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Self-Administrationof Oxycodone by Adolescent and Adult Mice Differentially Affects Hypothalamic Mitochondrial Metabolism Gene Expression

Yong Zhang[1*], Molly Deutsch-Feldman[1], Michele Buonora[1], Adam J Brownstein[1], Keiichi Niikura[1], Ann Ho[1], JurgOtt[2,3] and Mary Jeanne Kreek[1]

[1]*The Laboratory of the Biology of Addictive Diseases, The Rockefeller University, New York, NY 10065, USA*

[2]*The Laboratory of Statistical Genetics, The Rockefeller University, New York, USA*

[3]*Institute of Psychology, Chinese Academy of Sciences, Beijing, China*

Abstract

Objective: Illicit prescription drug use among adolescents is a pressing public health concern: 12% of high school students report using prescription opioids with many progressing to use heroin. However, little is known about the effects of these drugs on the adolescent brain compared to the adult brain. This study examined the effect of adolescent oxycodone self-administration on gene expression specifically related to mitochondrial energy metabolism in the hypothalamus as the hypothalamus is involved in the regulation of feeding, reproduction and stress-induced drug seeking behavior.

Methods: Adolescent and adult mice self-administered oxycodone (0.25mg/kg/infusion) or served as yoked saline controls 2 hours daily for 14 days. The hypothalamic mRNAs were analyzed with qPCR using a commercially available "mitochondrial energy metabolism" PCR array containing 84 genes.

Results: mRNA levels of the ubiquinol-cytochrome c reductase, complex III subunit VII (Uqcrq) gene showed an experiment-wise significant increase in adolescents that self-administered oxycodone compared with controls. This effect was not found in adult mice. We also found that mRNA levels of the oxidase assembly 1-like (Oxa1l) gene showed a point-wise significant decrease in adult mice that had self-administered oxycodone. Additionally, twenty-seven genes had increased expression in adolescents that self-administered oxycodone. Conversely, adults that self-administered oxycodone had eight genes with lower expression; none showed higher expression.

Conclusion: These findings demonstrate that prescription opioid use caused significant changes of gene expression related to mitochondrial metabolism. The differences between adolescents and adults demonstrate the importance of studying adolescents in order to develop effective treatments.

Keywords: Opioids; Hypothalamus; Adolescents; Adults; mRNA

Introduction

Illicit use of prescription opioids (mu opioid receptor agonists) is a growing problem in the United States, with special concern in adolescents. In 2012, 20 percent of high school seniors reported using prescription opioids [1]. This number indicates that prescription opioid abuse by adolescents presents a serious public health concern. Adolescent prescription abuse is particularly troublesome because little is known about how the developing adolescent brain is affected by exposure to prescription opioids [2].

Previous studies showed that acute or chronic exposure to drugs of abuse alters adult brain energy metabolism in specific brain regions. Specifically, there have been several studies associating opioid abuse with mitochondrial dysfunction. A recent study showed that the number of copies of mitochondrial DNA was lower in rats with opioid abuse [3]. Mitochondrial dysfunction has been observed as a result of alcohol consumption and drug abuse [4]. Additionally, mitochondrial enzymes have been identified as biomarkers of drug dependence [5]. However, the extent to which prescription opioids regulate brain metabolism remains poorly explored, especially in the adolescent brain.

The hypothalamus is one of the major components of the brain circuitry mediating food intake, reproduction, and stress-related drug seeking [6]. The orexin system, derived from the lateral hypothalamus, regulates a wide range of physiological processes, including feeding, energy metabolism, and arousal. Orexin may also play an important role in the reinforcing properties of most drugs of abuse [7,8]. Furthermore, as part of the Hypothalamic-Pituitary-Adrenal (HPA) axis, the hypothalamus also plays a key role in stress-induced drug-seeking behavior [9]. However, the role that the hypothalamus plays in energy metabolism under oxycodone self-administration conditions has not been studied. Specifically, whether and how oxycodone self-administration affects genes related to mitochondrial metabolism within the hypothalamus is unknown [10].

We have shown that adolescent mice self-administer less oxycodone than adult mice [11,12]. We hypothesized that this was due to differential neurobiological alterations in brain regions associated with reward in adolescents versus adults. Studies from our group found that adolescent mice that had self-administered oxycodone showed differences in gene expression in both the dorsal striatum [11] and in the hippocampus compared with adult mice that had also self-

***Corresponding author:** Yong Zhang, The Laboratory of the Biology of Addictive Diseases, The Rockefeller University, 1230 York Avenue, Box 171, New York, NY 10065, USA, Tel: 212-327-8490; Fax: 212-327-8574; E-mail: zhangyo@rockefeller.edu

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administered oxycodone [13]. The study described here expands on these findings by specifically examining genes related to mitochondrial energy metabolism in the hypothalamus of these mice.

Materials and Methods

Subjects

Male adolescent and adult (4 and 11 weeks old upon arrival, respectively) C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed in groups of five with free access to food and water in a light- (12:12 light/dark cycle, light on at 7:00 pm and off at 7:00 am) and temperature- (25 degree Celsius) controlled room. Animal care and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences 1996). The experimental protocols used were approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

Self-administration procedure

Catheter implantation: Following acclimation for 5 days, the mice were anesthetized with a combination of xylazine (8.0 mg/kg i.p.) and ketamine (80 mg/kg i.p.). After shaving and application of a 70% alcohol and iodine preparatory solution, incisions were made in the midscapular region and anteromedially to the forearm. A catheter of approximately 6 cm in length (ID: 0.31mm, OD: 0.64mm) (Helix Medical, Inc. CA) was passed sub-cutaneously from the dorsal to the ventral incision. After exposure of the right jugular vein, a 22-gauge needle was inserted into the vein to guide the catheter into the jugular vein. Once the catheter was inside the vein, the needle was removed and the catheter was inserted to the level of a silicone ball marker 1.1 cm from the end. The catheter was tied to the vein with surgical silk. Physiological saline was then flushed through the catheter to avoid clotting and the catheter then capped with a stopper. Antibiotic ointment was applied to the catheter exit wounds on the animal's back and forearm. Mice were individually housed after the surgery and were allowed 4 days of recovery (due to the limited period of adolescence in the mouse) before being placed in operant test chambers for the self-administration procedure [11].

Intravenous self-administration chamber: The self-administration chamber, ENV-307W (21.6 cm × 17.8 cm × 12.7 cm, Med. Associates, St. Albans, VT), was located inside a larger sound attenuation chamber (Med. Associates). The front, back and top were constructed of 5.6 mm polycarbonate. Each chamber contained a wall with two small holes (0.9 cm diameter, 4.2 cm apart, and 1.5 cm from the floor of the chamber). One hole was defined as active, the other was inactive.When the photocell in the active hole was triggered by a nose-poke, an infusion pump (Med. Associates) delivered an oxycodone infusion of 20 µl/3 sec from a 5 ml syringe. The syringe was connected by a swivel via Tygon tubing. The infusion pump and syringe were outside the chamber. During infusion, a cue light above the active hole was illuminated. Each injection was followed by a 20-sec "time-out" period during which poking responses were recorded but had no programmed consequences. All responses at the inactive hole were also recorded. Mice were tested during the dark phase of the diurnal cycle (all experiments were performed between 8:00 a.m. and 12:00 p.m.).

Oxycodone self-administration: A 2-hr self-administration session was conducted daily. Each day, mice were weighed and heparinized saline (0.02 ml of 30 IU / ml solution) was used to flush the catheter to maintain patency. During self-administration sessions, mice in the oxycodone (Sigma, St. Louis, MO) groups were placed in the self-administration chamber and a nose-poke through the active hole led to an infusion of oxycodone (0.25 mg/kg/infusion) under an FR1 schedule for 14 days. Drug volume was controlled by a computer for individual animals to follow daily changes in body weight. Mice in the control groups received yoked saline infusions during all sessions (saline was infused in the control mouse whenever the oxycodone mouse self-administered oxycodone). At the end of the experiment, only data from mice that passed a catheter patency test defined as loss of muscle tone within a few seconds after administration of the short-acting anesthetic ketamine (5mg/ml) (Fort Dodge, IA) were included in the statistical analyses.

RNA isolation

Mice were sacrificed within 1 hr after the last oxycodone or yoked saline self-administration session; the hypothalamus from each mouse was dissected from the brain and homogenized in Qiazol (Qiagen, Valencia, CA). Total RNA was isolated from homogenates of the hypothalamus using the miRNA easy kit (Qiagen, Valencia, CA). The quality and quantity of RNA from each sample was determined spectrophotometrically. Genomic DNA was removed from the isolated RNA using RT2 HT First Strand Kit (Qiagen, Valencia, CA). Complementary DNA was then synthesized from 500 ng of total RNA with the same kit (Qiagen, Valencia, CA).

Mouse mitochondrial energy metabolism RT2-Profiler™ PCR array

The Mitochondrial Energy Metabolism RT² Profiler™ PCR Array (PAMM-060ZE-4, SABioscience) profiles the expression of 84 genes involved in modulating ATP synthesis and oxidative phosphorylation. This includes genes that code for ATPase, ATP synthase, cytochrome c oxidase, and NADH oxidase enzymes. The assay used real-time PCR using the SYBR Green detection method. Total RNA (500 ng) was reverse transcribed using the First Strand cDNA Synthesis Kit (SABioscience, Frederick, MD, USA) following manufacturer's instructions. The generated cDNA was diluted with an appropriate volume of instrument-specific 2x SuperArray RT2 Real-Time™ SYBR Green PCR Master Mix (PA-012) and ultra-pure water, and 10 µl of this reaction mix was added to each well of the PCR array. The real-time PCR reaction was performed in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA), applying the following program: 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The ABI Prism 7900 HT Sequence Detection System was used to calculate the Ct value for each well. Data were normalized to the mean of the four housekeeping genes and analyzed by the comparative Ct-method (2-ΔΔCT). All significant changes in gene expression levels are reported; the complete list of genes assayed on the array can be found at the manufacturer's website (http://www.sabioscience.com/rt_pcr_product/HTML/PAMM-060ZE-4.html).

Statistical analysis

A total of 23 mice completed the study and passed the catheter patency test. There were 12 adolescents: 7 oxycodone self-administering mice and 5 yoked saline controls, and 11 adults: 5 oxycodone and 6 control mice. Several analytic approaches were taken.

First, differences in expression of genes were analyzed by two-way analysis of variance (Age × Drug Condition). This analysis yields two possible drug effects, a significant main effect of Drug Condition and a significant interaction that indicates that the drug effect is significantly different in the two Age groups.

In each of the two age groups, a t-test between mice self-administering oxycodone and their yoked saline controls was carried out for each of the 84 genes.

Finally, permutation tests were carried out using our sumstat program. This approach may be considered more appropriate than the ANOVA and t tests since it takes correlations among genes into account. The result is based on 20,000 permutation samples including the observed data. Calculations were carried out with a version for quantitative traits using the sumstat program (http://www.jurott.org/linkage/sumstat.html).

Below we refer to a point-wise significant result when it has a nominal p-value of 0.05 or less, and an experiment-wise significant result when it is corrected for all genes tested.

Results

Significant differences in gene expression by two-way ANOVA

Two-way ANOVA, Age×Drug Condition, revealed that there were no main effects of either Age or Drug Condition. However, seven genes showed a point-wise significant interaction of Age x Drug Condition (Table 1).

Gene expression altered in the adolescent or adult groups

T-tests revealed mRNA levels of numerous genes altered as a result of oxycodone self-administration in the adolescent and adult groups. For adolescents, twenty-seven genes showed higher expression levels in mice that had self-administered oxycodone compared to yoked saline controls (Table 2A); no genes showed lower expression in mice that had self-administered oxycodone compared to controls in adolescents. In sharp contrast, no gene showed up regulations in adult mice that had self-administered oxycodone; however, eight genes show lower expression levels in mice that had self-administered oxycodone compared with saline controls in adult groups (Table 2B).

A different analytical procedure: significant differences in gene expression by permutation analysis

Permutation analysis showed experiment-wise significance between the oxycodone and saline controls in one gene *Uqcrq* in the adolescent mice; this gene also showed a point-wise significant interaction. None of the genes examined from adult mice showed experiment-wise significant difference in expression between oxycodone and controls. In the adolescent groups, *Uqcrq* mRNA levels were significantly higher in oxycodone self-administering group than saline controls, $p < 0.05$ (Figure 1A). In the adult groups, Oxail mRNA, which had shown a point-wise significant interaction, showed a point-wise significant difference between oxycodone and saline groups in the permutation analysis, $p < 0.0098$, but this did not reach experiment-wise significance ($p = 0.079$) (Figure 1B).

Gene Symbol	Protein	P-value
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	0.0238
Cox4i1	Cytochrome c oxidase subunit IV isoform 1	0.0301
Cox6b2	Cytochrome c oxidase subunit VIb polypeptide 2	0.0371
Cyc1	Cytochrome c-1	0.0064
Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7	0.0220
Oxa1l	Oxidase assembly 1-like	0.0358*
Uqcrq	Ubiquinol-cytochrome c reductase, complex III subunit VII	0.0359*

Table 1: Genes showing significant Interaction of Age x Drug Condition

Discussion

In this study, as part of a larger investigation into gene expression levels in different brain regions, we compared mRNA levels of genes associated with mitochondrial metabolism in the hypothalamus of adolescent C57BL/6J mice versus adult C57BL/6J mice that had self-administered oxycodone for 14 consecutive days. We found that adolescent oxycodone self-administration led to an experiment-wise significant increase in mRNA level of ubiquinol-cytochrome c reductase, complex III subunit VII (*Uqcrq*) whereas adult oxycodone self-administration caused a point-wise significant decrease in mRNA levels of oxidase assembly 1-like (Oxa1l). Further, we found that adolescent oxycodone self-administration altered expression of more genes (showing point-wise significance) in the hypothalamus than adult self-administration did.

Uqcrq encodes for a ubiquinone-binding protein, a subunit of the ubiquinol-cytochome c reductase complex III.This enzyme is a part of the mitochondrial respiratory pathway. Mutation of this gene has been found to be associated with psychomotor retardation and marked global dementia with defects in verbal and expressive communication skills in humans [14]. We found that adolescent mice that had self-administered oxycodone showed a significantly higher level of *Uqcrq* mRNA than did the saline controls. The increase in *Uqcrq* mRNA may

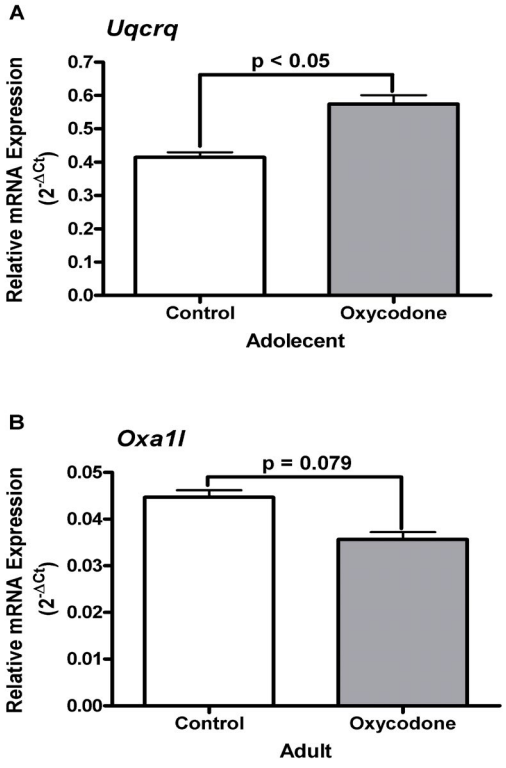

Figure 1: (A) Permutation analysis showed that UqcrqmRNA levels were significantly higher in oxycodone self-administering group than saline controls, $p < 0.05$, in the adolescent groups. (B) In the adult groups, OxailmRNA showed apoint-wise significant difference between oxycodone and saline groups by the permutation analysis, $p < 0.0098$, but did not reach experiment-wise significance ($p = 0.079$).

A Adolescent

Gene Symbol	Protein	P-value	Direction of Change
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	0.0409	↑
Atp5g2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)	0.0234	↑
Atp5g3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)	0.0178	↑
Atp5h	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	0.0390	↑
Atp5j	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F	0.0052	↑
Cox5a	Cytochrome c oxidase, subunit Va	0.0280	↑
Cox6a1	Cytochrome c oxidase, subunit VI a, polypeptide 1	0.0104	↑
Cox6b1	Cytochrome c oxidase, subunit VIb polypeptide 1	0.0304	↑
Cox6c	Cytochrome c oxidase, subunit VIc	0.0104	↑
Cox7b	Cytochrome c oxidase subunit VIIb	0.0094	↑
Ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	0.0466	↑
Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2	0.0391	↑
Ndufa3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3	0.0442	↑
Ndufa4	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4	0.0256	↑
Ndufa6	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (B14)	0.0074	↑
Ndufb2	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	0.0114	↑
Ndufb3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 3	0.0329	↑
Ndufb4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4	0.0375	↑
Ndufb5	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 5	0.0060	↑
Ndufb6	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 6	0.0374	↑
Ndufb7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7	0.0134	↑
Ndufc1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1	0.0151	↑
Ndufc2	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 2	0.0025	↑
Ndufs5	NADH dehydrogenase (ubiquinone) Fe-S protein 5	0.0156	↑
Ndufs6	NADH dehydrogenase (ubiquinone) Fe-S protein 6	0.0213	↑
Ndufv3	NADH dehydrogenase (ubiquinone) flavoprotein 3	0.0042	↑
Uqcrq	Ubiquinol-cytochrome c reductase, complex III subunit VII	0.0009	↑

B Adult

Gene Symbol	Protein	P-value	Direction of Change
Oxa1l	Oxidase assembly 1-like	0.002	↓
Atp5a1	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1	0.0118	↓
Ppa1	Pyrophosphatase (inorganic) 1	0.0165	↓
Ndufs7	NADH dehydrogenase (ubiquinone) Fe-S protein 7	0.0174	↓
Cyc1	Cytochrome c-1	0.0274	↓
Atp5b	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit	0.039	↓
Cox6b2	Cytochrome c oxidase subunit VIb polypeptide 2	0.0317	↓
Sdhc	Succinate dehydrogenase complex, subunit C, integral membrane protein	0.0426	↓

Table 2: Genes expression differing between oxycodone and yoked saline groups in adolescent (A) and Adult (B)

be seen as an increase in mitochondrial activity as a result of oxycodone self-administration in adolescent hypothalamus. This is the first study to report that oxycodone self-administration altered *Uqcrq* mRNA in the hypothalamus.

The gene product of Oxa1l is necessary for proper insertion of proteins into the mitochondrial membrane. These proteins are part of the oxidative phosphorylation pathway and thus Oxa1l is an integral part of proper ATP formation. Knockdown of Oxa1l genes in HEK293 cells resulted in markedly decreased ATP hydrolytic activity of ATP synthase and moderately reduced levels and activity of NADH: ubiquinone oxidoreductase (complex I) [15]. The decreased Oxa1l mRNA levels found in adult mice that had self-administered oxycodone suggest that oxycodone self-administration decreased ATP activity in the adult hypothalamus.

Interestingly, oxycodone self-administration led to altered expression of many more genes in the adolescent group (27 genes) than in the adults (8 genes) even though adolescent mice self-administered a lower total amount of oxycodone than adult mice did. Among the genes found to be altered, all showed higher expression levels in the adolescent mice that had self-administered oxycodone compared with saline controls, whereas in adult mice, all genes that differed showed lower expression levels in animals that had self-administered oxycodone compared to saline controls.

Previous studies found that drugs of abuse led to decreases (inhibition) of mitochondrial functions in adult rodents. For example, cytochrome c release showed a decrease upon treatment with street heroin as examined by immunocytochemistry, indicating a decrease in mitochondrial cytochrome content. Western blot showed that street heroin mediated a decrease in mitochondrial cytochrome c content by about 60% in the same study [16]. In a separate study, cocaine, morphine or a combination of both drugs have been found to decrease mitochondrial ATPase activity in rat liver [17]. Cocaine

or cocaine combined with morphine induced mitochondrial complex I dysfunction by decreasing state-3 respiration in brain mitochondria and decreased uncoupled respiration and mitochondrial potential in both isolated brain and liver mitochondria [18]. Also, complex I, II, II-III and IV activity were decreased after amphetamine administration in adult rats [19]. Furthermore, drugs of abuse have been shown to affect levels of mitochondrial enzymes. For example, decreased levels of mitochondrial enzymes were found in the adult rats exposed to cigarette smoke [20]. The level of the brain mitochondrial enzymes was found to be significantly lower in the morphine-treated rats compared with control animals [21].

Our finding that lower expression levels of genes altered as a result of oxycodone self-administration in the adult mice is in accord with previous reports, suggesting that oxycodone self-administration decreased mitochondrial function in the adult hypothalamus.

There has been little research on the impact of drugs of abuse on mitochondrial functions in the adolescent brain. For example, one study found that mitochondrial complexes I, II, II-III and IV and creatine kinase activities were increased after acute and chronic administration of fenproporex, an amphetamine-derived anorectic, in young rats [22]. Our finding that mice that had self-administered oxycodone during adolescence had increased expression of various genes related to mitochondrial metabolism is consistent with the report showing that some psycho stimulants increase brain energy metabolism in young rodents.

In summary, the current study identifies differences in gene expression associated with mitochondrial metabolism between adolescent and adult C57BL/6J mice, such as *Uqcrq* and Oxa1l. The opposite changes in gene expression associated with mitochondrial metabolism in the hypothalamus between the adolescent and adult mice are intriguing. Such differences may, in part, underlie the mechanism of difference in oxycodone self-administration behavior between the adolescent and adult mice [11,12], especially in view of important hypothalamic functions in the relationship of energy expenditure and reward (food and drug). The genes identified in this study also constitute appropriate candidates for further study, testing the mechanistic impact of these targets for pharmacotherapy, and their relationship to developmental stage of prescription opioid self-exposure.

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Disclosure / Conflict of Interest

The author(s) declare that, except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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