Neuroscience 228 (2013) 235–242

OPIATE-INDUCED CHANGES IN BRAIN ADENOSINE LEVELS AND NARCOTIC DRUG RESPONSES

M. WU,[a] P. SAHBAIE,[b] M. ZHENG,[a] R. LOBATO,[a] D. BOISON,[c] J. D. CLARK[a,b] AND G. PELTZ[a]*

[a] *Department of Anesthesia, Stanford University School of Medicine, Stanford, CA 94305, United States*

[b] *Veterans Affairs Palo Alto Healthcare System, Palo Alto, CA 94305, United States*

[c] *Robert S. Dow Neurobiology Laboratories, Legacy Research, Portland, OR 97232, United States*

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 twofold 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 receptor$_1$ (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.

Key words: 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. 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,b; Chu et al., 2009). By analyzing these inter-strain differences, we identified four genes affecting opioid responses (Liang et al., 2006a,b; Smith et al., 2008), 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-napthalene 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

*Corresponding author. Address: Department of Anesthesia, Stanford University School of Medicine, 300 Pasteur Drive L232, Stanford, CA 94305, United States.
E-mail address: gpeltz@stanford.edu (G. Peltz).
Abbreviations: Adk, adenosine kinase; A_1, adenosine A_1 receptor; A_{2a}, adenosine A_{2a} receptor; LC/MS, liquid chromatography with mass spectroscopy; LC/MS/MS, tandem mass spectroscopy; PBS, phosphate-buffered saline.

0306-4522/12 $36.00 © 2012 IBRO. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.neuroscience.2012.10.031

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-h 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, 20 mg/kg; Day 3, 30 mg/kg and Day 4, 40 mg/kg. Vehicle (Saline) injections followed the same twice-daily schedule. Nociceptive testing procedures began approximately 18 h after the final dose of morphine or saline. Naloxone-precipitated withdrawal was initiated by sub-cutaneous 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 A_1R 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 min 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 s 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 (2009). Fifty ml of the polar metabolite extract in 0.1 M sodium tetraborate buffer was combined with 50 ml of 20 mM dansyl chloride and vortexed. The mixture was incubated at room temperature for 30 min 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, 100 Å 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 min gradient at 0.5 ml/min was as follows: t = 0.5 min, 5% B; t = 20.5 min, 60% B; t = 25 min, 95% B; t = 30 min, 95% B. The column was balanced at 5% B for 5 min. 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 min. The collision energy was set at 28 V, isolation width 4 m/z, MS acquisition rate at 5 spectra/s and MS/MS acquisition rate at 3 spectra/s. 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 10 cm in diameter and 40 cm in height. After acclimation, fibers of sequentially increasing stiffness were applied approximately 1 mm lateral to the central wound edge, pressed upward to cause a slight bend in the fiber and left in place for 5 s. 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 trans-cardially 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