-of-origin allele-specific expression pattern of Kcnk9 in different brain regions of F1 hybrid animals from crosses between C57BL/6 (B6) and Mus musculus castaneus (Cast/Ei) mouse strains [(C57BL/6xCast/Ei) F1]. As expected, we observed a predominant expression of the maternal Kcnk9 allele in all analyzed brain regions (Fig. 1d). However, we also detected significant expression from the repressed paternal allele, which represented 1–14% of all
Fig. 1 Deletion of non-canonical imprinted Kcnk9 gene leads to impaired behavior of Kcnk9KO mice. 
a Schematic representation of the sequence of mouse behavioral experiments. 

b Y-maze percentage alternation analysis of WT (n = 23), Kcnk9KOmat (n = 27), and Kcnk9KOhom (n = 44) mice. A spontaneous alternation was defined as consecutive entries into all three arms without revisiting an arm. Kcnk9KO mice display a significant decrease in percentage alternation compared with WT mice. One-way ANOVA: F(2, 91) = 7.261, P = 0.0012; followed by Bonferroni’s multiple comparison post hoc test, *P < 0.05, **P < 0.01. 
c Total locomotor activity (distance traveled in the home cage) in light (12 h, sun symbol)/dark (12 h, moon symbol) phase. The left section shows no difference in distance traveled in the light phase of WT (n = 13), Kcnk9KOmat (n = 10) and Kcnk9KOhom (n = 9) mice. One-way ANOVA: F(2, 29) = 2.281, P = 0.1203. The right section depicts the nocturnal activity of WT (n = 18), Kcnk9KOmat (n = 13) and Kcnk9KOhom (n = 15) mice. Kcnk9KOmat and Kcnk9KOhom mice display significantly increased nocturnal hyperactivity compared with WT littermates with activity scores of Kcnk9KOmat mice intermediate between Kcnk9KOhom and WT mice. One-way ANOVA: F(2, 43) = 22.70, P < 0.0001; followed by Bonferroni’s multiple comparison post hoc test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. 

d Behavioral experiments were performed in seven independent sessions. 

d Non-canonical Kcnk9 imprinting in (C57BL/6xCast/Ei)F1 hybrid mice. Quantification of Allele-Specific Expression by Pyrosequencing (QUASEP) of several brain regions from (C57BL/6xCast/Ei)F1 hybrid mice; maternal allele (red) and paternal allele (blue). Cerebellum n = 14, pons n = 10, olfactory bulb n = 12, cortex n = 14, hippocampus n = 14, hypothalamus n = 9, striatum n = 5, midbrain n = 5, medulla n = 5, and locus coeruleus n = 4; n = biologically independent samples. 

Data are means ± 1 SEM (standard error of the mean). Statistical analyses and approaches are provided in Supplementary Table 1. Source data are provided as a Source Data file. The mouse images in this figure were created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License; https://smart.servier.com.
transcripts depending on the brain region analyzed (Fig. 1d, suppl. Fig. 2c). Highest paternal expression was observed in the LC (Fig. 1d). Accuracy of the LC tissue-punches was demonstrated through elevated gene expression levels of tyrosine hydroxylase (TH) in LC samples (Supplementary Fig. 2a, b).

These data demonstrate a brain region-specific leakiness of the imprint on the Kcnk9pat allele with a peak in the LC. This leakiness might be responsible for the observed intermediate phenotype in Kcnk9KOmat animals and suggested a particular importance of LC Kcnk9 expression for locomotor activity during the active (dark) phase.

**Kcnk9 knockdown in the LC induces elevated nocturnal activity.** To test the role of functional Kcnk9 expression in LC neurons for the control of nocturnal activity, we bilaterally infused AAV vectors for expression of eGFP and shRNAmir sequences, either scrambled (pAAV-Syn-shRNAmir-scrambled-EF1a-eGFP) or specifically targeting Kcnk9 mRNA (pAAV-Syn-shRNAmir-Kcnk9-EF1a-eGFP), into the LC of WT mice under stereotactic control (Fig. 2a). Efficiency of the used shRNA sequence was pre-tested in vitro (Fig. 2b) and validated by RT-qPCR from mouse brain tissue (Fig. 2c). Targeting efficiency and selectivity of stereotactically guided infusion was demonstrated by eGFP immunohistochemistry (Fig. 2a). Twenty-one days after injection, animals were tested for circadian activity and working memory. A significant and selective increase of nocturnal activity was detected in the shRNAmir-Kcnk9 injected animals compared with age-matched control animals injected with the scrambled shRNAmir virus (Fig. 2d). These results demonstrated that altering local expression of Kcnk9 in the LC was sufficient to selectively affect dark-phase activity in mice. Furthermore, it identified LC as an important neural hub for mediating the behavioral effects of altered Kcnk9 expression, which warranted further mechanistic analysis of these neurons. Interestingly, a clear trend toward impaired working memory was also observed in shRNAmir-Kcnk9 injected animals (Fig. 2e), suggesting that Kcnk9 expression also controls working memory-related activity of LC neurons.

**Increased dark-phase LC pacemaking activity in Kcnk9KOhom but not Kcnk9KOmat mice.** Electrical activity of LC neurons drives periods of wakefulness and arousal21–23. Indeed, selective optogenetic activation of LC neurons revealed their causal role in sleep-to-wake transitions and locomotor arousal by demonstrating that sustained 3-Hz LC neuronal stimulation enhanced spontaneous locomotion (total track length) by about 50%24. As we detected a similar increase of spontaneous locomotion during the active (dark) phase of Kcnk9KOhom mice compared with WT controls, we reasoned that KCNK9-dependent differences in LC pacemaking activity might contribute to this phenotype.

By recording from synaptically isolated (including inhibition of somatodendritic alpha2-antagonists), spontaneously active LC neurons in brainstem slices from adult mice, we observed a dark phase-selective, about 70% increase of pacemaker frequency in LC neurons from Kcnk9KOhom mice (Fig. 3a–d). In contrast, no significant dark-phase increase of LC pacemaker frequency was observed in WT animals (Fig. 3c, d). These results demonstrate that some degree of KCNK9 channel expression is necessary to selectively dampen enhanced pacemaker activity during the dark phase, which is present in Kcnk9KOhom mice. Interestingly, LC neurons from Kcnk9KOmat animals displayed no significant increase in pacemaker activity in the dark phase (Fig. 3c, d), but a significant difference in pacemaker activity in the dark was observed between Kcnk9KOhom and Kcnk9KOmat animals. This indicated that a certain level of functional expression of KCNK9 channels from the Kcnk9pat allele in LC is present in Kcnk9KOmat animals to prevent the full nocturnal in vitro pacemaker frequency increase. This level of Kcnk9 expression might at the same time not be sufficient to dampen in vivo LC activity, which is also driven by synaptic inputs from neuronal networks, in line with the observed intermediate behavioral phenotype regarding dark-phase locomotion in Kcnk9KOmat mice. In line with this argument, we show below that boosting further expression of KCNK9 channel subunits from paternal alleles indeed also restores the WT motor activity phenotype.

**CI-994 activates the paternally repressed Kcnk9 allele.** The paternally inherited Kcnk9/KCNK9 gene is epigenetically silenced yet structurally unimpaired. Our behavioral and gene expression experiments suggest a contribution of paternally expressed Kcnk9 to the intermediate phenotype of the Kcnk9KOmat mice and the possibility of upregulation of the paternal allele in the case of loss of the maternal allele. We, therefore, speculated that exogenous application of epigenetic modulators might alter the structure at the Kcnk9 promoter region and result in further derepression of the Kcnk9pat allele. We hypothesized that this could fully compensate for the loss of the maternal allele and rescue the BBIDS-like phenotype of Kcnk9KOmat mice.

To investigate the paternally derived Kcnk9 transcript levels after epigenetic drug treatment, we isolated E14 mouse primary cortical neurons (mPCNs) from WT and Kcnk9KOmat animals from crosses between WT males and Kcnk9KOhom females (Fig. 4a). We then chose six different compounds representing different classes of epigenetic modulators for treatment (Fig. 4b).

Murine PCNs were treated for 3 days with the epigenetic modulators and Kcnk9 expression was analyzed by RT-qPCR. Kcnk9 expression was determined to be activated by CI-994 and other acetamide-based compounds (Fig. 4c, d). This result suggests a contribution of paternally expressed Kcnk9/KCNK9 expression for locomotor activity during the active (dark) phase of Kcnk9KOhom mice compared with control conditions (Supplementary Fig. 3a). For further investigations, we focused on the benzamide-based second-generation histone deacetylase inhibitor (HDACi) CI-994, a selective inhibitor of class I HDACs, which is a potent inhibitor of HDAC1 and 3 isoenzymes relative to HDAC6 and HDAC825. CI-994 showed the most efficient upregulation of Kcnk9pat expression in Kcnk9KOmat mPCNs (Fig. 4c). Furthermore, a previous study reported that intraperitoneal administration of CI-994 in WT mice resulted in long-lasting CI-994 levels in the brain without affecting the overall behavioral phenotype of mice26.

The effect of CI-994 in Kcnk9KOmat mPCNs was further tested in a dose-response experiment. Kcnk9KOmat mPCNs were treated with increasing concentrations of CI-994 for 24 h, and Kcnk9 expression was analyzed by RT-qPCR in comparison to DMSO-treated Kcnk9KOmat and WT control cells (Fig. 4d). As expected, the Kcnk9pat expression in DMSO-treated Kcnk9KOmat mPCNs was significantly reduced compared with that of WT mPCNs. After CI-994 treatment of Kcnk9KOmat mPCNs, we observed a strong linear correlation between the increase of Kcnk9pat expression and CI-994 dosage suggesting a specific, dose-dependent effect of CI-994. Notably, Kcnk9 expression in 80 μM CI-994-treated Kcnk9KOmat mPCNs exceeded that of DMSO-treated WT mPCNs in vitro (Fig. 4d). Treatment of Kcnk9KOmat mPCNs with 20 μM CI-994 showed a consistent increase of Kcnk9pat expression even after 10 days suggesting a prolonged and stable in vivo effectiveness (Fig. 4e). Cell viability analysis after treatment of Kcnk9KOmat mPCNs with 10 or 20 μM
CI-994 did not reveal any toxic effect (Supplementary Fig. 4c). Intriguingly, CI-994 treatment did not affect the expression of other nearby imprinted genes within the imprinted cluster on mouse chromosome 15 in vitro (Supplementary Fig. 3b).

**CI-994 rescues the behavioral phenotype of Kcnk9KO<sup>mat</sup> animals.** The detection of Kcnk9<sup>mat</sup> allele expression in several brain regions, prominently among them the LC, as well as the observation of intermediate phenotypes in Kcnk9KO<sup>mat</sup> animals led us to hypothesize that epigenetic manipulation could further stimulate paternal gene expression and thereby boost the phenotypic rescue.

For testing, either DMSO (100%) or CI-994 (30 mg/kg of body weight in 100% DMSO) were injected daily over 14 days in the peritoneum (Fig. 5a). After injections, Kcnk9<sup>pat</sup> expression was induced up to ~3-fold compared with DMSO-treated controls in several brain regions including the cerebellum, hippocampus,
Kcnk9 increase of spontaneous alternation in the signal (green), neurobiotin-positive signal (red) and a merge of both signals (yellow). Upper images show an overview of the LC at a low-magnification (×4) and lower images display signals of analyzed single-cell at high-magnification (×60 oil). 

Electrophysiological traces of single cells in the night phase of WT, Kcnk9KOhom and Kcnk9KOmat animals. Recordings from synaptically isolated spontaneously active LC neurons reveal increased pacemaker frequency in LC neurons from Kcnk9KOhom mice during dark phase. 

Scatter dot-plot of mean frequencies including all analyzed cells in the day and night phase of WT (light n = 46, N = 5, dark n = 23, N = 3), Kcnk9KOmat (light n = 41, N = 5, dark n = 26, N = 4) and Kcnk9KOhom (light n = 22, N = 3, dark n = 30, N = 5) animals. Ordinary one-way ANOVA with Bonferroni’s multiple comparison post hoc test, *P < 0.05, **P < 0.01. Values are means ± SEM. N = number of mice, n = total number of cells. Statistical analyses and approaches are provided in Supplementary Table 1. Source data are provided as a Source Data file.

CI-994 interferes with H3K27 acetylation at the Kcnk9 locus. Imprinting of the Kcnk9/KCNK9 gene is thought to be regulated by a maternally methylated germline differentially methylated region (DMR) in the promoter of the Peg13/PEG13 gene3.

CI-994 treatment with either DMSO or CI-994 did not exhibit deficits in working memory and significant differences in the Y-maze. By contrast, no increase of spontaneous alternation was seen in the CI-994 treated Kcnk9KOhom animals suggesting that the paternal allele silenced in the Kcnk9KOmat animals carries the effect of the treatment (Supplementary Fig. 5a).

We then asked whether CI-994 treatment also alters nocturnal activity in Kcnk9KOmat or in Kcnk9KOhom mice. Indeed, Kcnk9KOmat mice showed a significant decrease in horizontal nocturnal activity during the 12-h dark phase after CI-994 treatment (Fig. 5f). Again, post hoc analysis of multiple comparisons showed no significant difference between CI-994-treated Kcnk9KOmat and DMSO- or CI-994-treated WT animals suggesting full rescue of the nocturnal hyperactivity phenotype in the Kcnk9KOmat animals. No drug effect was observed in Kcnk9KOhom animals and total locomotor activity in the light phase was not altered by CI-994 treatment irrespective of the genotype of the animals (Fig. 5f, Supplementary Fig. 5b).

CI-994-mediated enhancement of Kcnk9 expression also promotes behavioral recovery, WT, Kcnk9KOmat, and Kcnk9KOhom mice were subjected to behavioral testing after treatment with either DMSO (100%) or CI-994 (30 mg/kg of body weight). In the Y-maze task, CI-994 treatment led to a significant increase of spontaneous alternation in the Kcnk9KOmat mice compared with DMSO-treated Kcnk9KOmat control mice (Fig. 5e). Post hoc testing for multiple comparisons showed no difference between CI-994-treated Kcnk9KOmat animals and either DMSO- or CI-994-treated WT animals (Fig. 5e). WT mice
Acetylation of histone 3 lysine 27 (H3K27ac), monomethylation of histone 3 lysine 4 (H3K4me1) as well as DNA methylation at the maternally methylated DMR in the human PEG13 promoter region have been suggested to control human KCNK9 expression4 (Fig. 6a, Supplementary Fig. 6a-d). CI-994 is a second-generation class I histone deacetylase inhibitor inhibiting HDACs1 and 3 with high specificity25. To clarify the epigenetic mechanism underlying the induction of the Kcnk9pat allele through CI-994 we investigated the methylation status of two subregions of the Peg13-DMR (Peg13DMR1 and Peg13DMR2) in adult hippocampus and LC of DMSO- and CI-994-treated Kcnk9KOmat mice using bisulfite pyrosequencing. Methylation levels of about 40–50% typical for imprinted gene DMRs in somatic cells were determined for both DMRs in the two brain regions of Kcnk9KOmat mice. These methylation levels were not significantly altered by CI-994 treatment of Kcnk9KOmat mice (Supplementary Fig. 6c, d). Our results indicate that the upregulation of Kcnk9 mRNA in various brain regions of Kcnk9KOmat mice after CI-994 treatment was independent of the DNA methylation status at the Peg13-DMR.

The human PEG13-DMR has been further demonstrated to bind CTCF-cohesin which conveys chromatin looping between a brain-specific enhancer region, marked by H3K27ac and H3K4me1, and the Peg13 and KCNK9 promoters4. To determine the effect of CI-994 on the Kcnk9 locus in the hippocampus and LC of Kcnk9KOmat mice, we performed Chromatin immunoprecipitation (ChIP)-qPCR following CI-994 or vehicle treatment. Using several public mouse brain ChIP-seq datasets, we did not find an orthologous region in the
Fig. 4 Identification of epigenetic modulators upregulating Kcnk9\textsuperscript{mat} expression in mPCNs. \textbf{a} Workflow of murine primary cortical neurons (mPCN) isolation, drug treatment, and RT-qPCR analysis. \textbf{b} Epigenetic modulators used for treatment of mPCNs (left column) with enzymatic activities inhibited by them (right column). \textbf{c} Significant upregulation of paternal allele-derived Kcnk9 mRNA in DZnep-, SAHA-, VPA- and CI-994-treated E14 Kcnk9\textsuperscript{mat} mPCNs compared with DMSO-treated Kcnk9\textsuperscript{mat} mPCNs detected by RT-qPCR (3-day treatment, normalization to Impa2, DMSO n = 7 cultures/group, DZnep (20 µM) SAHA (30 µM), VPA (5 mM) and CI-994 (40 µM) n = 3 cultures/group). One-way ANOVA: F(5, 22) = 75.51, P < 0.0001; followed by Bonferroni’s multiple comparison post hoc test to Kcnk9\textsuperscript{mat} DMSO control, ***P < 0.0001. \textbf{d} Upregulation of paternal allele-derived Kcnk9 mRNA after treatment of Kcnk9\textsuperscript{mat} mPCNs with different CI-994 concentrations compared with paternal allele-derived Kcnk9 mRNA of DMSO-treated Kcnk9\textsuperscript{mat} mPCNs and Kcnk9 mRNA of DMSO-treated WT mPCNs detected by RT-qPCR (1 day treatment, normalization to Impa2, Kcnk9\textsuperscript{mat} DMSO n = 9, 4 µM n = 2; 20–80 µM n = 4 and WT DMSO n = 5 cultures/group). One-way ANOVA: F(5, 22) = 75.51, P < 0.0001; corrected with Bonferroni’s multiple comparison post hoc test to Kcnk9\textsuperscript{mat} DMSO mPCNs, ***P < 0.0001**, P < 0.0001, *P < 0.0001. \textbf{e} performed in 2–4 independent experiments. \textbf{f} Duration of Kcnk9 derepression in vitro. Work flow is illustrated in schematic representation (left). After 1 and 10 days, Kcnk9 is significantly upregulated after CI-994 treatment in mPCN compared with DMSO controls. One day DMSO n = 2, 1 day CI-994 n = 3, day 10 DMSO n = 6, day 10 CI-994; n = 4; n = samples (3–5 wells/sample; 6 embryos pooled). Day 1: P = 0.0297, day 10: P = 0.0001, Mann–Whitney U. \textbf{c–f} Values are means ± SEM. Statistical analyses and approaches are provided in Supplementary Table 1. Source data are provided as a Source Data file. Images displaying the mouse, the embryo and the neurons in this figure were created using Servier Medical Art templates, which are licensed under a Creative Commons Attribution 3.0 Unported License. https://smart.servier.com.

Mouse displaying a brain-specific enhancer chromatin signature similar to that identified at the human imprinted domain on chromosome 8q24. Thus, we focused our analysis on the promoter and intronic region of the Kcnk9 locus, which are enriched for both marks in the mouse brain ChIP-seq datasets (Fig. 6a). Due to the small size of the LC, we opted to use a novel low-input ChIP-method\textsuperscript{27} for both regions (LC and hippocampus) and validated this by conventional ChIP-qPCR in the hippocampus (Supplementary Fig. 7b). Upon CI-994 treatment, we observed a significant more than two-fold increase in H3K27ac depositions at the two Kcnk9 regions both in the hippocampus and LC of Kcnk9\textsuperscript{mat} mice (Fig. 6b). The levels of H3K27ac were also increased in the hippocampus of WT littermates however the increase was not as prominent as in the Kcnk9\textsuperscript{mat} animals (Supplementary Fig. 7i, j). An intergenic region, lacking H3K27ac or H3K4me1 modifications, was used as negative control (Supplementary Fig. 8c-j). Looking at allele-specific H3K27ac deposition in the hippocampus of (C57BL/6xCast/Ei)F\textsubscript{1} hybrid animals using two different SNPs in intron 1 of Kcnk9, we found that, as expected, the maternal allele had a higher degree of H3K27ac association than the paternal allele. CI-994 treatment of Kcnk9\textsuperscript{mat} mice, however, led to a higher degree of H3K27ac deposition at the paternal than at the maternal Kcnk9 allele (Fig. 6c).

In addition, we tested the deposition of H3K4me1 both in the LC and hippocampus to assess if histone acetylation inhibition leads to additional changes in other histone marks. Treatment with CI-994 led to only a slight, but not significant increase of H3K4me1 deposition at the Kcnk9 promoter region (Supplementary Fig. 7f-h). Our findings indicate that CI-994 treatment affects histone acetylation consistently at the promoter and intronic regions of Kcnk9 of the investigated brain tissues and has diverging effects on H3K4me1 deposition at the investigated Kcnk9 locus.

Taken together, our findings demonstrate that the paternal allele of Kcnk9 is not fully silenced in the brain, further derepressed in the case of loss of the maternal allele and substantially activated upon exogenous epigenetic modulation. Our data provide evidence for a promising therapeutic effect of the class I HDAC inhibitor CI-994, in a mouse model for BBIDS with maternal loss of Kcnk9.

**Discussion**

Kcnk9/KNK9 is an imprinted gene in mouse and man that is expressed from the maternal allele. Pathogenic variants present on only the maternal allele, therefore, cause disease in patients.\textsuperscript{12} In a study comparing full Kcnk9 knockout animals (Kcnk9\textsuperscript{hom}) with maternal Kcnk9 knockout animals (Kcnk9\textsuperscript{mat}) and WT littermates in a behavioral battery, we surprisingly found an intermediate phenotype in nocturnal locomotor activity in Kcnk9\textsuperscript{mat} animals. Furthermore, electrophysiological analysis of tyrosine hydroxylase (TH)-positive neurons in the LC, a region involved in regulation of arousal and locomotor activity in mice, displayed significantly increased spontaneous firing frequencies only during the active dark phase in full knock-outs in contrast to WT, while it was fully rescued in the Kcnk9\textsuperscript{mat} animals. Allele-specific expression analysis of Kcnk9 mRNA in (C57BL/6xCast/Ei)F\textsubscript{1} hybrid animals revealed significant residual paternal expression of up to 14% of total Kcnk9 expression in the LC in the Kcnk9\textsuperscript{mat} animals. Daily i.p. injection of the second-generation HDAC inhibitor CI-994 could increase this expression up to three-fold and resulted in a full rescue of the behavioral phenotypes of Kcnk9\textsuperscript{mat} animals.

We show here that an increase in dark-phase locomotion in the Kcnk9\textsuperscript{hom} animals is associated with an increase of in vitro pacemaker frequency in the LC. Deletion of the active maternal Kcnk9 allele in the mouse is supposed to lead to a phenotype comparable to the full knockout. Surprisingly, however, our study identified an intermediate behavioral phenotype in nocturnal (hyper)activity in the Kcnk9\textsuperscript{mat} animals, as well as a fully rescued cellular phenotype (LC firing frequency) (Figs. 1c, 3). Extended allele-specific expression analysis using (C57BL/6xCast/Ei)F\textsubscript{1} mouse hybrids demonstrated residual Kcnk9 expression from the paternal allele in several brain regions with a significant peak in the LC of 14% of total Kcnk9 expression coming from the paternal allele.

The LC plays an essential role in arousal and sleep-wake transitions\textsuperscript{28} and, as the origin of noradrenergic neurons projecting into the hippocampus and the prefrontal cortex, is also involved in the regulation of long-term and working memory, two essential building blocks of higher cognition/brain functions.\textsuperscript{29} In a knockdown experiment using shRNA\textsubscript{AMir} containing AAVs targeting the Kcnk9 mRNA specifically injected into the LC we found a similar behavior phenotype in circadian activity as seen in the full and maternal Kcnk9 knockout animals demonstrating an essential and causal contribution of Kcnk9 expression within the LC in the control of nocturnal locomotor activity. Interestingly, our electrophysiological data show full rescue of in vitro pacemaker firing frequency through Kcnk9\textsuperscript{mat} expression in the LC in Kcnk9\textsuperscript{mat} animals on the one hand and only a partial rescue of dark-phase activity on the other. This might indicate different levels of Kcnk9 expression in order to control on one hand isolated pacemaker function, which typically operates with very small ionic currents of a few picombs, compared...
Fig. 5 Effects of CI-994 histone deacetylase inhibitor treatment in vivo. a Experimental design of the mouse study. CI-994 or DMSO was intraperitoneally injected. b Significant upregulation of Kcnk9 after CI-994 treatment in several brain regions normalized to DMSO treatment, Mann–Whitney U test. Regions from left to right, DMSO/CI-994, n = 12/13; 6/9; 12/14; 7/8; 9/12; 6/6; 9/9; 6/6; 7/8 and 7/4. c Upregulation of Kcnk9 in the cerebellum of CI-994-treated Kcnk9KOmat mice (n = 13), but not of CI-994-treated WT mice (n = 12), each compared with DMSO-treated Kcnk9KOmat mice (n = 13) mice in the distance traveled in the light phase. No differences between DMSO-treated WT (n = 10), CI-994-treated WT mice (n = 12), CI-994-treated Kcnk9KOmat mice (n = 12) and CI-994-treated Kcnk9KOmat mice did not exhibit deficits in working memory. CI-994-treated Kcnk9KOmat mice showed a significant increase in percent spontaneous alteration compared with DMSO-treated Kcnk9KOmat mice. Two-way ANOVA, Tukey post hoc test, **P < 0.01, *P < 0.05. d-f Distance traveled in the home cage in light (12 h) phase (left section) and the dark (12 h) phase (right section). No differences between DMSO-treated WT (n = 10), CI-994-treated WT (n = 10), DMSO-treated Kcnk9KOmat (n = 12) and CI-994-treated Kcnk9KOmat (n = 12) mice displayed a significant increase of nocturnal activity compared with DMSO-treated WT mice (n = 24) and CI-994-treated Kcnk9KOmat (n = 13) mice as well as a visible, but not significant increase of nocturnal activity compared with CI-994-treated WT mice (n = 15). Two-way ANOVA, Bonferroni’s post hoc test, **P < 0.01, *P < 0.05. b–f n = sample or data arising from biologically independent animals. e, f Behavioral experiments were performed in nine independent sessions. b–f Values are means ± SEM (n indicates the standard error). Statistical analyses and approaches are provided in Supplementary Table 1. Source data are provided as a Source Data file.

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with in vivo function for behavioral control, where neurons are embedded in active networks and larger currents are necessary to alter their firing patterns. Our data suggest that the influence of KCNK9 on pacemaker frequency and potentially in vivo activity could be feasible as a therapeutical strategy for attention deficit and hyperactivity disorder (ADHD). However, future studies should directly record from LC neurons in vivo in awake and hyperactivity disorder (ADHD). However, future studies could be feasible as a therapeutical strategy for attention deficit within the same experiment but not across the different experiments performed.

A recent study has suggested histone methylation as a potential therapeutic target for another imprinting disorder, the Prader-Willi syndrome (PWS)\textsuperscript{31}. The authors showed that UNC0642, a selective inhibitor of euchromatic histone-lysine N-methyltransferase-2, derepressed the maternal copies of paternally expressed PWS candidate genes and promoted the growth and survival of mouse pups with a paternal deletion of the PWS region without altering DNA methylation at the PWS-ICR. We show here that derepression of the paternal allele in 

Kcnk9\textsuperscript{mat}\textsuperscript{KO} mice with CI-994 for the revised manuscript version. In light of these limitations, we strongly argue that a comparison of nocturnal activity values makes only sense within the same experiment but not across the different experiments performed.

Kcnk9\textsuperscript{mat}\textsuperscript{KO} for the revised manuscript version. In light of these limitations, we strongly argue that a comparison of nocturnal activity values makes only sense within the same experiment but not across the different experiments performed.

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Kcnk9\textsuperscript{mat}\textsuperscript{KO} for the revised manuscript version. In light of these limitations, we strongly argue that a comparison of nocturnal activity values makes only sense within the same experiment but not across the different experiments performed.
for treatment of imprinting disorders such as BBIDS. CI-994 treatment of Kcnk9KOmat mice as a mouse model of BBIDS substantially activated the repressed paternal allele of Kcnk9 in several brain areas resulting in a significant increase in Kcnk9 expression. This led to the full rescue of specific domains of brain function impaired in BBIDS. Our data for the first time shows successful phenotypic rescue of impaired brain function in a mouse model for an imprinting disorder using an epigenetic modulator.

Similar to the findings in the UNCG042-treated mouse model of PWS31, activation of the Kcnk9pm allele was not accompanied by changes of DNA methylation at the Peg13 DMR, which is assumed to play a key role in regulation of Kcnk9 imprinting also in the mouse4. Notably, the bisulphite technique did not allow us to discriminate between 5-methylcytosine and 5-hydroxymethylcytosine levels possibly affected by CI-994. Though, our data strongly argue that CI-994-mediated derepression of the Kcnk9pm allele is primarily caused by an increase of H3K27ac at regulatory elements in the promoter and intronic region of the Kcnk9 gene. However, the treatment did not display significant differences in H3K27me3 deposition between the LC and hippocampus tissues suggesting a region-independent effect of the HDAC inhibitor. Such effects have commonly been reported in multiple genome-wide studies investigating the effect of HDAC inhibitors32. Interestingly, CI-994 has been used in anti-epileptic therapy for decades without severe tumorigenic or metabolic long-term effects. CI-994 is a second-generation, specific class I HDAC inhibitor has been used in anti-epileptic therapy for decades and provides neuroprotective effects in the central nervous system and cell survival in vitro36. Interestingly, CI-994 treatment of imprinting disorders.

In summary, we show here that brain region-specific upregulation of the paternally silenced Kcnk9 allele particularly in the LC of Kcnk9KOmat animals modulates nocturnal hyperactivity, a central phenotype of Kcnk9 knockout animals. We further show that the class I HDAC inhibitor CI-994 leads to a significant additional increase of Kcnk9pm expression in several brain regions and fully rescues behavioral alterations in Kcnk9KOmat animals. This rescue is associated with an increase of H3K27 histone acetylation at the promoter and intronic region of the Kcnk9 gene but not with changes in DNA methylation at the differentially methylated region in the Peg13/Kcnk9 locus suggesting epigenetic modulation by CI-994 as a promising therapeutic strategy in patients with BBIDS and, more generally, derepression of imprint gene alleles as a sustainable approach for the treatment of imprinting disorders.

Methods

Mice. All experimental procedures were performed in accordance with institutional animal welfare guidelines and were approved by the ethical committee of the state government of Rhineland-Palatinate, Germany (ID: 23.177-07/G 17-1-022). Kcnk9KOmice were provided by Florian Lesage, Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France. The gene targeting strategy of Kcnk9KOmice was based on a cre-mediated deletion of exon 2 encoding pore domains P1 and P2, transmembrane domains M2-M4, and C-terminus32. Breeding of two heterozygous animals revealed homozgyous, heterozygous and WT animals at a proportion of 1:1:2. WT littersmates were used as controls. WT and heterozygous Kcnk9KO mice with inactivation of the maternal Kcnk9 allele (Kcnk9KOmat) were obtained by crossing male WT mice with female Kcnk9KOmat mice. Finally, breeding of homozygous female and heterozygous male mice resulted in Kcnk9KOmat and Kcnk9KOjuno mice. The mice were kept under specific-pathogen-free (SPF) conditions on a 12 h light/12 h darkness cycle in standard polystyrene cages with free access to water and food. Using tail tip DNA, mice were genotyped by assessing exon 2 excision using the primers flanking this exon: 5′-TGGAATTCGCGGAAGAGGATG-3′ and R, 5′-ATGCTCTTAACTTCAGCTGTGCTG-3′ producing fragments for WT and mutant alleles. Additional primers within exon 2 were applied to generate a control product for the WT allele (F, 5′-CCACCCAGGTATCTTCTCCTT-3′ and R, 5′-GGACGGGAATGTAGTGTCCTC-3′). Male mice at 8–10 weeks of age were used for expression analysis and to investigate the behavioral phenotype with and without drug treatment. Allele-specific expression analyses were carried out with total RNA from F1 offspring derived from crosses between female WT C57BL/6J or KcnK9juno mice and male WT Mus musculus castaneus (Cast/Ei) mice.

Behavioral testing. Littermate WT and Kcnk9KO mice were tested in 4–8 cohorts of mice, with testing beginning at 8–10 weeks of age. All experimenters were blinded to the genotype of the mice throughout the studies and behavioral analyses. Immediately after the behavioral tests, animals were sacrificed and whole brains were rapidly removed and incubated in RNAlater® (Sigma) for subsequent expression analysis.

Drug administration in vivo. Nine weeks old male Kcnk9KOmat and WT mice were injected intraperitoneally once daily with CI-994 (ApexBio) or dimethyl sulfoxide (DMSO) (control vehicle) for 7 consecutive days before behavior experiments were initiated (Fig. 1c). Mice were injected 2 h before behavior experiments. A single injection contained either 20 μl DMSO or 20 μl CI-994 (35 mg/kg) dissolved in 100 % DMSO.

Toxicity/ viability test in CI-994 treated mPCNs. mPCNs were washed with PBS and fixed with 4% paraformaldehyde for 2 h. Subsequently, cells were incubated for 20 min in 0.4% Triton X-100 in PBS at 37 °C and further rinsed three times with PBS. After blocking with 10% sheep serum at 37 °C for 1 h, cells were washed for a further three times with PBS. Next, neurons were incubated overnight at 4 °C with anti-neuron-specific nuclear protein NeuN monoclonal antibody (cat. # ab104225; dilution 1:200 in PBS), followed by five washes with PBS for 5 min each. The
neurons were subsequently incubated with an Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (cat # ab150577, dilution 1:200 in PBS) for 1 h at 37 °C. After three times of PBS rinses, the dishes were mounted onto microscope slides and counterstained with mounting medium containing 2 µg/mL Hoechst 333258 (Cat # H3569).

**Tissue collection.** Whole mouse brains were removed and incubated in RNAlater® (Sigma) and stored for 2 days at 4 °C. After the dehydration process, several brain regions were further processed with Lovejoy® knives, coronal brain sections (80 µm) between bregma -5.40 and +5.52 were generated, and 0.51 µm punches bilaterally of each brain region were collected with the Brain Punch Tissue Set (Leica). The accuracy of the LC tissue collection was validated by performing tyrosine hydroxylase (TH) expression analyses and comparing the expression levels of TH among the WT cerebellum and hippocampus samples. Samples with minimal TH expression TH signals were excluded from analysis. Tyrosine hydroxylase, which serves as a norepinephrine marker, was expressed –400-fold higher compared with hippocampus samples, indicating a precise tissue dissection (Supplementary Fig. 2).

**DNA methylation analysis.** Approximately 500 ng DNA was subjected to sodium bisulfite treatment and purified using the EZ DNA Methylation Direct® kit (Zymo Research). Bisulfite PCR primers for the Peg13-DMR1 and Peg13-DMR2 (located from +195 to +833 bp relative to the 5’ end of the Peg13 transcript) were designed using the Pyrosequencing Assay Design Software (Qiagen). PCR amplification was performed with 1 µg bisulfite treated DNA and specific primers at 42 cycles in a following bisulfite PCR analysis. We amplified two Cpg-rich regions, Peg13-DMR1 and Peg13-DMR2, associated with the mouse Kcnk9 gene regulation with the following primers: Peg13-DMR1, forward 5′-TTGGATGAGTTATTTAATGAAGTTTGA-3′ and reverse 5′-ACAACTATCCACCTACCACTC-3′-biomethylated (product size: 175 bp), sequencing 5′-AATTTAAATAGTGGATT3′- A3′-T3′-PEG13-DMR2, forward 5′-AGATTTGGAATATTGGTAGTTA-3′ and reverse 5′-CC TCAAATACCCACCTTAACTCACT-3′-biomethylated (product size: 198 bp), sequencing 5′-GGAATTTTGAGTGGACAGATTA-3′. The pyrosequencing was carried out on the PyroMark Q96 instrument (Qiagen).

**Chromatin Immunoprecipitation (ChIP)-qPCR.** ChIP experiments were performed as described in Akhtar et al. with minor modifications for brain tissue processing26. Briefly, brain tissues were dissected in cold PBS, homogenized with a pestle. Following dissection, tissues were fixed with 1% formaldehyde in PBS for 10 min at room temperature, followed by quenching of the fix with 125 mM glycine for 5 min. Fixed cells were resuspended in 140 mM RIPA (10 mM Tris-Cl pH 8.0, 140 mM NaCl, 0.1% Triton X-100, and 0.1% SDS) and subjected to 18 cycles of sonication on Bioruptor Pico (Diagenode), with 30 secs “ON”/“OFF” at high settings. After sonication, samples were centrifuged at 14,000 × g for 10 min at 4 °C and supernatant was transferred to a fresh tube. The extracts were neutralized with overnight incubation in 1 µg (1:500 dilution) of H3K27ac (Abcam, Cat # ab4729), H3K4me1 (Active motif, Cat # 39297), H3 (Abcam, Cat # ab7197) or Peg13 K9me3 (Biolegend, Cat # S9412) for 4 h at 4 °C (2000 rpm). After overnight incubations, 20 µl of blocked Protein A and G Dynabeads (Diagenode) were added to the tubes and further incubated for 3 h to capture the antibodies. Bead separation was carried out using a magnetic rack and washed 8 times once with 140 mM RIPA (10 mM Tris-Cl pH 8.0, 140 mM NaCl, 0.1% Triton X-100, and 0.1% SDS) with head-over tail washes. For reverse of cross-linking, samples were RNase-treated (NEB) and subjected to Proteinase K treatment for 12 h at 37 °C and at least 6 h at 56 °C after the immunoprecipitation. After protease K treatment, the DNA was extracted using the phenol-chloroform method. After precipitating and pelleting, DNA was dissolved in 30 µl of TE buffer pH 8. For the real-time PCR was performed using SYBR Green chemistry (ABI) on the ABI StepOnePlus™ Real-Time PCR System.

**RNA isolation, DNA synthesis, and RT-qPCR.** The total RNA extraction with TRIzol reagent from brain tissue was prepared as suggested by Invitrogen Life Technologies. High Pure RNA Tissue Kit (Roche) and High Pure RNA Isolation Kit (Roche) were applied to extract total RNA from small tissue and cell samples using spin columns. The purity, quantity, and integrity of the RNA were measured with a NanoDrop 1000 spectrophotometer and an Agilent 2100 Bioanalyzer. The cDNA samples were synthesized from 200 to 1000 ng total RNA using the Pri-meScript™ RT Master Mix cDNA (Takara) as recommended by the manufacturer. Quantitative real-time PCR (qPCR) was carried out on an ABI StepOnePlus™ Real-Time PCR System using exon–exon junction primer with the following conditions: 95 °C/10 s, 40 cycles of 95 °C/5 s, 68 °C/30 s. All reactions were performed in triplicates, and median cycles to threshold (Ct) values were used for analysis. The housekeeping gene Impa2 was used to normalize against experimental genes, and relative gene expression was determined using the 2-ΔΔCt method28. Impa2 forward primer 5′-GCGGGACAAACATCTCA-3′ and reverse primer 5′-CGTGGGCAAAATCATCAG-3′.

**Allele-specific expression analysis by QUASEP.** For allele-specific expression analysis, the synthesized cDNA of (C57BL/6J×Cast/Ei)F1 hybrid mice was amplified in a PCR applying one non-biometric forward primer: 5′-GCGTCTGACTCTCACTCTAC3′ and one biomethylated reverse primer: 5′-GACACTATCAGGATA TGGACATG-3′. To distinguish the origin of the alleles we subsequently analyzed the RT-PCR products in allele-specific expression analysis using the C/T-SNP rs225149059 (Ensembl; GRCm38.p5) and QUASEP method as previously described29. A Kcnk9-specific pyrosequencing primer: 5′-TGGCGGGGCTTGTGTC-3′, was designed flanking the allele-specific SNP and pyrosequencing was carried out on a PyroMark Q96 instrument (Qiagen).
manner, using two neighboring SNPs, rs240891437 and rs47242380 (Ensembl; GRCm38.p5). The quantification of enrichment was performed using the pyrosequencing technique. A Kcnk9-specific pyrosequencing primer-5′-AAAGAATGGGTGTCGC-3′ was designed and pyrosequencing was carried out on a PyroMark Q96 instrument (Qiagen).

Behavioral analyses. Brief descriptions of the behavioral tests are provided below. Male mice between 8 and 10 weeks of age were used for behavioral tests, as described below.

Circadian locomotor activity. Mice were individually placed in transparent polypropylene cages (38 × 22 × 15 cm) with standard beddings. Food and water were available and accessible at all times. The cages were placed in a sound-prove room under dimed light during the light phase and under infrared LED lights during the dark cycle. The horizontal locomotor activity of the mice was recorded in the light and in the dark phase using a video tracking software (EthovisionXT, Noldus software). The circadian rhythm activity was measured after 48 h acclimatization for 24 h and analyzed with EthovisionXT, Noldus software. The results show the distance traveled (in cm) in the light and in the dark phase.

Spontaneous alternation. Spatial working memory was assessed in a Y-maze test as described previously40. During a 10-min session in a Y-maze the spontaneous alternation behavior was recorded. The spontaneous alternation test is based on the willingness of rodents to explore new environments, therefore to enter the arm of a Y-maze that had not been recently explored, i.e., the arm that was not entered in the previous choice. Mice were placed in a gray plexiglass Y maze in a sound proof room. Each maze consisted of three arms. Each arm was 40-cm long, 20-cm high, and 10-cm wide, and the arms converged to an equilateral triangular central area. Each mouse was placed at the end of the start arm, in which the mouse starts to explore and move freely through the maze during the 10-min session. The series and order of arm entries was observed manually and considered to be completed when the hind paws of the mouse had completely entered the arm. Transitions between arms were scored as either correct or incorrect alternations by the observer. Healthy animals (controls) show a high change rate. This shows that the animal can remember which arm was last entered. The percentage of alternation was calculated as the ratio of actual to possible alternations multiplied by 100, either for a 10-min session or 24 h, respectively. The wins of the arm of the Y maze was cleaned with 70% ethanol solution between trials.

In vitro brain slice patch-clamp recordings of LC neurons. Adult mice were anesthetized and perfused with ice-cold ACSF as previously described41. The brainstem slices containing the LC were sectioned after intracardiac perfusion using ice-cold ACSF (50 mM NaCl, 25 mM KCl, 25 mM NaHCO3, 1.25 mM NaH2PO4, 2.5 mM glucose, 6.2 mM MgCl2, 0.1 mM CaCl2, and 0.9% kynurenic acid; Sigma-Aldrich GmbH), oxygenated with 95% O2/5% CO2. Slices were continuously perfused with oxygenated ACSF (2-4 ml min−1; 36 °C; 22.5 mM NaCl, 3.5 mM KCl, 25 mM NaHCO3, 1.25 mM NaH2PO4, 2.5 mM glucose, 2 mM MgCl2, 0.1 mM CaCl2, 6.2 mM MgCl2, and 0.9% kynurenic acid) for 45 min and then transferred to a recording chamber. CNQX (12.5 mM; Biotrend), and yohimbine (1 µM; Biotrend) were added to inhibit fast excitatory and inhibitory synaptic transmission as well as activity of somatodendritic alpha2 adrenoceptors, respectively. The electrophysiological recordings (whole-cell patch-clamp recordings) and data acquisition were performed as previously described42.

Histology. Animals were injected with phenobarbital and perfused with fixed solution containing 4% paraformaldehyde, 13% picric acid in phosphate buffer solution (pH 7.4). The removal brains after post-fixed overnight, post-fixed sections were sliced using a microtome (VT1000S, Leica). Free-floating sections were washed in PBS and incubated with blocking solution (10% horse serum, 0.5% Triton X-100 and 0.2% BSA in PBS) for 1 h at room temperature. Then, sections were incubated with primary antibodies in carrier solution (1% horse serum, 0.5% Triton X-100 and 0.2% BSA in PBS) at room temperature overnight. After washing the sections with PBS, they were incubated with secondary antibodies in carrier solution overnight. The following primary antibodies were used: monoclonal mouse anti-tyrosine hydroxylase (TH, catalog #MAB318, 1:1000, Millipore), polyclonal rabbit anti-GFP (catalog #A11212, 1:1000, Life Technology). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit (catalog #A11008, 1:1000, Thermo Fisher Scientific, Invitrogen) and Alexa Fluor 568 goat anti-mouse (catalog A11004, Thermo Fisher Scientific, Invitrogen). On the third day, sections were washed with PBS, incubated in PBS containing 0.02% 4,6-diamidin-2-phenylindol (DAPI, catalog #D1306, Molecular Probes, Invitrogen) for 5 min and washed in PBS for 10 min. Thereafter, sections were mounted on slides, coverslipped and stored at 4 °C until confocal images were taken.
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Author contributions
A.C. carried out most experiments and wrote parts of the manuscript; J.R., S.u.S. and U.Z. have designed the project, supervised the experiments and wrote the paper; J.W. supervised and helped with the ChIP experiments; T.B. conducted the ChIP experiments; N.H. did the AAV infusions into the LC; S.J. conducted in vitro brain slice patch-clamp recordings of LC neurons; D.L.F.G. and K.R. helped with the behavior experiments; I.A. has established the low-input ChIP; F.L. provided the KrosKO animals; S.S. helped with microscopy.

Competing interests
The authors declare no competing interests.

Additional information
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