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Opiate-induced Changes in Brain Adenosine Levels and Narcotic Drug Responses

Manhong Wu¹, Peyman Sahbaie², Ming Zheng¹, Robert Lobato¹, Detlev Boison³, J. David Clark^{1,2}, and Gary Peltz¹

¹Department of Anesthesia, Stanford University School of Medicine, Stanford CA 94305

²Veterans Affairs Palo Alto Healthcare System, Palo Alto, CA, USA 94305

³Robert S. Dow Neurobiology Laboratories, Legacy Research, Portland OR 97232

Abstract

We have very little information about the metabolomic changes that mediate neurobehavioral responses, including addiction. It was possible that opioid-induced metabolomic changes in brain could mediate some of the pharmacodynamic effects of opioids. To investigate this, opiate-induced brain metabolomic responses were profiled using a semi-targeted method in C57BL/6 and 129Sv1 mice, which exhibit extreme differences in their tendency to become opiate dependent. Escalating morphine doses (10–40 mg/kg) administered over a 4-day period selectively induced a two-fold decrease ($p < 0.00005$) in adenosine abundance in the brainstem of C57BL/6 mice, which exhibited symptoms of narcotic drug dependence; but did not decrease adenosine abundance in 129Sv1 mice, which do not exhibit symptoms of dependence. Based on this finding, the effect of adenosine on dependence was investigated in genetically engineered mice with alterations in adenosine tone in the brain and in pharmacologic experiments. Morphine withdrawal behaviors were significantly diminished ($P < 0.0004$) in genetically engineered mice with reduced adenosine tone in the brainstem, and by treatment with an adenosine receptor₁ (A_1) agonist (2-chloro-N6-cyclopentyladenosine, 0.5 mg/kg) or an A_{2a} receptor (A_{2a}) antagonist (SCH 58261 1 mg/kg). These results indicate that adenosine homeostasis plays a crucial role in narcotic drug responses. Opiate-induced changes in brain adenosine levels may explain many important neurobehavioral features associated with opiate addiction and withdrawal.

Keywords

opiates; metabolomic analysis

Introduction

Efforts to improve the care of patients with chronic pain conditions have led to a marked increase in the use of opioid medications. Unfortunately, prescription opioid analgesics are more commonly misused than all other illicit drugs combined, including marijuana [reviewed in (Dodrill et al., 2011)]. New approaches and a re-evaluation of our current understanding of the mechanisms involved in narcotic drug addiction are urgently needed.

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Address correspondence to: gpeltz@stanford.edu. 300 Pasteur Drive L232 Stanford CA, 94305.

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To develop new strategies for addressing this public health problem, we have been analyzing a murine model of opiate dependence. Mice can be made physically dependent upon morphine, and inbred strains dramatically differ in the extent to which they manifest various features of narcotic drug addiction, which resemble those observed in humans (Liang et al., 2006a, Liang et al., 2006b, Chu et al., 2009). By analyzing these inter-strain differences, we identified four genes affecting opioid responses (Smith et al., 2008) (Liang et al., 2006a) (Liang et al., 2006b), including the *Htr3a/5-HT3* serotonin receptor (Chu et al., 2009). We also demonstrated that administration of a commonly used 5-HT3 antagonist (ondansetron) reduced narcotic drug withdrawal symptoms in mice and in normal human subjects (Chu et al., 2009) (Liang et al., 2011).

In addition to genetic data, metabolomic analysis can reveal a great deal about the physiological state of a tissue. We have very little information about metabolomic changes mediating neurobehavioral responses or diseases. It is likely that clinically important opiate responses could be mediated (at least in part) by metabolomic changes that are induced by opiates. However, the extreme differences in physicochemical properties make it impossible to accurately measure changes in all cellular metabolites with a single analytic method. Therefore, we coupled a recently developed Dansyl [5-(dimethylamino)-1-naphthalene sulfonamide] derivatization method (Guo and Li, 2009) with LC/MS analysis to analyze changes in a large number of metabolites in brainstem after opiate administration. Dansylation increases metabolite detection sensitivity by 10–1000 fold, and improves metabolite retention and separation on reversed phase columns. It enables changes in many metabolites that have primary or secondary amino or other groups, to be evaluated in an unbiased fashion. This semi-targeted method was used to characterize opiate-induced metabolomic changes in a brain region that is critical for opiate responses in two inbred mouse strains, which exhibit extreme differences in the extent of physical dependence developing after opiate administration. Metabolomic changes in the brainstem were analyzed, since this region has been shown to regulate narcotic drug dependence (Gulati and Bhargava, 1989, Costall et al., 1990, Tao et al., 1998).

Experimental Procedures

Animal studies

All experiments were performed according to protocols that were approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Palo Alto Healthcare System. Male C57BL/6J and 129/SvImJ mice strains (7–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, MA) and kept in our facility for a minimum of 1 week prior to initiation of the experiments. Mice with genetically engineered alterations in adenosine kinase (*Adk*) expression (which are referred to as *Adk-tg* and *fb-Adk-def* mice) were provided by Dr. Detlev Boison (Legacy Research Institute, Portland, OR 97232) and kept in our facility for 2 weeks prior to initiation of experiments. All mice were kept under standard conditions with a 12 hr light/dark cycle and an ambient temperature of 22±1°C. Animals were allowed food and water *ad libitum*. All experiments were performed using 7–8 mice per group, as determined by power analyses using pilot data and from previous experiments.

Morphine (Sigma Chemicals, St. Louis, MO) was administered subcutaneously to different groups of mice twice per day on an ascending schedule: Day 1, 10 mg/kg; Day 2, 20mg/kg; Day 3, 30mg/kg and Day 4, 40 mg/kg. Vehicle (Saline) injections followed the same twice-daily schedule. Nociceptive testing procedures began approximately 18 hours after the final dose of morphine or saline. Naloxone-precipitated withdrawal was initiated by subcutaneous injection of Naloxone (10 mg/kg) one hour after the last dose of morphine on day 4 (Chu et al., 2009). To study the effect of selective adenosine receptor agents on

withdrawal in morphine-treated mice, the A₁R agonist 2-chloro-N⁶-cyclopentyladenosine 0.5 mg/kg (Tocris Bioscience, Ellisville, MI) or the A_{2A}R selective antagonist SCH 58261 (Tocris Bioscience, Ellisville, MI) 1 mg/kg or vehicle (saline) was injected intra-peritoneally 15 minutes prior to naloxone administration.

Brain tissue preparation

Since rapid tissue isolation is critical for metabolomic analysis, the total time for brainstem and frontal cortex isolation after sacrifice was 90 seconds using the following procedure: after decapitation by guillotine, the skull was exposed and opened along the sagittal and lambdoid sutures; the brain was then transferred to a cold plate (4 °C); the olfactory bulbs and cerebellar hemispheres were removed, and the areas of interest were separated before snap freezing on dry ice. The areas of interest included the hindbrain (medulla and pons) and midbrain (tectum and cerebral peduncle minus the cerebellum).

Dansylation

Dansylation was performed using a modification of the procedures developed by Guo and Li (Guo and Li, 2009). Fifty ml of the polar metabolite extract in 0.1M sodium tetraborate buffer was combined with 50 ml of 20 mM dansyl chloride and vortexed. The mixture was incubated at room temperature for 30 minutes before addition of 50 ml of 0.5% formic acid to stop the reaction. The supernatant of the reaction mixture was then placed into an autosampler vial.

LC/MS analysis

All samples were analyzed on an Agilent (Santa Clara, CA) accurate mass Q-TOF 6520 coupled with an Agilent UHPLC infinity 1290 system. The chromatography runs were performed using a Phenomenex (Torrance, CA) Kinetex reversed phase C18 column (dimension 2.1×100 mm, 2.6 mm particles, 100Å pore size). Solvent A was HPLC water with 0.1% formic acid and Solvent B was LC/MS grade acetonitrile with 0.1% formic acid. A 30 minute gradient at 0.5 ml/min was as follows: t=0.5 minute, 5% B; t=20.5 minutes, 60% B; t=25 minutes, 95% B; t=30 minute, 95% B. The column was balanced at 5% B for 5 minutes. All data were acquired by positive ESI (electrospray ionization) with MassHunter acquisition software. Molecular feature extraction on all data was performed using MassHunter qual software. The metabolite abundance, which is a measure of the metabolite concentration in an extract, was determined using software that integrates the peak area for the indicated metabolite on the extracted ion chromatogram for each sample. To confirm the identity of adenosine, a targeted MS/MS spectrum was acquired on the QTOF 6520 using the above HPLC gradient and specified retention time with window of 0.6 minutes. The collision energy was set at 28V, isolation width 4 m/z, MS acquisition rate at 5 spectra/second and MS/MS acquisition rate at 3 spectra/second. To compare metabolite abundances between samples in different groups, the signal intensities for each metabolite was log-transformed, and a two-sample two-tail t test was applied to the log-transformed data.

Assessment of Mechanical Sensitivity

Mechanical sensitivity was assayed using nylon von Frey filaments according to the “up-down” algorithm as described previously to detect allodynia in mice (Liang et al., 2006a). In these experiments, mice were placed on wire mesh platforms in clear cylindrical plastic enclosures 10cm in diameter and 40cm in height. After acclimation, fibers of sequentially increasing stiffness were applied approximately 1mm lateral to the central wound edge, pressed upward to cause a slight bend in the fiber and left in place for 5 sec. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained the next stiffest fiber in the series was applied to the same paw; if a response was obtained a less

stiff fiber was applied. Testing proceeded in this manner until 4 fibers had been applied after the first one causing a withdrawal response. The mechanical withdrawal threshold was estimated using a data fitting algorithm that permitted the use of parametric statistics for analysis (Poree et al., 1998).

Immunohistochemistry

Adult male *Adk-tg*, *fb-Adk-def*, and wild type (C57BL/6J) mice (n = 3, each) were transcardially perfused with 0.15 M phosphate buffered saline (PBS), followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed in the same fixative at 4°C for 1 day before being cut into 40 µm sagittal sections using a vibratome. For the immunohistochemical detection of ADK, we followed our published procedures (Studer et al., 2006). Digital images of ADK immunohistochemistry on 3,3'-diaminobenzidine (DAB) stained slices were acquired using a Zeiss AxioPlan inverted microscope equipped with an AxioCam 1Cc1 camera (Carl Zeiss MicroImaging, Inc., Thornwood, NY).

Results

C57BL/6J mice become morphine-dependent after 4 days of administration of increasing doses of morphine; the morphine-dependent mice develop signs of withdrawal within 18 hours of their last morphine dose, and naloxone administration rapidly induces substantial withdrawal symptoms (Chu et al., 2009). To characterize opiate-induced metabolomic changes, brainstem tissue was prepared from C57BL/6J mice placed into 4 treatment groups (n=8 per group) (Fig. 1A): 1) Dependence: tissue was harvested 1 hour after the last morphine dose on day 4; 2) Naloxone-induced withdrawal: the opiate receptor antagonist naloxone (10 mg/kg s.c.) was administered to morphine-dependent mice 1 hour after their last morphine dose on day 4, and then tissue was harvested 10 minutes later; 3) Natural withdrawal: tissue was harvested 18 hours after their last morphine dose on day 4, which is when withdrawal symptoms are maximal; 4) Control: saline injections were administered over the 4 day period. Within the 32-brainstem tissues analyzed, 1300 metabolite peaks were identified. When the dependence, naloxone-induced withdrawal, or natural withdrawal samples were compared to the control samples, adenosine was the only metabolite whose abundance was significantly altered in all 3 conditions after morphine administration (Fig. 1B). The identity of adenosine as the differentially reduced metabolite in all 3 conditions was confirmed using a chemical standard and by LC/MS/MS analysis (Fig. 2). Brainstem adenosine abundance was significantly lower (2-fold, $p < 0.00005$) in all 3 conditions associated with morphine administration relative to that of control mice. Thus, opiate exposure induces a 50% decrease in brainstem adenosine abundance. This represents a very significant metabolomic response, since adenosine is an important neuromodulator whose intracellular and extra-cellular concentration is very tightly controlled by transporters and by enzymes regulating adenosine metabolism [reviewed in (Boison, 2006)]. Adenosine abundance was significantly decreased during the period of morphine dependence, and this decrease is maintained during the 18-hour period of narcotic drug abstinence when these mice manifest maximal signs of withdrawal.

Since 129Sv1 mice do not develop physical dependence after 4 days of morphine administration (Chu et al., 2009), metabolomic changes in brainstem tissues in these mice were also examined during the dependence and naloxone-induced withdrawal states. In contrast to C57BL/6J mice, no metabolite exhibited a significant change in 129Sv1 brainstem tissue. In particular, adenosine abundance was un-altered (Fig. 1C) in 129Sv1 mice; while adenosine abundance was decreased in the brainstem of C57BL/6J mice during the dependent state, which were subject to the same morphine administration protocol and at the same time as the 129Sv1 mice. We previously demonstrated that co-administration of ondansetron (1 mg/kg IP with morphine prevented morphine tolerance and dependence in

C57BL/6J mice (Chu et al., 2009). However, ondansetron co-administration with morphine did not alter the decrease in brainstem adenosine abundance that is induced by morphine and naloxone treatment (Fig. 1C). Thus, there is a specific decrease in brainstem adenosine levels in C57BL/6J mice during the morphine dependence and withdrawal states; this metabolomic change specifically occurs in the opiate-dependent strain. While ondansetron reduces behavioral aspects of dependence, it acts distal to the site where opiates alter adenosine abundance.

Adenosine deaminase and adenosine kinase (ADK) can reduce adenosine by forming inosine and AMP, respectively. However, ADK is the key regulator of adenosine metabolism in the adult brain (Boison, 2006). Consistent with ADK playing an important role in the opiate response, inosine abundance in brainstem tissues during the periods of morphine dependence or during the two different withdrawal states was not altered (Fig. 3). To study the role of adenosine and ADK in opiate responses, we characterized opiate responses in two mouse strains with altered levels of ADK expression (Li et al., 2008). In both of these lines, the endogenous ADK, which is subject to dynamic expression changes (Masino et al., 2011) (Studer et al., 2006), has been replaced by a constitutively over-expressed transgene (Li et al., 2007) (Li et al., 2008). Both lines were maintained on the C57BL/6J background that exhibits a high degree of opiate dependence. *Adk-tg* mice have globally increased ADK expression in brain, including brainstem; while *fb-Adk-def* have an identical level of ADK over-expression of ADK throughout the entire basal and midbrain regions, but have reduced ADK expression within the entire dorsal telencephalon (Li et al., 2008) (Fig. 4A). We first measured the jumping behavior precipitated by naloxone administration to opiate dependent control, *Adk-tg*, and *fb-Adk-def* mice. There was a very significant reduction ($P < 0.0004$) in the withdrawal response exhibited by *Adk-tg* and *fb-Adk-def* mice relative to control C57BL/6J mice (Fig. 4B). The similarly reduced withdrawal response in transgenic mice with increased (*Adk-tg*) or decreased (*fb-Adk-def*) forebrain ADK expression ($p > 0.05$) indicates that other brain regions affect the withdrawal response.

Then, adenosine abundance in the brainstem of control C57BL/6J and *fb-Adk-def* mice was measured in the basal state and in morphine dependent mice after naloxone-induced withdrawal. As before, adenosine abundance was decreased in C57BL/6J mice during naloxone-precipitated withdrawal ($p = 0.028$) relative to basal. Although adenosine abundance in brainstem was reduced in *fb-Adk-def* mice in the basal state relative to control C57BL/6J mice, it was not further decreased in the withdrawal state, which is consistent with the elimination of the *endogenous Adk-gene* (Fig. 4C). This indicates that ADK activity affects the development of narcotic drug dependence and the level of adenosine in the brainstem. It is also noteworthy that the basal adenosine levels in both 129Sv1 and *fb-Adk-def* mice were lower than those in C57BL/6J mice, which exhibited a greater level of narcotic dependence after four days of opiate administration (Figs. 1C and 4C). Furthermore, unlike C57BL/6 mice, the brainstem adenosine levels in 129Sv1 and *fb-Adk-def* mice were not changed after morphine administration.

It was surprising that the opiate-induced change in the brainstem adenosine level was not altered by ondansetron, especially since ondansetron alleviated opiate dependence and withdrawal symptoms. However, it was possible that ondansetron could act downstream of the opiate-induced change in adenosine, possibly through an effect on adenosine receptors. To investigate this possibility, we examined the effect that drugs acting on adenosine receptors had on opiate-induced behaviors. Consistent with the results observed by others (Zarrindast et al., 1999), administration of an adenosine receptor₁ (**A₁**) agonist significantly attenuated behavioral evidence of naloxone-induced opioid withdrawal in our protocol. Somewhat surprisingly, administration of an adenosine A_{2a} receptor (**A_{2a}**) antagonist also

decreased opioid withdrawal symptoms (Fig. 4D). Moreover, both agents significantly ameliorated morphine induced hyperalgesia, another opioid withdrawal complication (Fig. 4E). These results demonstrate that adenosine receptors have a strong effect on reducing withdrawal behaviors.

Discussion

This study demonstrates that semi-targeted metabolomic profiling data can provide important insight into neurobehavioral responses. It also provides the first demonstration that systemic opiate administration decreases adenosine abundance in brainstem. The decrease in narcotic drug withdrawal behavior in mice with genetically engineered changes in brainstem adenosine levels or after administration of pharmacologic agents acting on adenosine receptors demonstrates the importance of this opiate-induced metabolomic change. These results indicate that brainstem adenosine contributes (at least in part) to the neurobehavioral features of addiction and withdrawal. Adenosine is an important inhibitory neuromodulator; it inhibits glutamate release (Brambilla et al., 2005) and the post-synaptic action of excitatory neurotransmitters via activation of A₁R (Dunwiddie and Masino, 2001), which are densely expressed in the brainstem (Reppert et al., 1991) (Dixon et al., 1996). Adenosine (Salem and Hope, 1999) (Kaplan and Coyle, 1998) (Ahlijanian and Takemori, 1985), adenosine receptors (Salem and Hope, 1997) (Kaplan and Sears, 1996) (Kaplan et al., 1994) and A_{2a} function (Latini and Pedata, 2001, Brown and Short, 2008) (Yao et al., 2006) (Castane et al., 2008, Brown et al., 2009) have been previously linked with opiate responses and addiction. Although the relationship is more complex and variable, partly because A_{2a} expression is primarily in the striatum (Dixon et al., 1996), A_{2a} function has also been linked with opiate responses and addiction (Yao et al., 2006) (Brown et al., 2009) (Castane et al., 2008) (Stella et al., 2003). The opiate-induced decrease in adenosine occurs within the brainstem, a region that has been previously associated with addiction (Gulati and Bhargava, 1989) (Tao et al., 1998). The withdrawal response from diazepam, ethanol, nicotine or cocaine in mice was previously shown to be antagonized by ondansetron injection into the amygdala and dorsal raphe nucleus, while injections into the nucleus accumbens and striatum were ineffective (Costall et al., 1990).

The extra-cellular adenosine concentration is highly dynamic, and can increase in response to hypoxia or metabolic stress. The extra-cellular adenosine concentration is determined by the rate of inward adenosine flux, which is mediated by equilibrative transporters whose activity depends upon the intracellular adenosine levels. The intracellular adenosine concentration depends largely on metabolic clearance through ADK, which converts adenosine to AMP. Thus, the rate of intracellular adenosine metabolism and the intracellular adenosine concentration is controlled by ADK activity. By this mechanism, intracellular adenosine is the primary determinant of extra-cellular adenosine concentration (Greene, 2011).

Although we do not fully understand the mechanism (or even the required magnitude) through which a change in brain adenosine tone mediates opiate-induced behaviors, three key observations emerge from the metabolomic data presented here. (i) Morphine induces a decrease in brainstem adenosine tone in C57BL/6J mice, which become morphine dependent. (ii) The basal brainstem adenosine levels in 129Sv1 and *fb-Adk-def* mice, which do not become morphine dependent, were lower than those in C57BL/6 mice. (iii) Brainstem adenosine levels in 129Sv1 and *fb-Adk-def* mice did not change after opiate administration. These observations can be unified by the concept that drug-induced changes in adenosine tone affect dependence. In C57BL/6J mice, which are dependence prone, morphine strongly down-regulates brain adenosine tone. This could facilitate the development of dependence via reduced A₁ receptor signaling and enhanced neuronal excitability (Dunwiddie and

Masino, 2001) (Greene, 2011). In contrast, little dependence develops in ADK transgenic or 129/Sv mice that have a low basal adenosine tone, which is not altered after morphine administration. Since these mice do not experience a morphine-induced change in brain adenosine tone, this could make them less likely to develop dependence. The decrease in dependence observed after treatment with an A₁R agonist is also consistent with this concept; agonist-induced adenosine signaling could also decrease neuronal excitability. The effect that A_{2a} receptor antagonists have on decreasing morphine withdrawal reveals that there may be more complexity in the effect of adenosine on morphine responses. Conflicting results have been obtained using other A_{2a} antagonists, and some were reported to augment opioid dependence (Kaplan and Sears, 1996), but they lacked specificity for adenosine receptors. Morphine withdrawal in rats was also attenuated after administration of the A_{2a} antagonist used here (Stella et al., 2003). Of note, Halimi et al. (Halimi et al., 2000) reported a very modest (20%) increase in adenosine levels in striatal tissue after morphine administration, which is where A_{2a} receptors are expressed; but this region was not analyzed here. It is possible that there is regional variation in the effects of adenosine signaling, which could be due to regional variation in A_{2a} expression.

Of note, adenosine has an important role in sleep regulation; increased adenosine levels promote sleep, while adenosine receptor antagonists have been shown to induce wakefulness (Porkka-Heiskanen et al., 1997, Bjorness and Greene, 2009, Palchykova et al., 2010). In rats, opioids have been shown to disrupt sleep, and to decrease adenosine levels in brain regions that regulate sleep (Nelson et al., 2009, Gauthier et al., 2011). Similarly, opioids disrupt human sleep (Kay et al., 1981), and major disruptions of sleep occur during human opiate withdrawal (Beswick et al., 2003) (Oyefeso et al., 1997). Adenosine also plays an important role in the regulation of nociception (Sawynok, 1998), and opioid withdrawal stimulates a very well characterized hyperalgesic response. Seizures are also an important part of narcotic drug withdrawal, and are especially prominent in neonates born to mothers that chronically use narcotic drugs (O'Grady et al., 2009). Increased adenosine clearance due to over-expression of transgenic ADK in mouse brain has also been shown to induce spontaneous seizure activity (Fedele et al., 2005) (Li et al., 2008), which is dependent upon A₁R function (Masino et al., 2011). Further studies are required to better define the affect of opiates on adenosine and other metabolites. However, these results raise the intriguing possibility that opiate-induced alterations in brain adenosine levels could contribute to the hyperalgesia, disrupted sleep and seizures that are characteristic of opiate withdrawal.

Our results demonstrate metabolomic analysis can provide insight into the mechanisms mediating opiate responses. Furthermore, our results also indicate that opiate-induced changes in brain adenosine tone may mediate clinically important opiate responses. Although further studies are required, an increased understanding of this mechanism could produce new strategies for addressing the public health concern created by narcotic drug addiction.

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Abbreviations

Adk	adenosine kinase
A₁	adenosine A ₁ receptor
A_{2a}	Adenosine A _{2a} receptor

LC/MS liquid chromatography with mass spectroscopy
LC/MS/MS tandem mass spectroscopy

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Morphine decreased brainstem adenosine in morphine-dependent mice

Withdrawal was diminished in genetically engineered mice with reduced adenosine tone

Opiate-induced changes in adenosine may explain addiction-associated behaviors.

Figure 1.

A) The 4-day protocol for developing morphine dependence, and for inducing natural or naloxone-induced withdrawal is shown. **B)** Adenosine abundance (+ SEM) in brainstem tissue obtained from 4 groups of C57BL/6J mice (n=8 per group). **C)** Adenosine abundance (+ SEM) in brainstem tissue obtained from the indicated groups (n=8 per group) of 8-week old male C57BL/6J or 129Sv1 mice is shown. Ondansetron (4 mg/kg IP) was also co-administered with each morphine dose to C57BL/6J mice. The p-value, which is calculated relative to control mice for each of the groups, is also shown. The data presented in each of panel was produced in independent experiments with different LC/MS analyses. Due to differences in metabolite extraction efficiency and in detection sensitivity, the measurements obtained in one experiment cannot be directly compared across different experiments, which are shown in different panels. However, the adenosine abundance in brainstem tissue obtained from 129Sv1 mice in the basal state was significantly below the level in basal C57BL/6J mice.

Figure 2.

Confirmation of the identity of adenosine in extracts using a chemical standard. **A)** The extracted ion chromatogram of adenosine in a brainstem extract (red) overlapped with that of an adenosine standard (black), which indicates that they have identical retention times. **B)** Confirmation of the identity of adenosine by LC/MS/MS analysis. The upper (red) and lower (black) panels show the collision induced dissociation (CID) spectrum of adenosine in a brainstem sample and of an adenosine standard, respectively. The collision energy was set at 28V.

Figure 3.

Inosine abundance ($\pm$ SEM) in C57BL/6J brainstem tissue obtained from the indicated 4 groups of mice ($n=8$ per group). The p-value and fold change, which is calculated relative to that in the control mice for each of the 3 groups analyzed, is also shown.

Figure 4A

Figure 4.

A) Representative immunohistochemical images of sagittal brain sections from wildtype (C57BL/6J), *fb-Adk-def* and *Adk-tg* mice. The sections were stained with diamino-

benzidine hydrochloride (DAB) for ADK immunoreactivity. **B)** C57BL/6J, *Adk-tg*, and *fb-adk-def* mice (which over-express ADK in basal and midbrain regions but have reduced ADK expression in forebrain) were treated for 4 days with morphine to establish physical dependence (n=8–9 mice per group). On day 4, the number of jumps made during the 15-min period after naloxone injection was measured as an indicator of opioid dependence. The data represent the mean number of jumps for each indicated group $\pm$ SEM. **C)** Adenosine abundance (+ SEM) in the brainstem (**B**) **D,E)** The effect of selective adenosine receptor agents in morphine dependent mice on (**D**) naloxone-precipitated withdrawal behavior or (**E**) withdrawal-induced hyperalgesia. A₁R agonist 2-Chloro-N⁶-cyclopentyladenosine (CCPA 0.5mg/kg i.p.), A_{2A}R selective antagonist SCH 58261 (1mg/kg i.p.) or vehicle (saline) was administered 15 minutes prior to behavior testing. Data represent the Mean $\pm$ SEM for n=8 mice per group; and **p<0.01, ***p<0.001.