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## **The effect of morphine upon DNA methylation in ten regions of the rat brain**

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## Abstract

Morphine is one of the most effective analgesics in medicine. However, its use is associated with the development of tolerance and dependence. Recent studies demonstrating epigenetic changes in the brain after exposure to opiates have provided insight into mechanisms possibly underlying addiction. In this study, we sought to identify epigenetic changes in ten regions of the rat brain following acute and chronic morphine exposure. We analyzed DNA methylation of six nuclear-encoded genes implicated in brain function (*Bdnf*, *Comt*, *Il1b*, *Il6*, *Nr3c1* and *Tnf*) and three mitochondrially-encoded genes (*Mtco1*, *Mtco2* and *Mtco3*), and measured global 5-methylcytosine (5-mc) and 5-hydroxymethylcytosine (5-hmc) levels. We observed differential methylation of *Bdnf* and *Il6* in the pons, *Nr3c1* in the cerebellum, and *Il1b* in the hippocampus in response to acute morphine exposure (all  $p < 0.05$ ). Chronic exposure was associated with differential methylation of *Bdnf* and *Comt* in the pons, *Nr3c1* in the hippocampus and *Il1b* in the medulla oblongata (all  $p < 0.05$ ). Global 5-mc levels significantly decreased in the superior colliculus following both acute and chronic morphine exposure, and increased in the hypothalamus following chronic exposure. Chronic exposure was also associated with significantly increased global 5-hmc levels in the cerebral cortex, hippocampus and hypothalamus, but significantly decreased in the midbrain. Our results demonstrate, for the first time, highly localized epigenetic changes in the rat brain following acute and chronic morphine exposure. Further work is required to elucidate the potential role of these changes in the formation of tolerance and dependence.

Keywords: Morphine, addiction, DNA methylation, 5-methylcytosine, 5-hydroxymethylcytosine, mitochondrial epigenetics, BDNF, opiates

## Introduction

Morphine is one of the most effective analgesic medications. It, along with other opioids, relieves acute and chronic pain by acting at mu opioid receptors found throughout the central nervous system. Alongside its therapeutic effects, morphine use is also associated with adverse effects such as tolerance and dependence.

Morphine tolerance may originate in part through neuronal processes via activation of mu opioid receptors and subsequent neuroexcitation<sup>1</sup>. Preclinical gene expression profiling studies have demonstrated widespread changes in the transcriptome of the nucleus accumbens and striatum following chronic morphine exposure<sup>2,3</sup>, including to genes associated with circadian rhythms, neurotransmitter release, and glucocorticoid receptor signalling. There is also increasing evidence for a role of microglia in tolerance. Microglia are macrophages present within the central nervous system that produce pro-inflammatory cytokines such as interleukin 1 $\beta$  (IL-1 $\beta$ ), interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) following their activation by morphine<sup>4</sup>. IL-1 $\beta$ , IL-6 and TNF- $\alpha$  have been shown to reduce morphine analgesia within five minutes of administration<sup>4</sup>, and blockade of IL-1 $\beta$  signalling prolongs morphine-induced analgesia<sup>5</sup>, thereby potentially implicating these three cytokines in morphine tolerance. Microglial activation is further implicated in the development of tolerance through increased expression of brain derived neurotrophic factor (*BDNF*) in the ventral tegmental area (VTA) that facilitates the switch to the dopamine-dependent reward system present in addiction<sup>6</sup>. Notably, *Bdnf*<sup>-/-</sup> knockout mice do not develop tolerance to morphine<sup>7</sup>. Other genes implicated in morphine tolerance include the catechol-O-methyltransferase (*COMT*) gene through its role in dopamine elimination and whose activity therefore regulates morphine response in mice<sup>8</sup>, and the glucocorticoid receptor gene *NR3C1* that is epigenetically silenced in the HPA axis following chronic, but not acute, morphine exposure<sup>9</sup>.

Exposure to opiates is also known to induce mitochondrial dysfunction in neuronal and glioma cells<sup>10,11</sup>, and chronic exposure is associated with reduced mitochondrial DNA copy number in the rat hippocampus<sup>12</sup>. Furthermore, morphine may induce oxidative stress in the brain<sup>13</sup>, and this is known to induce the expression of mitochondrial DNA methyltransferase 1 (mtDNMT1) that regulates the mitochondrial epigenome<sup>14</sup>.

Morphine exposure has been demonstrated to affect the epigenetic regulation of genes implicated in addiction and tolerance, both in human studies and in animal models. Differential methylation of the *BDNF*<sup>15,16</sup> and opioid receptor Mu 1 (*OPRM1*)<sup>17,18</sup> gene promoters have been reported in the blood of opiate-dependent individuals. Epigenetic changes in the brain may be highly localized, as studies utilizing whole brain tissue have often reported no significant change in global DNA methylation levels following morphine exposure<sup>19,20</sup>. Targeted approaches have proven more insightful, identifying changes in regions of the brain associated with addiction such as the hippocampus, medial prefrontal cortex and VTA in a rat model of heroin self-administration and reward devaluation<sup>21</sup> and post-mortem orbitofrontal cortex brain tissue from former heroin users<sup>22</sup>.

However, there remains a significant knowledge gap regarding the spatial and temporal epigenetic regulation of genes in the brain that are implicated in tolerance and dependence. In this study, we analyzed epigenetic changes in response to acute and chronic morphine induction in 10 regions of the rat brain reported to be implicated in response to opiates and the formation of addiction: the midbrain contains the VTA that has been consistently implicated in addiction<sup>23,24</sup>; the pons contains the locus coeruleus that is key in the integration of opioid and stress signalling and which is served by innervation from the paraventricular nucleus of the hypothalamus<sup>25</sup>; the inferior and superior colliculus regulate response to opiate withdrawal through reduced activation of mu-opioid receptor signalling<sup>26,27</sup>; the cerebral cortex (containing the dorsomedial prefrontal cortex),

hippocampus and cerebellum are implicated in reinforcement of drug-seeking behaviors<sup>28-30</sup>; the medulla oblongata is crucial in pain modulation and opiate withdrawal behaviors<sup>31</sup>; and the thalamus whose function is disrupted in opiate dependence<sup>32,33</sup>. We measured global 5-methylcytosine and 5-hydroxymethylcytosine levels, and analyzed the DNA methylation of genes previously identified as implicated in morphine tolerance (*Bdnf*, *Comt*, *Il1b*, *Il6*, *Nr3c1* and *Tnf*) and mitochondrially-encoded genes potentially implicated in mitochondrial dysfunction (*Mt-co1*, *Mt-co2*, and *Mt-co3*).

## **Results**

### ***Study overview***

An overview of the study is provided in Figure 1. Male rats were assigned to control, acute challenge and chronic induction groups (all n=5). After 10 days, brain tissue was dissected and DNA extracted for epigenetic analysis.

### ***Gene-specific DNA methylation variability by region of the rat brain***

The nuclear-encoded genes *Bdnf*, *Comt*, *Il1b*, *Il6*, *Nr3c1* and *Tnf* displayed differential variability by region of the brain (Figure 2A-F). *Bdnf* and *Tnf* displayed highly conserved methylation levels between the regions, with mean *Bdnf* methylation levels ranging between 3.0 and 4.6% (Figure 2A), and *Tnf* methylation between 54.0 and 62.5% (Figure 2F). The greatest variability was observed in *Il6* methylation, which displayed low levels (<6.5%) in the cerebellum, cerebral cortex and hippocampus, but markedly higher methylation (>20%) in the hypothalamus, inferior colliculus, superior colliculus, and the thalamus (Figure 2D). Global DNA methylation levels, estimated using LINE-1 as a surrogate marker (*L1-5utr* and *L1-orf*), were highly consistent between brain regions (Figure 2G-H).

***Morphine induction is associated with gene-specific and global DNA methylation changes in the rat brain***

Acute morphine exposure (10 mg/kg, one hour post injection) was associated with significantly increased methylation of *Il6* in the pons (11.6 vs 14.3%,  $p<0.05$ ) and *Nr3c1* in the cerebellum (1.0 vs 1.2%,  $p=0.03$ ), and significantly decreased methylation of *Bdnf* in the pons (3.9 vs 3.3%,  $p=0.03$ ) and *Il1b* in the hippocampus (76.5 vs 71.8%,  $p=0.02$ ) (Table 2, Figure 3). Chronic morphine exposure (10 mg/kg/day bid for 10 days) was associated with significantly increased methylation of *Il1b* in the medulla oblongata (64.8 vs 68.4%,  $p=0.03$ ) and *Nr3c1* in the hippocampus (1.0 vs 1.6%,  $p=0.02$ ), and significantly decreased methylation of *Bdnf* (3.9 vs 3.1,  $p=0.02$ ) and *Comt* in the pons (77.3 vs 66.9%,  $p<0.0001$ ).

Global DNA methylation levels, estimated through measurement of *Line1* methylation, were significantly lower in the superior colliculus following both acute (*L1-orf*, 82.5 vs 81.9%,  $p<0.05$ ) and chronic morphine exposures (*L1-orf*, 82.5 vs 81.3%,  $p=0.003$ ), and higher in the hypothalamus following chronic exposure (*L1-5utr*, 53.7 vs 54.9%,  $p=0.04$ ) (Figure 4).

***Nuclear 5-hydroxymethylcytosine levels are altered following morphine induction***

We measured global 5-hydroxymethylcytosine (5-hmc) levels in each of the ten regions of the brain following acute and chronic morphine induction. In the control rats, 5-hmc levels varied by brain region (Table 2). The lowest levels of 5-hmc were observed in the hippocampus (0.34%) and thalamus (0.37%), with the highest in the midbrain (0.79%) and pons (0.69%). Significant changes in 5-hmc levels were identified following chronic morphine induction, but none following acute exposure. 5-hmc levels increased in the cerebral cortex (0.50% vs 0.78%,  $p=0.002$ ), hippocampus (0.34% vs 0.43%,  $p=0.01$ ) and



hypothalamus (0.40% vs 0.54%,  $p=0.008$ ), while they decreased in the midbrain (0.79% vs 0.36%,  $p<0.0001$ ) (Figure 5).

***Morphine induction is not associated with alterations in DNA methylation of mitochondrially-encoded genes***

We measured DNA methylation of three mitochondrially-encoded genes (*Mtco1*, *Mtco2* and *Mtco3*) following morphine exposure. Methylation levels of the three genes were uniformly low across the 10 brain regions, with mean levels ranging between 0.8 (*Mtco2*, cerebral cortex and pons) and 2.7% (*Mtco1*, cerebral cortex) (Table 3). No significant effect of acute or chronic morphine induction was observed on methylation of the three genes in any of the brain regions. The most substantial changes in methylation were observed for *Mtco1* in the cerebral cortex following acute (2.7 vs 1.8%,  $p=0.23$ ) and chronic (2.7 vs 1.9%,  $p=0.29$ ) morphine induction.

**Discussion**

In this study, we investigated the effect of acute and chronic morphine exposures upon nuclear and mitochondrial DNA methylation in ten regions of the rat brain known to be affected by opiates. Brain region-specific changes were identified in global 5-methylcytosine and 5-hydroxymethylcytosine content, and the *Bdnf*, *Comt*, *Il1b*, *Il6* and *Nr3c1* genes in response to morphine exposure. We did not observe differential methylation within the mitochondrial genome. To our knowledge, our study is the first to identify region-specific epigenetic changes in the brain in response to morphine exposure.

Morphine induces the dopaminergic mesolimbic pathway in the ventral tegmental area (VTA) within the midbrain. This region of the brain has a high concentration of dopaminergic neurons and is considered to be crucial in addiction. We observed significantly

decreased global DNA methylation in the superior colliculus, estimated through analysis of *Line1*, and a highly significant decrease in 5-hmc content in the midbrain. Together, these observations demonstrate substantial epigenetic changes in this important region of the brain with morphine exposure.

5-hmc is approximately 10-fold more abundant in the brain than other tissues<sup>34</sup>, increasing during neuronal development but also implicated in age-related neurodegeneration.<sup>35</sup> While 5-hmc is an intermediate in active DNA demethylation, 5-mc and 5-hmc levels are not inherently correlated<sup>36</sup> and therefore our observations of decreased 5-mc and 5-hmc are not contradictory. Furthermore, our results could also be the product of the direct role of 5-hmc in RNA splicing<sup>37</sup> and gene expression through inhibition of chromatin remodelling<sup>38</sup>, which may offer an alternative explanation for our observations. TET1 has been demonstrated to be down-regulated in response to cocaine administration, leading to locus-specific decreases in 5-hmc.<sup>39</sup> Whilst other studies have reported no change in global 5-hmc content in response to heroin and cocaine exposure<sup>19,20,39</sup>, they have focussed upon a single region (nucleus accumbens) or utilized homogenized brain tissue and therefore could not exclude the possibility of localized changes in 5-hmc in specific regions of the brain. The limitations of such approaches were addressed in the current study. Indeed, a particular strength of this work is the analysis of ten different regions of the brain that has facilitated the identification of region-specific changes in DNA methylation. Our findings demonstrated significantly increased 5-hmc levels in the cerebral cortex, hippocampus and hypothalamus, in contrast to observations elsewhere of increased hippocampal 5-hmc levels following the cessation of cocaine.<sup>40</sup> Further work is required to elucidate the functional implications of these observations.

We identified differential methylation of three genes (*Bdnf*, *Comt* and *Il6*) in the pons following morphine exposure. This region, located inferior to the midbrain, regulates

respiration and sleep, which are commonly disturbed with morphine use. Animal studies have demonstrated a morphine-induced reduction in acetylcholine in the pons and medulla oblongata<sup>41</sup>, thereby further implicating this region in response to the drug. *BDNF* expression facilitates opiate-associated neural plasticity<sup>42</sup> and the dopamine-dependent response to opiates.<sup>6</sup> Chronic exposure to opiates is associated with increased *BDNF* levels in the VTA<sup>23</sup>, and it has been demonstrated that this increase begins rapidly after initial usage.<sup>43</sup> Other studies have reported increased expression of *BDNF* in the VTA following morphine withdrawal.<sup>24</sup> Our results provide further evidence for the role of *BDNF* in the acute response to morphine exposure and suggest that this may not be limited to the VTA. Catechol-O-methyltransferase (COMT) is implicated in dopamine elimination, and subsequently COMT activity reduces response to morphine<sup>8</sup>, while genetic variants in the COMT gene are associated with differential response to morphine as an analgesic.<sup>44</sup> Our observation of decreased *Comt* methylation with chronic exposure may be in response to increased dopamine signalling and a role in its catabolism. The changes seen in *Il6* in the pons may be in accordance with the modulating role that opioids have in inflammatory pathways<sup>45</sup>. Our observation of increased *Il6* promoter methylation is in contrast to reports of increased secretion in acute response to morphine in the spinal cord<sup>4</sup> and plasma<sup>46,47</sup>. Notably, however, we also identified regional-specificity in *Il6* methylation levels, and therefore the difference in measured methylation levels may represent alterations in cell composition, such as increased microglial infiltration. As IL-1 $\beta$  and TNF- $\alpha$  are also secreted by activated macrophages in response to morphine<sup>48</sup>, but were not differentially methylated in the pons, it is unclear how IL-6 alone may be implicated in response to morphine.

Morphine administration can induce the generation of reactive oxygen species (ROS) by increasing the metabolism of substrates such as dopamine and xanthine oxidase.<sup>49</sup> Elevated ROS levels are known to induce site-specific alterations in DNA methylation by

mechanisms such as modulation of the expression of DNA methyltransferases (DNMTs).<sup>50</sup> While the observed gene-specific changes in DNA methylation are likely to be directly induced by morphine administration, global changes in 5-mc and 5-hmc content could in part be non-specific effects through increased ROS production.

The study of the mitochondrial epigenome is an emerging field of interest. Alterations in mitochondrial DNA methylation have been demonstrated in cardiovascular disease<sup>51</sup> and in response to environmental exposures.<sup>52,53</sup> It has been speculated that such epigenetic changes may give rise to the altered mitochondrial function observed in drug addiction.<sup>14</sup> However, our study did not identify differential methylation of mitochondrial genes in response to morphine induction. We cannot exclude the possibility that other regions of the mitochondrial epigenome may be differentially methylated in response to exposure, and therefore more comprehensive analysis is required.

Our study contains certain limitations that restrict the inferences that can be made from our observations. In particular, the absence of rat behavioral studies and the lack of RNA and protein samples to analyze gene expression have precluded the study of the functional impact of the observed epigenetic changes. The number of cells obtained from some of the dissected tissue specimens was only sufficient to extract DNA from, and therefore no analysis of gene expression was possible. Gene-specific analysis of 5-hmc content could also have proven highly insightful, but is currently prohibitively expensive. Nonetheless, our study offers several important strengths. Firstly, the study of ten different regions of the brain has enabled us to identify region-specific epigenetic changes in response to drug exposure. Furthermore, we have performed a range of epigenetic analyses (nuclear and mitochondrial gene-specific DNA methylation, global 5-mc and 5-hmc content) that have facilitated a broader study of the epigenome in response to morphine exposure. Finally, our

study design has enabled the delineation of epigenetic changes associated with acute and chronic morphine exposure.

In conclusion, we have identified gene-specific and global changes in DNA methylation in response to acute and chronic morphine exposure, which were highly localized to specific regions of the brain. Of especial interest, we identified significant changes in 5-hmc content in four of the ten regions, suggestive of wider epigenetic remodelling in response to morphine exposure. It is hoped that our findings will help to inform further studies in the treatment of dependence, including elucidation of the functional consequences of the observed epigenetic changes and how they relate to long-term response to opiates. Additionally, our findings may help to facilitate the production of biomarkers of drug tolerance. Given the increases in deaths from opiate overdose within the past decade<sup>54,55</sup>, there is an urgent clinical need for tolerance biomarkers that could help to reduce thousands of avoidable deaths.

## **Materials and Methods**

### ***Animals and Housing Conditions***

Male Wistar rats (180-200 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Following shipment, rats were housed in a temperature- (22-24 °C) and humidity- (60%) controlled environment on a 12-hour light/dark cycle (lights on at 7:00 AM) for three days to allow for acclimatization prior to experimental studies. The animals were given free access to food and water *ad libitum*. All efforts were made to reduce the number of animals used and to minimize their suffering. Experimental study groups were assigned randomly. All procedures were approved by the Laboratory Animal Use Committee of Shanghai Jiao Tong University School of Pharmacy.

## ***Drugs***

Morphine hydrochloride was purchased from Shenyang First Pharmaceutical Co., Ltd. (Shenyang, Liaoning, China) and Maybridge Chemicals (Cornwall, U.K.). CBIO was freshly dissolved in sterile normal saline solution (Sinopharm Group Chemical Reagent Co., Ltd.) with the pH adjusted to 7.3-7.5 with 1 M NaOH solution.

## ***Morphine induction***

Male rats were assigned into three groups (each n=5) receiving alternative treatment regimens: saline/saline (Group A, control); saline/morphine (Group B, acute challenge, 10 mg/kg); and morphine/morphine (Group C, chronic induction). For morphine chronic induction, the first treatment period consisted of subcutaneous injection of saline (Groups A and B, 10 ml/kg) or morphine (Group C, 10 mg/kg) twice daily at 12-hour intervals for nine consecutive days<sup>56</sup>. On Day 10, the second treatment was performed by a single subcutaneous injection of saline (Group A) or morphine (Groups B and C, 10 mg/kg) (Figure 1). Previous work has demonstrated tolerance to morphine is developed within 7-10 days, with epigenetic changes observable in the rodent brain 5-7 days following first treatment<sup>57,58</sup>.

## ***Sample Preparation and DNA Extraction***

The rats were decapitated on Day 10, one hour after the last administration. Brains were removed from the skull and ten brain regions (cerebellum, cerebral cortex, hippocampus, hypothalamus, inferior colliculus, midbrain, pons, medulla oblongata, superior colliculus and thalamus) were rapidly dissected, frozen on dry ice, and kept at -80°C until processed. DNA was extracted from the tissues using the Wizard Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. Purified DNA was stored at -20°C until analysis.

### ***DNA Methylation Analysis***

Gene-specific and global 5-mc DNA methylation were analyzed by pyrosequencing. Global methylation levels were estimated using two assays to interrogate Line1 methylation (*LI-utr* and *LI-orf*) that have been widely utilized for this purpose since their first development<sup>59</sup> and which correlate well with measures of global 5-mc content by high-performance liquid chromatography<sup>60</sup>. Gene-specific assays were designed to interrogate promoter regions for the *Bdnf*, *Comt*, *Il1b*, *Il6*, *Nr3c1* and *Tnf* genes. Specifically, the assays interrogated *Bdnf* promoter IV that influences response to levomethadone<sup>15</sup>, the *Comt* P1 promoter that regulates expression of the shorter tissue-specific S-COMT isoform that influences dopamine metabolism in the mouse brain<sup>61</sup>, and the *Nr3c1* GR1<sub>10</sub> promoter that we have previously demonstrated to be differentially methylated in the rat brain in response to environmental exposures<sup>62</sup>.

Bisulfite conversion was performed using 1 µg of genomic DNA and the EZ-96 DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. 30 µl M-Elution Buffer was used for the elution of bisulfite-converted DNA. Following amplification of target regions by polymerase chain reaction (PCR), DNA methylation was analyzed by pyrosequencing. Details of the primers and thermocycling conditions are shown in Table 1. In brief, a 30 µl-PCR was carried out using 15 µl GoTaq Hot Start Green Master Mix (Promega, Madison, WI), 10 pmol forward primer, 10 pmol reverse primer, 1 µl bisulfite-treated DNA, and water. Pyrosequencing was performed using the PyroMark Q96 MD Pyrosequencing System (QIAGEN, Germantown, MD). The percentage of methylated cytosines was quantified at three CpG sites for *Bdnf*, two for *Comt*, two for *Il1b*, one for *Il6*, six for *Nr3c1*, one for *Tnf*, two for *LI-utr* and one for *LI-orf*. For the mitochondrially-encoded genes, the percentage-methylation was measured at three CpG sites

for each of *Mtco1*, *Mtco2* and *Mtco3*. Pyrosequencing reactions were performed in duplicate and the mean of the replicates taken forward for analysis. The correlation coefficient between replicate pyrosequencing runs ranged from 0.77 (*L1-orf*) to 0.97 (*Comt*).

#### ***Analysis of global 5-hydroxymethylcytosine levels***

5-hydroxymethylcytosine was measured in total DNA using the MethylFlash Global DNA Hydroxymethylation ELISA Easy Kit (Epigentek) according to the manufacturer's protocol. The correlation coefficient between replicates was 0.97.

#### ***Statistical Analysis***

Analysis was performed using GraphPad Prism version 7.0b. Differences in DNA methylation were determined by t-test, with significance defined as  $p < 0.05$ .

#### **Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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## Figure legends

Figure 1. Overview of experimental approach.

Rats were assigned to control (A), acute morphine challenge (10 mg/kg one hour post injection, B) and chronic morphine induction (10 mg/kg/day bid for 10 days, C) groups. Saline or morphine were administered twice daily until tissue collection at Day 10.

Figure 2: Variation in DNA methylation by region of the rat brain. A-F: DNA methylation of *Bdnf*, *Comt*, *Il1b*, *Il6*, *Nrc31* and *Tnf* in the cerebellum (Ce), cerebral cortex (CE), hippocampus (Hi), hypothalamus (Hy), inferior colliculus (IC), medulla oblongata (MO), midbrain (Mi), pons (Po), superior colliculus (SC), and thalamus (Th). G-H: global DNA methylation estimated by measurement of *L1-5utr* and *L1-orf* elements in the same 10 regions of the brain.

Figure 3: Differential gene-specific DNA methylation in response to morphine exposure. Gene-specific changes in DNA methylation by morphine exposure (control, acute and chronic) are illustrated, with significant differences indicated (\* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ ).

Figure 4: Global DNA methylation levels following acute and chronic morphine exposure. Global DNA methylation levels were estimated through measurement of *L1-5utr* and *L1-orf* elements in 10 regions of the rat brain. Regions displaying significant changes are illustrated (\* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ ).

Figure 5: Global 5-hydroxymethylcytosine levels following acute and chronic morphine exposure. Global 5-hmc levels were measured by ELISA in 10 regions of the rat brain, with significant changes illustrated (\* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ ; \*\*\*\* =  $p < 0.0001$ ).

Table 1: PCR and pyrosequencing primer sequences.

Table 2: Nuclear DNA methylation in the rat brain following morphine induction. Mean DNA methylation levels of six genes (*Bdnf*, *Comt*, *Il1b*, *Il6*, *Nr3c1* and *Tnf*), global DNA methylcytosine (*L1-5utr* and *L1-orf*) and global 5-hydroxymethylcytosine (*5-hmc*) are provided for the 10 regions of the rat brain that were analyzed, with standard error of the mean in brackets.

Table 3: DNA methylation of mitochondrial-encoded genes in the rat brain following morphine induction. Mean DNA methylation levels of three genes (*Mtco1*, *Mtco2* and *Mtco3*) are provided, with standard error the mean in brackets.

Figure 1. Overview of experimental approach

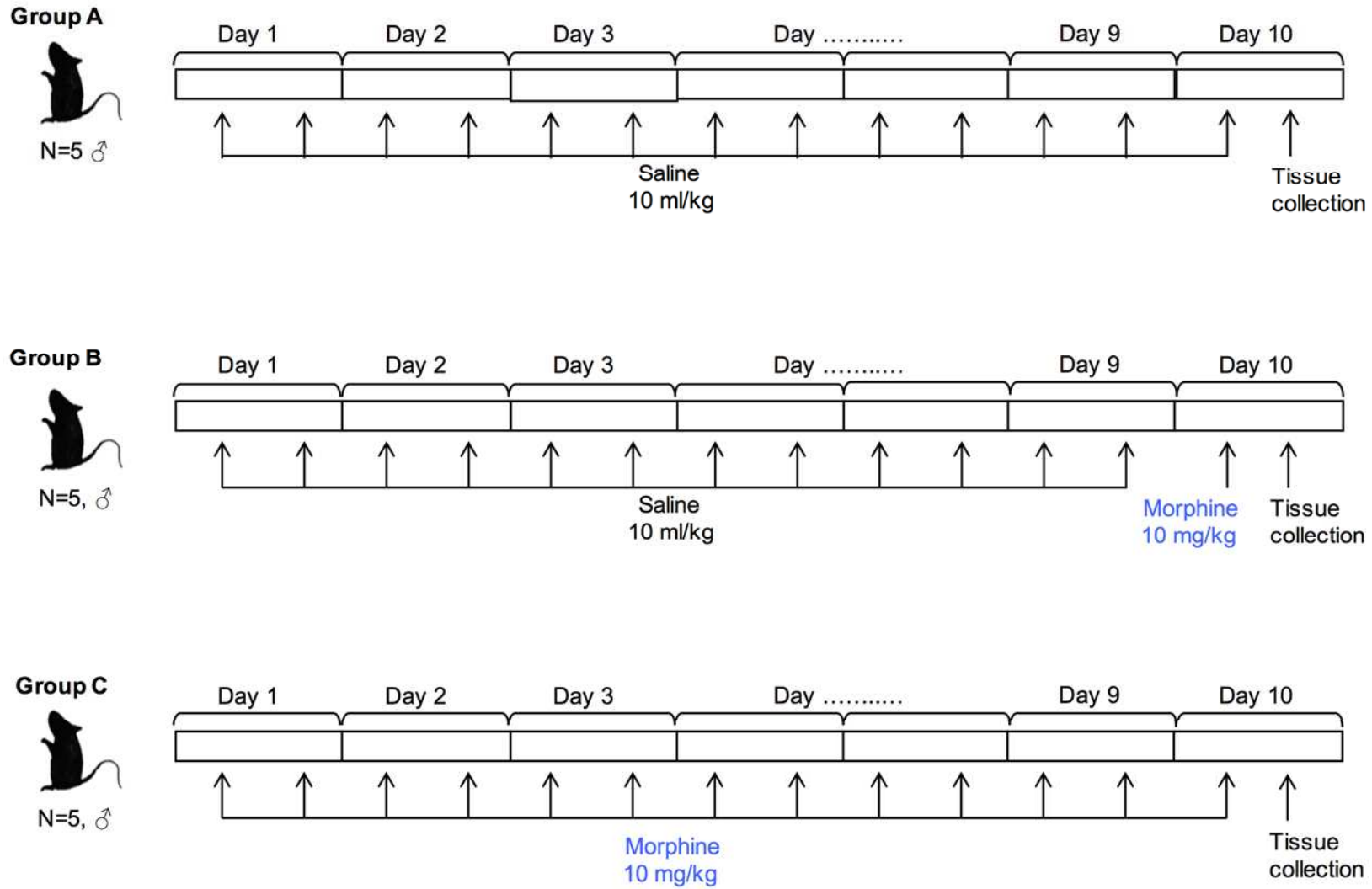


Figure 2: Variation in DNA methylation by region of the rat brain

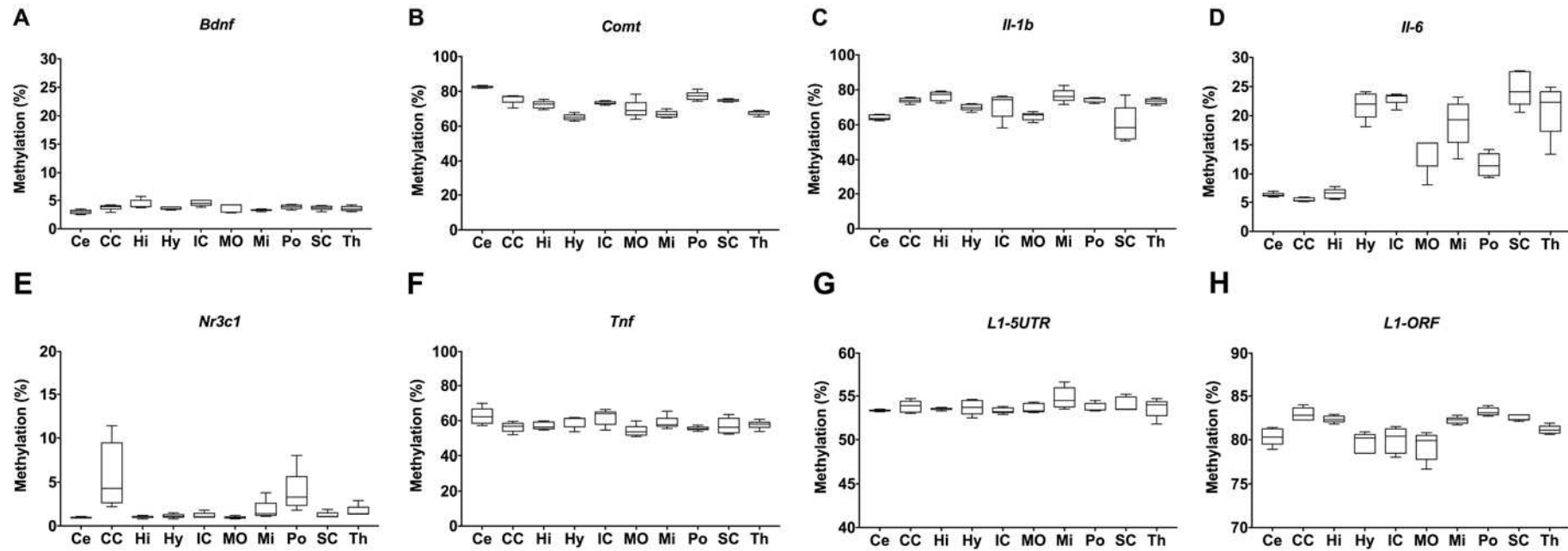


Figure 3: Differential gene-specific DNA methylation in response to morphine exposure

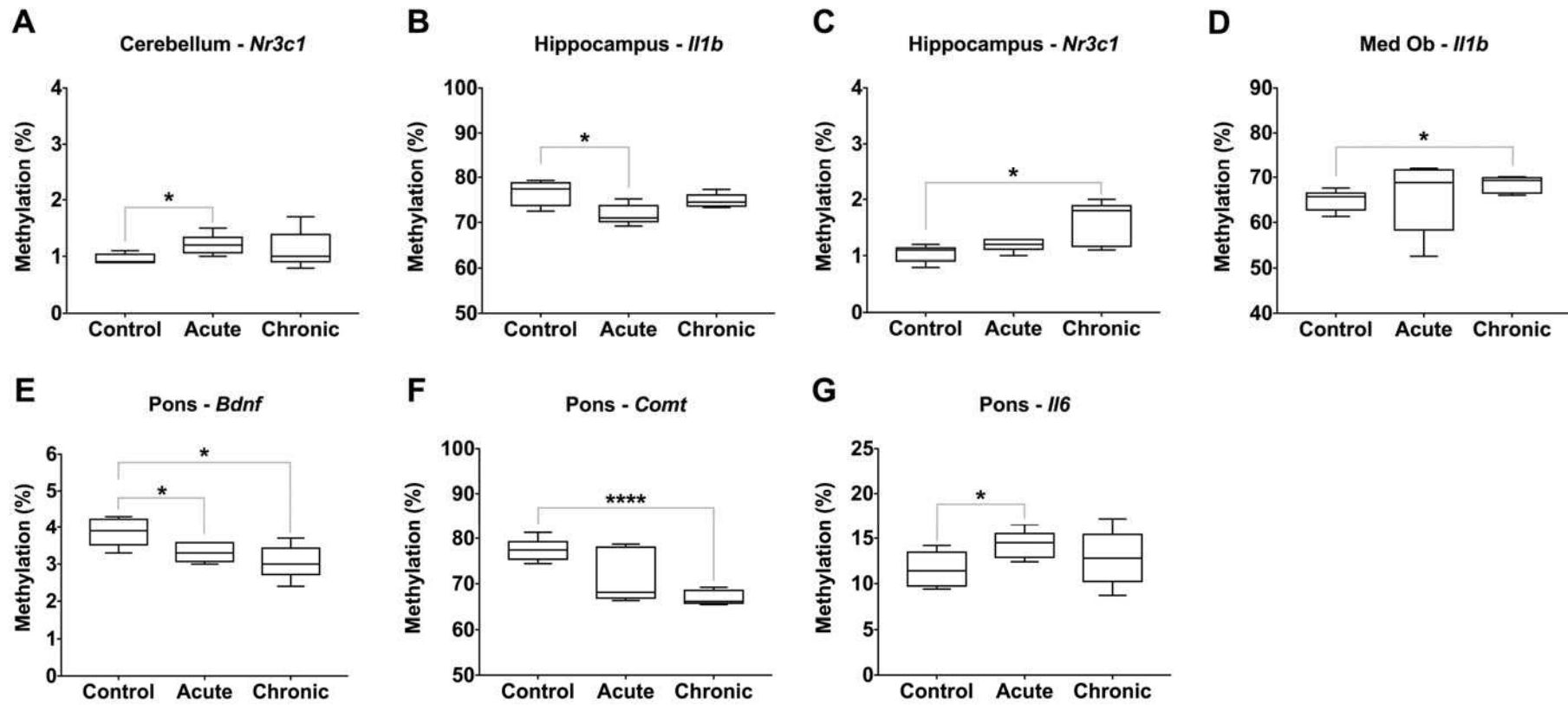


Figure 4: Global DNA methylation levels following acute and chronic morphine exposure

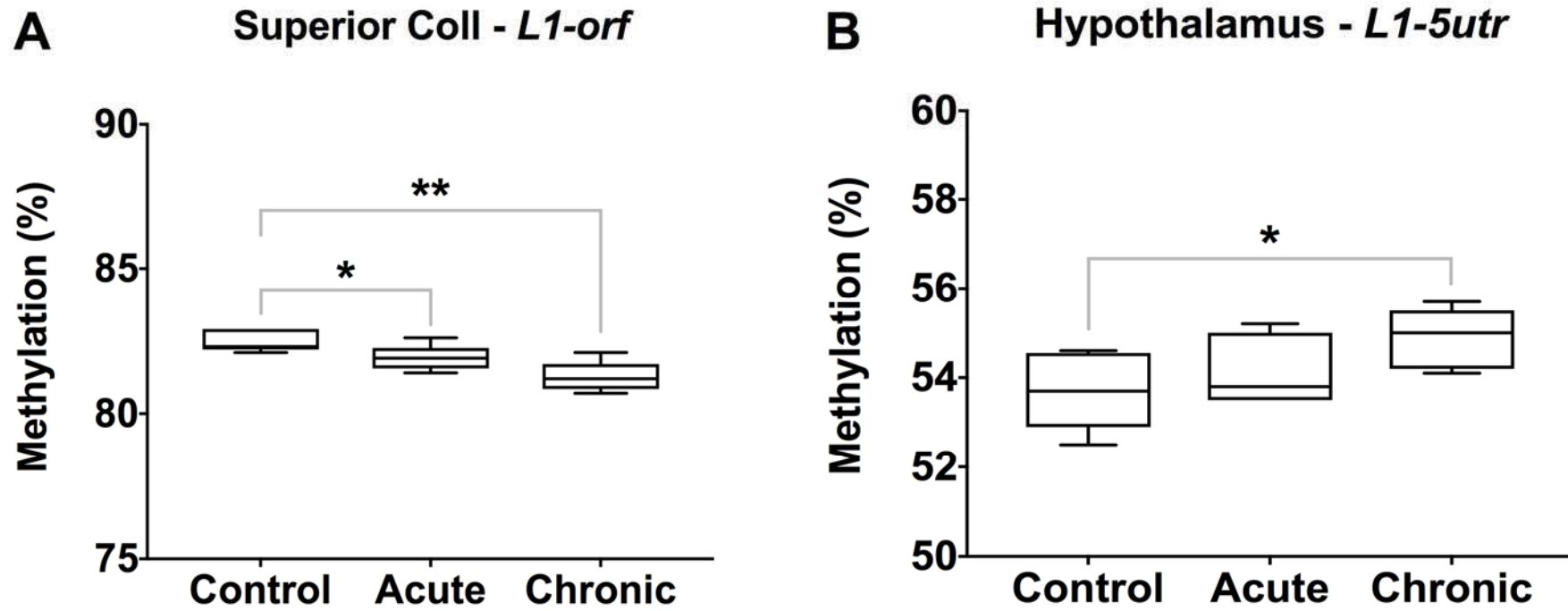


Figure 5: Global 5-hydroxymethylcytosine levels following acute and chronic morphine exposure

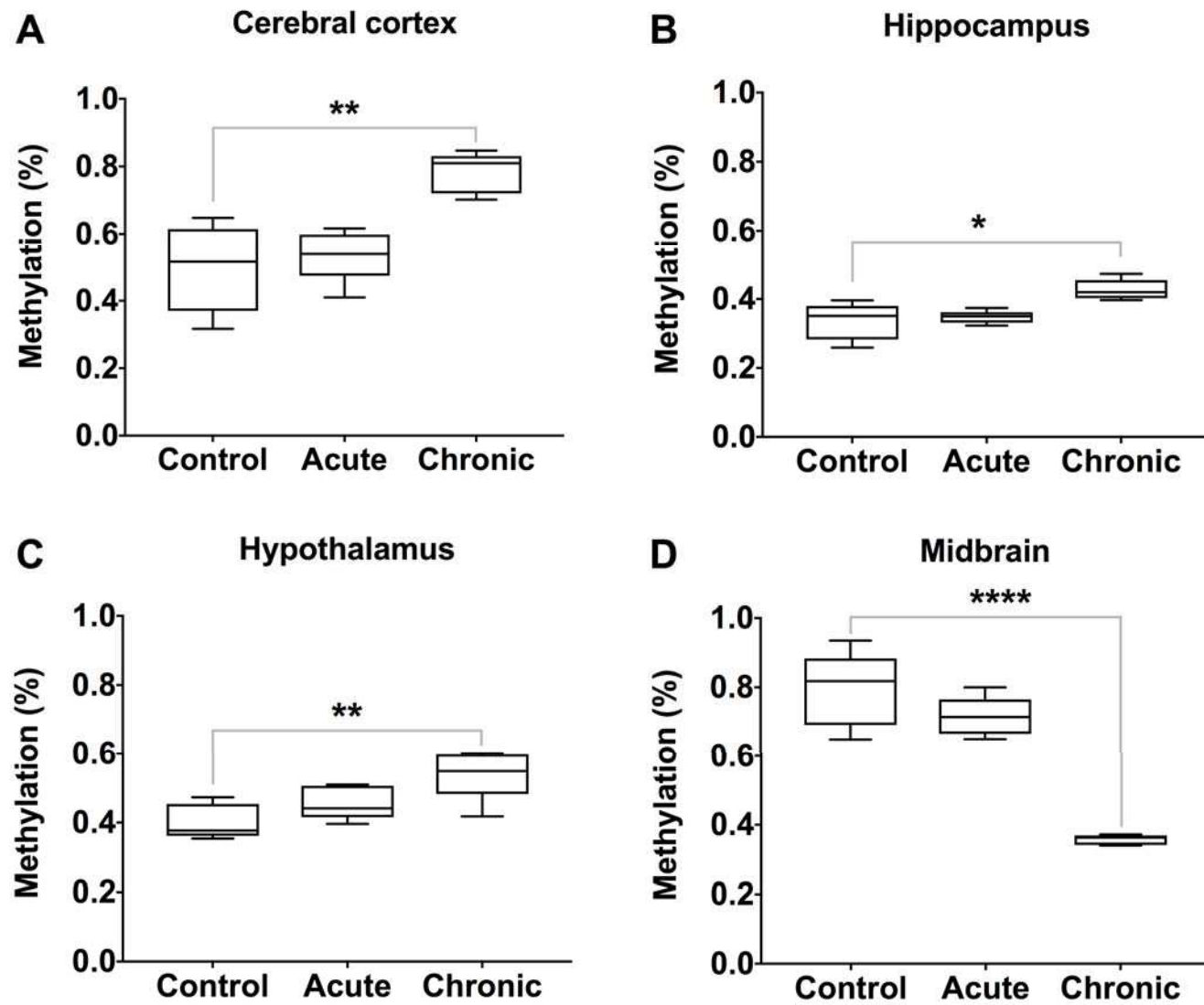




Table 1: PCR and pyrosequencing primer sequences

Gene name	Primer	Sequence
<i>Bdnf</i>	Forward primer (5' to 3')	TTTTTAGTTTTTGTTTAGATTAATGGAGT
	Reverse biotin primer (5' to 3')	CAACAAAAAATTAATTAATAATAAAA
	Sequencing primer (5' to 3')	GTTTTTATGAAGG
	Sequence analyzed	C/TGTGC/TGAGTATTATTTTC/TGTTATG
<i>Comt</i>	Forward primer (5' to 3')	GTTTGTGTAGTTTGGAGTTAGGT
	Reverse biotin primer (5' to 3')	CTCATCCTCCCCATTACCT
	Sequencing primer (5' to 3')	TATGGTTTAGTGTGG
	Sequence analyzed	C/TGGTTGTGGGGTGTAGGGGGC/TGGGGGGGT
<i>Nr3c1</i>	Forward primer (5' to 3')	GGTTGGTAAAAGTTTGTTAAGTT
	Reverse biotin primer (5' to 3')	CTAAAACTCTCCCTCCCC
	Sequencing primer (5' to 3')	GTTTATTTTAGTATT
	Sequence analyzed	C/TGC/TGC/TGTTTC/TGTTTC/TGTTTC/TGC
<i>Il1b</i>	Forward primer (5' to 3')	TGTATAAGGAAGTTTGATTGGAGAG
	Reverse biotin primer (5' to 3')	ATAAAATCAATTAACCCAAAAA
	Sequencing primer (5' to 3')	ATGTTTTGAATTATT
	Sequence analyzed	C/TGGGGTTTGCTGTCCACTAGTTTTCTCTCCCTC/TGTTTTA
<i>Il6-1</i>	Forward primer (5' to 3')	TTGTGATTTTTTGGATGTTAAATGA
	Reverse biotin primer (5' to 3')	CAAACATCCCCAATCTCATATTTAT
	Sequencing primer (5' to 3')	TTGTGATTTTTTGGATGTTAAATGA
	Sequence analyzed	C/TGTTATAT
<i>Il6-2</i>	Forward primer (5' to 3')	TTGTGATTTTTTGGATGTTAAATGA
	Reverse biotin primer (5' to 3')	CAAACATCCCCAATCTCATATTTAT
	Sequencing primer (5' to 3')	TTAAAAGTAGAGAGT
	Sequence analyzed	C/TGATTTTAA
<i>Tnf</i>	Forward primer (5' to 3')	GGATTGTTATAGAATTTTGGTGAGG
	Reverse biotin primer (5' to 3')	ACTTCCTTAATAAAAAAACCATAATCTC
	Sequencing primer (5' to 3')	TTAAATTTTTGTTTT

	Sequence analyzed	C/TGTATTGGAGAAGAAATTGA
<i>MT-co1</i>	Forward primer (5' to 3')	AGTTGGAGTTGGAATAGGATGAATA
	Reverse biotin primer (5' to 3')	TAACCTCTAAAATAAAAAACACCCC
	Sequencing primer (5' to 3')	ATATTTTTTTTTAGT
	Sequence analyzed	C/TGGAAATTTAGTTTATGTTGGGG/ATATTC/TGTAGATTTAA
<i>MT-co2</i>	Forward primer (5' to 3')	TAATGATTTAAAATTAGGTGAATTT
	Reverse biotin primer (5' to 3')	TAACCCTAATAAAAAATAACTCATAAATA
	Sequencing primer (5' to 3')	ATAGAATTTTTAATT
	Sequence analyzed	C/TGTATATTAATTTTATTC/TGAAGAC/TGTTTTG
<i>MT-co3</i>	Forward primer (5' to 3')	GTTATTATATTTTTATTGTATAAAAAGGTT
	Reverse biotin primer (5' to 3')	AAATAATAAAATACTCAAAAAAATCC
	Sequencing primer (5' to 3')	GTATAAAAAGGTTTT
	Sequence analyzed	C/TGATAC/TGGAATAATTTTGTATTGTTTTTC/TGAAGT
L1 UTR	Forward primer (5' to 3')	GGTGTATAGGTTTTTTGGTTGTTG
	Reverse biotin primer (5' to 3')	AAATTCACCAAACAACCTTCTTACAA
	Sequencing primer (5' to 3')	TTTTTTGGTTGTTGT
	Sequence analyzed	C/TGTTGTAGAGAGTTC/TGTGGTAGTATTTTA
L1 ORF	Forward primer (5' to 3')	AGAAAGAATATTTAAAATAGTAAGGGAAAA
	Reverse biotin primer (5' to 3')	ATCAATCCAAAATCTTCTAACCTTC
	Sequencing primer (5' to 3')	TTATATTAGATTTTT
	Sequence analyzed	C/TGTTAGAAATTAT

Table 2: Nuclear DNA methylation in the rat brain following morphine induction

Region	Treatment	<i>Bdnf</i>	<i>Comt</i>	<i>Il1b</i>	<i>Il6-1</i>	<i>Nr3c1</i>	<i>Tnf</i>	<i>L1-5utr</i>	<i>L1-orf</i>	<i>5-hmc</i>
Cerebellum	Control	3.0 (0.2)	82.5 (0.3)	64.2 (0.7)	6.3 (0.2)	1.0 (0.0)	62.5 (2.2)	53.3 (0.1)	80.4 (0.5)	0.48 (0.07)
	Acute	3.0 (0.2)	83.6 (0.5)	66.2 (1.3)	5.9 (0.3)	1.2 (0.1)	63.2 (2.8)	53.8 (0.3)	82.9 (0.3)	0.51 (0.06)
	Chronic	3.5 (0.4)	82.4 (0.3)	64.8 (0.4)	6.0 (0.2)	1.1 (0.2)	65.3 (0.9)	53.5 (0.1)	82.4 (0.2)	0.63 (0.03)
Cerebral cortex	Control	3.8 (0.2)	75.8 (1.3)	74.1 (0.7)	5.5 (0.2)	5.7 (1.7)	56.4 (1.3)	53.7 (0.4)	79.7 (0.5)	0.50 (0.06)
	Acute	4.1 (0.4)	74.8 (0.9)	76.1 (1.0)	8.2 (1.8)	3.1 (1.4)	55.3 (1.4)	53.3 (0.2)	80.0 (0.7)	0.54 (0.03)
	Chronic	3.8 (0.4)	77.5 (0.5)	74.6 (2.2)	5.5 (0.5)	4.7 (0.9)	52.8 (1.2)	53.6 (0.2)	79.3 (0.7)	0.78 (0.03)
Hippocampus	Control	4.3 (0.4)	72.4 (1.0)	76.5 (1.3)	6.5 (0.4)	1.0 (0.1)	57.1 (1.0)	54.8 (0.6)	82.2 (0.2)	0.34 (0.02)
	Acute	3.5 (0.2)	70.0 (1.1)	71.8 (1.0)	5.7 (0.5)	1.2 (0.1)	57.5 (1.8)	53.7 (0.2)	83.2 (0.2)	0.35 (0.01)
	Chronic	3.4 (0.2)	71.9 (0.2)	74.8 (0.7)	6.4 (0.3)	1.6 (0.2)	58.9 (1.0)	54.0 (0.4)	82.5 (0.2)	0.43 (0.01)
Hypothalamus	Control	3.6 (0.1)	65.1 (0.8)	70.1 (0.9)	21.8 (1.1)	1.1 (0.1)	59.4 (1.5)	53.6 (0.5)	81.1 (0.2)	0.40 (0.02)
	Acute	3.8 (0.2)	65.2 (1.5)	68.9 (2.1)	16.4 (2.7)	1.3 (0.2)	55.9 (1.4)	54.0 (0.6)	81.5 (0.4)	0.46 (0.02)
	Chronic	3.7 (0.3)	65.0 (0.8)	68.0 (1.2)	20.4 (0.9)	1.1 (0.1)	58.9 (1.1)	54.3 (0.7)	83.4 (0.3)	0.54 (0.03)
Inferior colliculus	Control	4.6 (0.3)	73.4 (0.5)	71.1 (3.4)	23.0 (0.5)	1.2 (0.2)	62.0 (2.1)	54.5 (0.5)	82.6 (0.1)	0.57 (0.1)
	Acute	3.9 (0.4)	72.6 (0.9)	72.4 (3.8)	22.5 (1.2)	1.3 (0.1)	62.5 (1.7)	54.2 (0.4)	80.0 (0.5)	0.62 (0.07)
	Chronic	4.0 (0.4)	72.7 (0.5)	76.1 (0.7)	24.0 (0.5)	1.3 (0.1)	63.7 (1.3)	53.6 (0.6)	77.5 (2.2)	0.79 (0.04)
Medulla oblongata	Control	3.7 (0.4)	69.8 (2.3)	64.8 (1.1)	13.7 (1.4)	1.0 (0.1)	54.0 (1.5)	53.4 (0.3)	80.1 (0.5)	0.51 (0.05)
	Acute	3.4 (0.3)	69.1 (1.1)	65.7 (3.6)	13.2 (0.9)	1.1 (0.1)	53.0 (1.5)	54.5 (0.6)	81.8 (0.2)	0.56 (0.07)
	Chronic	3.8 (0.2)	67.3 (0.8)	68.4 (0.9)	14.6 (0.3)	0.9 (0.1)	56.5 (0.4)	53.9 (0.4)	82.7 (0.1)	0.66 (0.08)
Midbrain	Control	3.3 (0.1)	66.8 (0.9)	76.6 (1.7)	18.8 (1.8)	1.8 (0.5)	58.8 (1.7)	55.4 (0.6)	81.9 (0.2)	0.79 (0.05)
	Acute	3.7 (0.4)	68.1 (1.1)	72.0 (1.8)	19.3 (0.9)	1.9 (0.4)	57.2 (1.8)	53.3 (0.4)	81.8 (0.4)	0.72 (0.03)
	Chronic	3.2 (0.3)	68.2 (1.0)	73.6 (2.0)	21.3 (1.2)	1.6 (0.3)	60.6 (2.1)	54.6 (0.7)	80.7 (0.1)	0.36 (0.01)
Pons	Control	3.9 (0.2)	77.3 (1.1)	74.3 (0.7)	11.6 (0.9)	3.9 (1.1)	55.5 (0.6)	53.6 (0.4)	83.1 (0.3)	0.69 (0.09)
	Acute	3.3 (0.1)	71.6 (2.7)	75.0 (1.4)	14.3 (0.7)	3.6 (0.8)	54.3 (1.1)	53.8 (0.3)	82.3 (0.1)	0.58 (0.05)
	Chronic	3.1 (0.2)	66.9 (0.8)	72.5 (1.6)	12.8 (1.4)	3.7 (1.1)	56.6 (2.5)	54.9 (0.3)	78.5 (0.2)	0.57 (0.06)
Superior colliculus	Control	3.7 (0.2)	74.8 (0.4)	60.3 (4.7)	24.6 (1.4)	1.2 (0.2)	57.0 (2.1)	53.9 (0.4)	79.7 (0.3)	0.54 (0.04)
	Acute	3.3 (0.3)	73.4 (1.8)	59.4 (3.6)	21.4 (1.8)	1.8 (0.5)	57.9 (1.6)	53.9 (0.3)	79.0 (0.2)	0.53 (0.03)
	Chronic	3.2 (0.3)	74.1 (0.7)	58.4 (2.2)	25.1 (0.6)	1.2 (0.1)	60.8 (1.1)	53.6 (0.0)	81.9 (0.2)	0.51 (0.03)

	Control	3.6 (0.2)	67.7 (0.6)	73.5 (0.8)	21.0 (2.0)	1.7 (0.3)	57.7 (1.1)	54.4 (0.3)	82.8 (0.1)	0.37 (0.01)
Thalamus	Acute	3.4 (0.1)	67.1 (1.0)	72.9 (2.4)	20.1 (2.2)	1.8 (0.4)	57.9 (2.4)	54.7 (0.1)	81.3 (0.2)	0.36 (<0.01)
	Chronic	3.6 (0.3)	67.9 (0.8)	72.2 (1.0)	22.2 (0.8)	2.0 (0.4)	60.1 (1.3)	53.5 (0.2)	81.7 (0.4)	0.37 (<0.01)

Table 3: DNA methylation of mitochondrial-encoded genes in the rat brain following morphine induction

Region	Treatment	<i>Mtco1</i>	<i>Mtco2</i>	<i>Mtco3</i>
Cerebellum	Control	2.1 (0.1)	0.9 (0.1)	1.1 (0.1)
	Acute	2.0 (0.2)	1.0 (0.1)	1.2 (0.1)
	Chronic	2.1 (0.1)	0.9 (0.1)	1.1 (0.1)
Cerebral cortex	Control	2.7 (0.6)	0.8 (0.1)	1.1 (0.1)
	Acute	1.8 (0.3)	0.9 (0.2)	1.1 (<0.01)
	Chronic	1.9 (0.4)	0.8 (0.1)	1 (0.1)
Hippocampus	Control	1.9 (0.1)	1.0 (0.1)	1.2 (0.1)
	Acute	1.9 (0.2)	0.8 (0.1)	1.3 (0.1)
	Chronic	1.8 (0.1)	0.8 (0.1)	1.1 (0.1)
Hypothalamus	Control	2.0 (0.1)	0.9 (0.1)	1.1 (0.1)
	Acute	2.1 (0.2)	0.9 (0.1)	1.1 (0.1)
	Chronic	1.9 (0.2)	0.7 (0.1)	1.0 (0.1)
Inferior colliculus	Control	2.2 (0.1)	1.0 (0.1)	1.3 (0.2)
	Acute	2.2 (0.1)	0.9 (0.1)	1.2 (0.1)
	Chronic	2.0 (0.1)	0.8 (0.1)	1.1 (0.1)
Medulla oblongata	Control	2.4 (0.3)	1.0 (0.1)	1.1 (0.1)
	Acute	2.0 (0.2)	0.8 (0.1)	1.1 (0.1)
	Chronic	2.0 (0.2)	0.8 (0.1)	1.1 (0.1)
Midbrain	Control	1.9 (0.2)	0.9 (0.1)	1.2 (0.2)
	Acute	2.2 (0.4)	0.9 (0.1)	1.1 (0.1)
	Chronic	2.0 (0.2)	1.0 (0.1)	1.1 (0.1)
Pons	Control	1.9 (0.3)	0.8 (0.1)	1.0 (0.2)
	Acute	2.3 (0.4)	0.8 (0.1)	1.6 (0.5)
	Chronic	2.6 (1.0)	0.8 (0.1)	1.1 (0.1)
Superior colliculus	Control	2.1 (0.1)	1.0 (0.1)	1.3 (0.1)
	Acute	2.4 (0.2)	0.8 (0.1)	1.3 (<0.01)
	Chronic	2.1 (0.2)	0.9 (0.1)	1.3 (0.1)
Thalamus	Control	2.1 (0.1)	1.0 (0.1)	1.3 (0.2)
	Acute	1.9 (0.1)	0.9 (0.1)	1.1 (<0.01)
	Chronic	1.8 (0.2)	1.0 (0.3)	0.9 (0.1)