Regulation of Gene Expression in Brain Tissues of Rats Repeatedly Treated by the Highly Abused Opioid Agonist, Oxycodone: Microarray Profiling and Gene Mapping Analysis

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ABSTRACT:

Although oxycodone is the most often used opioid agonist, it remains one of the most understudied drugs. We used microarray analysis to better understand the global changes in gene expression in brain tissues of rats repeatedly treated with oxycodone. Many genes were significantly regulated by oxycodone (e.g., *Fkbp5*, *Per2*, *Rt1.Dα*, *Slc16a1*, and *Abcg2*). Validation of the microarray data by quantitative real-time-polymerase chain reaction (Q-PCR) indicated that there was a strong significant correlation ($r = 0.979$, $p < 0.0000001$) between the Q-PCR and the microarray data. Using MetaCore (a computational platform), many biological processes were identified [e.g., organic anion transport ($p = 7.251 \times 10^{-4}$) and regulation of immune response ($p = 5.090 \times 10^{-4}$)]. Among the regulated genes, *Abcg2* mRNA was up-regulated by 2.1-fold, which was further confirmed by immunoblotting (1.8-fold up-regulation). Testing the *Abcg2* affinity status of oxycodone using an *Abcg2* ATPase assay suggests that oxycodone

behaves as an *Abcg2* substrate only at higher concentrations ($\geq 500 \mu\text{M}$). Furthermore, brain uptake studies demonstrated that oxycodone-induced *Abcg2* up-regulation resulted in a significant ($p < 0.05$) decrease (~ 2 -fold) in brain/plasma ratios of mitoxantrone. These results highlight markers/mediators of neuronal responses and identify regulatory pathways involved in the pharmacological action of oxycodone. These results also identify genes that potentially modulate tolerance, dependence, immune response, and drug-drug interactions. Finally, our findings suggest that oxycodone-induced up-regulation of *Abcg2* enhanced the efflux of the *Abcg2* substrate, mitoxantrone, limiting its brain accumulation and resulting in an undesirable drug-drug interaction. Extrapolating these results to other *Abcg2* substrates (e.g., daunorubicin and doxorubicin) indicates that the brain uptake of these agents may be affected if they are administered concomitantly with oxycodone.

Oxycodone is a semisynthetic derivative of thebaine that is widely used in clinical settings for management of cancer and postoperative pain. Oxycodone has been thought to exert its pharmacological activity through activation of κ -opioid receptors (Nielsen et al., 2000); however, other studies suggested that the analgesic and abuse effects of oxycodone are probably mediated through μ -opioid receptors

(Beardsley et al., 2004; Nozaki and Kamei, 2007). Oxycodone is the most often prescribed opioid agonist in the United States (Hays, 2004). In 1996, the legal distribution of oxycodone in the United States alone reached 2 million grams (Joranson et al., 2000). Its use has increased almost exponentially with approximately 1 billion prescriptions written in 2004 alone (Cicero et al., 2005). The severity of the prescription abuse problem associated with oxycodone has been well documented by the Drug Abuse Warning Network, which reported a total of 1014 oxycodone-related deaths between 1999 and 2002 in 23 states (Cone et al., 2003). Despite its widespread use, the mediators that underlie the pharmacological actions, the abuse potential, and the drug-drug interactions observed upon repeated oxycodone administration are yet not clearly understood, and oxycodone remains one of the most understudied drugs.

Repeated opioid administration was shown to induce the MDR transporter, P-gp (Abcb1) in brain tissues of rats (Aquilante et al.,

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ABBREVIATIONS: MDR, multidrug resistance protein; P-gp (Abcb1), P-glycoprotein; MHC, major histocompatibility complex; Q-PCR, quantitative real-time polymerase chain reaction; C_t , threshold cycle; ANOVA, analysis of variance; GO, Gene Ontology; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Bcrp (Abcg2), breast cancer-related protein; PAGE, polyacrylamide gel electrophoresis; PDVF, polyvinylidene difluoride; TBS, Tris-buffered saline; TBS-T, TBS Tween 20; Mrp (Abcc), multidrug resistance-associated protein; MCT, monocarboxylate transporter; CNS, central nervous system; BBB, blood-brain barrier; PK, pharmacokinetics; PD, pharmacodynamics.

2000). In mice, *Abcb1* was demonstrated to modulate the antinociceptive activity and the brain uptake of many opioids that are MDR substrates (Dagenais et al., 2004). In clinical settings, opioids are concomitantly administered with many chemotherapeutic agents that are MDR substrates (e.g., paclitaxel, mitoxantrone, doxorubicin, and methotrexate). Modulation of MDR transporters by repeated opioid administration may result in transporter-mediated drug-drug interactions (Lin, 2003; Mao and Unadkat, 2005; Endres et al., 2006). We have demonstrated in our laboratory (Hassan et al., 2007) that oxycodone is a P-gp substrate and that repeated oxycodone administration resulted in induction of P-gp in different tissues. We further observed that oxycodone-mediated P-gp up-regulation had a direct impact on the tissue distribution of paclitaxel, a P-gp substrate. Our study supports the hypothesis that repeated opioid administration results in up-regulation of MDR transporters and mediates drug-drug interactions between opioids and other agents that are MDR substrates. In expansion of our work, we used microarray analysis to determine oxycodone-regulated brain transporters and to determine markers/mediators that govern the pharmacological phenomena associated with oxycodone.

With the use of microarray analysis, extensive studies have been focused on identifying morphine-induced changes in gene expression (e.g., *Fkbp5*, *Rt1.Dβ*, *Rt1.Bα*, and *Rt1.Bβ*) (Loguinov et al., 2001; Ammon et al., 2003; Beagles et al., 2004; McClung et al., 2005; Stefano et al., 2005). Up-regulation of *Fkbp5* by repeated morphine administration is believed to mediate both morphine tolerance and dependence (McClung et al., 2005), whereas down-regulation of the major histocompatibility complex (MHC) class II molecules (e.g., *Rt1.Dβ*, *Rt1.Bα*, and *Rt1.Bβ*) is believed to mediate immune responses in morphine-treated rats (Beagles et al., 2004). Although oxycodone is the most often used opioid agonist, a comprehensive gene expression profile in brain tissues of animals repeatedly treated with oxycodone is still lacking, and it is not known whether any of the aforementioned genes is regulated upon its repeated administration. The consequences of repeated oxycodone administration are manifested by increased drug craving, tolerance development, and expression of withdrawal symptoms when oxycodone is discontinued (Beardsley et al., 2004). Each of these features is a result of adaptive changes in the expression levels of several genes. Identifying target genes that are regulated upon repeated oxycodone administration will aid in better understanding of the pharmacological phenomena that are governed by these adaptive changes. In addition, extensive and quantitative characterization of gene expression will provide insight on markers/mediators of neuronal responses and identify indicators of regulatory pathways involved in the pharmacological action of oxycodone.

In this study, we used Affymetrix microarrays to obtain a global profile of genes regulated by repeated oxycodone administration in rats. Regulated genes were then imported to MetaCore, a Web-based computational platform, to build a highly interconnected gene network and to determine the oxycodone-regulated pathways in the context of rat regulatory networks and pathways reported in the literature. To verify the results, 22 selected genes were analyzed by quantitative real-time PCR (Q-PCR). We were intrigued by the finding that oxycodone up-regulated the efflux transporter *Abcg2* mRNA, so the work was expanded to determine the *Abcg2* protein expression levels and the *Abcg2* affinity status (substrate, nonsubstrate, or inhibitor) of oxycodone. Finally, functional drug-drug interaction studies were conducted to determine the influence of the up-regulated *Abcg2* on the brain uptake of a representative *Abcg2* substrate, mitoxantrone.

Materials and Methods

Experimental Animals. Male Sprague-Dawley rats (270–300 g) were purchased from Harlan Indianapolis, IN). They were fed chow and water ad libitum and maintained on a 12-h light/dark cycle. The animals were housed individually and allowed to acclimate for at least 1 week before the experiments were conducted. The protocol for the animal studies was approved by the School of Pharmacy, University of Maryland, Institutional Animal Care and Use Committee.

Repeated Administration of Oxycodone. Sprague-Dawley rats were assigned to one of two groups ($n = 18/\text{group}$) administered either oxycodone hydrochloride (obtained from Dr. Andrew Coop, School of Pharmacy, University of Maryland, Baltimore) dissolved in saline at a dosage regimen of 15 mg/kg i.p. every 12 h for 8 days or saline at a dosage regimen of 1 ml/kg i.p. every 12 h for 8 days. This dosage regimen was precisely selected and is pharmacologically relevant because oxycodone was reported to be 1.5 times more potent than morphine (Kalso et al., 1990; Heiskanen et al., 1998; Nielsen et al., 2000). Thus, a dose of 15 mg/kg oxycodone is almost equivalent to a dose of 20 mg/kg morphine, which is one of the most common doses for morphine administration in rats (Guitart and Nestler, 1989; Pöyhä and Kalso, 1992; Aquilante et al., 2000; Ammon et al., 2003). In addition, twice-daily dosing of oxycodone is commonly reported in the literature (Holtman and Wala, 2006; Hassan et al., 2007) because oxycodone has an elimination half-life ($t_{1/2}$) of 3.0 ± 0.5 h and needs five half-lives (>15 h) for ~95% of the drug to be eliminated (Chan et al., 2008). Thus, dosing every 12 h ensures that enough oxycodone is administered just before its complete elimination from the rats' bodies. Finally, an 8-day dosing regimen was adopted because rats under similar experimental conditions developed tolerance to the analgesic effect of oxycodone (Hassan et al., 2007). The oxycodone-treated group was compared directly to the saline-treated control group, which was handled, treated, and sacrificed at the same time and under the same conditions.

RNA Extraction from Brain Tissues of Oxycodone- and Saline-Treated Rats. Rats in both groups ($n = 6/\text{group}$) were sacrificed by CO_2 asphyxiation and decapitated 12 h after administration of the last dose. Whole-brain tissues (including olfactory tubercles) were harvested, and the cerebellums were excluded. Then, total RNAs were immediately isolated from the fresh brain tissues using TRIzol (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The concentration of the total RNA was determined by UV spectrophotometry at 260/280 nm, and the total RNA (30 μg) was subjected to DNase treatment for 10 min using an RNase-free DNase set (QIAGEN). Samples were then purified using an RNeasy MinElute Kit (QIAGEN). Concentration of the total purified RNA was determined again by UV spectrophotometry at 260/280 nm, and RNA quality was assessed by electrophoresis on a 1% agarose gel. Only samples with a 260/280 nm ratio of >1.8 and no signs of RNA degradation based on the gel electrophoresis were used. Further assessment of the integrity of the RNA was performed using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). RNA samples with sharp and defined 28S and 18S ribosomal peaks validated good RNA integrity and were used for subsequent studies. RNA samples with poorly defined or missing peaks indicated degraded RNA and were not used.

Microarray Hybridization and Staining. We used the GeneChip rat genome 230 2.0 array (Affymetrix, Santa Clara, CA) containing 31,000 probe sets that analyze the expression levels of more than 30,000 transcripts encoding for more than 28,000 well substantiated rat genes. Many genes were represented by more than one probe set, which gave confidence in concluding whether a gene was regulated or not. RNA samples isolated from six brain tissues/group were combined so that each two RNA samples were pooled (2×1) to have a total of three pooled RNA samples per group. Pooled RNA samples from both groups were labeled and run on the arrays at the same time for each experiment ($n = 3$ arrays/group). In brief, total RNA (5 $\mu\text{g}/\text{array}$) was converted to cDNA, amplified, and labeled according to the Affymetrix protocols. Hybridization and washing were performed using the Affymetrix Fluidics Station 450 and Hybridization Oven 640 under standard conditions. The arrays were then scanned with a GeneChip Scanner 3000 (Affymetrix). RNA labeling, hybridization, washing, and scanning were performed according to the manufacturer's instructions (Affymetrix) at the Biopolymer/Genomics core facility at the University of Maryland, Baltimore.

Microarray Data Analysis. Based on six individual CEL files (three files/group), gene expression measures were calculated using the Background Adjusted Robust Multiple Average method (Wu et al., 2004) and implemented in the Bioconductor R packages *Affy* and *gcrma* (Gentleman et al., 2005). This process includes background correction, probe-level quantile normalization and expression measures calculated by using median polish. A detection p value by Affymetrix Microarray suite version 5 was used to make a reliable prediction of gene expression (present, marginal, or absent). In this context, present indicates that the detection p value is <0.04 , marginal means that the detection p value is between 0.04 and 0.06, and absent means that the detection p value is >0.06 . Affymetrix Microarray suite version 5 also quantitatively determines raw signal intensities for each transcript: the higher the raw signal intensity (>40) for each transcript, the more reliable are the estimated detection calls. -Fold changes in the transcript levels and statistical analysis were calculated using Spotfire DecisionSite 8.2.1 algorithm (Spotfire, Inc., Somerville, MA), which relies on the Profile ANOVA algorithm for determining mean differences between groups. The following criteria were used to select candidate genes for further analysis: 1) genes must have present or marginal calls in all arrays, 2) genes must have raw signals of >40 in all arrays, 3) genes must be up- or down-regulated by ≥ 1.5 -fold relative to the control, and 4) genes must have mean differences that are significantly different ($p < 0.05$) from the control based on Spotfire statistical analysis. Only genes that met the above criteria were considered for further analysis. Under the above conditions, Affymetrix arrays have been shown to achieve a statistical power of >0.8 to detect 1.5-fold changes and >0.99 to detect 2.0-fold changes (Shippy et al., 2004). Special attention was paid to the significantly regulated transporters because of their manifested importance in drug-drug interactions (Lin, 2003; Mao and Unadkat, 2005; Endres et al., 2006). As a result, a list of transporters that met the above criteria (with the exception of the 1.5 cutoff -fold change) was generated.

Q-PCR. Expression levels of 22 genes [13 significantly regulated ($p < 0.05$) and 9 nonsignificantly regulated ($p > 0.05$)] (Table 1) were tested by Q-PCR to validate the microarray findings. Genes were randomly selected to represent the following groups: 1) genes significantly ($p < 0.05$) up- or down-regulated ($n = 13$); 2) genes nonsignificantly ($p > 0.05$) up- or down-regulated ($n = 9$); 3) genes of different magnitudes of -fold changes (low, medium, and high) covering the range of ($1.5 \leq \text{fold change} \leq 8.2$); and 4) genes belonging to different classes (e.g., transporters, metabolizing enzymes, housekeeping genes, protein folding genes, rhythmic-process regula-

tors, and others). This approach was adopted to test the reliability of the normalization and the statistical methods used for the microarray data analysis.

Q-PCR was conducted following the manufacturer's recommendation using the iCycler instrument (Bio-Rad Laboratories, Hercules, CA). In brief, 1 μg of total RNA was used to synthesize cDNA in a final reaction volume of 20 μl , using the iScript reverse transcription kit (Bio-Rad Laboratories). Ten percent of the volume of the reverse transcription reaction (2 μl) was used as a template for Q-PCR reactions, which were performed in a total volume of 25 μl , using iQ SYBR Supermix (Bio-Rad Laboratories) with a final primer concentration of 200 nM. Primers for Q-PCR were designed using the Beacon Designer software (Biosoft International, Palo Alto, CA). Primers were then tested for specificity by blasting the candidate sequence against the whole rat database (Annereau et al., 2004). Designing of primers was performed in an iterative fashion until a specific primer for each gene was obtained. After an initial denaturation step at 95°C for 3 min, PCR cycles ($n = 40$) consisted of a 30-s melt at 95°C, followed by annealing and extension at 60°C for 45 s. All reactions were conducted in triplicate. The threshold cycles (C_t) were calculated by using the second derivative of the reaction and were automatically set by the iCycler iQ real-time detection system software. The C_t of each gene was normalized against that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), which showed no change upon oxycodone treatment in our microarray study. -Fold changes were determined by using the Levak method ($\Delta\Delta C_t$ method). Student's t test was used to determine the statistical significance ($p < 0.05$) of the normalized C_t using the SigmaStat 2.03 statistical package (Systat Software Inc., San Jose, CA).

Identification of Relevant Biological Processes and Gene Network Visualization by MetaCore. The MetaCore software (GeneGo, St. Joseph, MI) is a computational resource that uses logic operations for identifying biological processes that are altered because of changes in gene expression. Genes with altered expression were mapped to Gene Ontology (GO) using MetaCore algorithm. GO annotations were used as indicators of the biological functions. GO describes gene products in terms of their associated biological processes, cellular components, and molecular functions. The GO entries are hierarchically linked, thus allowing construction of cluster genes of crossed pathways. A statistically significant gene network was also developed by MetaCore and represents genes' interactions compiled from a curated database of rat protein interactions, metabolism, and bioactive compounds. These networks represent an approach to identify new interactions/relationships using data obtained from numerous genomic studies (Ekins et al., 2006).

TABLE 1
Oligonucleotide primers used for Q-PCR in 5'-3' direction

	Gene Symbol	Sense	Antisense	Accession No.
Significantly regulated genes ($p < 0.05$)				
1	<i>Sgk</i>	CCTCAGGAGCCCGAAGCTTATG	AGGATGGACCCAGGTGATTTG	NM_019232
2	<i>Gpd1</i>	AACCCGTATTCTTGACTCTACCC	AGCCTATACAGATACCTTGAGCG	NM_022215
3	<i>Fkbp5</i> predicted	TGGTCTGACTCTCGTGTTCCTTG	CGCAGGGTGTACGCCAAC	BI284255
4	<i>Abcg2</i>	CTGAAAAGGATGTCTAAACAGGGC	CAGGGTTGTTGTAGGGCTCAC	AB094089
5	<i>Sult1a1</i>	GGTTCCTCAGGTCTTGAAACT	ATGTAGATCACCTTGACCTTCTGA	AF394783
6	<i>Mt1a</i>	GGTGCCCTCGGACAAGTGC	TTAGTAACTGGGTGGAGGTGTAC	AF411318
7	<i>Per2</i>	TGCAGACAAGAAGGCCAAGG	CTTCACAATATACTCGGAGGTAATGC	NM_031678
8	<i>Slc16a1</i>	GCAACGACCAGTGAAGTGTC	GTGTTACAGAAAAGAGCTGCAATCA	NM_012716
9	<i>Tnfrsf11b</i>	CCTTGCCCTGACTACTCTTATACA	GCTTCAAGCAGAATTCGAGCT	NM_012870
10	<i>Adamts1</i>	ATGAATTGGGCCATGTGTTTAAAC	ATGTAGGCACATGCAAGGAGAC	NM_024400
11	<i>Tm4sf1</i> predicted	TTATGAAAGCAGAAAGGGTGGGATA	CTCTCGCCCAACGCAATATAACTG	AW435343
12	<i>S100a8</i>	GCCCTCAGTTTGTGCAGAATAAA	TGTTACTCCTTGTGGCTGTCTTT	NM_053822
13	<i>S100a9</i>	ATGAGAAGCTGCATGAGAACAAC	CATGGCGACCTCTTAATTACTTCC	NM_053587
Nonsignificantly regulated genes ($p > 0.05$)				
14	<i>Abcb1</i>	CTGCTCAAGTGAAAGGGGCTACA	AGCATTTCTGTATGGTATCTGCAAGC	AY582535
15	<i>Slc10a1</i>	AAGGGGACTGACTGAAGAAAACAT	CCTCTGGCACAGCGCAACAC	NM_017047
16	<i>Slc38a4</i>	TTTTCGTCAAGTGTGGTGATTTACAA	GTCAGGTTCCTGTTATTTGTGATC	NM_130748
17	<i>Slc21a10</i>	GTAGACCTGAACAGTGTCCGAAT	TAATAGTCCATTACAATGAAGCTGAG	NM_031650
18	<i>Cyp2d1</i>	GTGCAGGAAGTGCTGGTGAC	CGCAGGGTGGACACAGAGA	NM_153313
19	<i>Cyp2d2</i>	GGAAGCTGTGGTGACCTATGG	CAGGGTGGACACAGAGAATCG	NM_012730
20	<i>Cyp3a1</i>	AGAGGAGTAATTTGCTGACAGACC	GTGTGCGGGTCCCAATTC	NM_173144
21	<i>Cyp3a2</i>	GAATGCTTTTCTGTCTTACAAACC	TTTACCAGAAATGTCCTCACTGTTCA	NM_153312
22	<i>Gapdh</i>	CCCATCACCATCTTCCAGGAG	GTTGTCATGGATGACCTTGGC	NM_017008

TABLE 2

*Up-regulated genes in brain tissues of oxycodone-treated rats*MetaCore assigned gene symbols/names are indicated in parentheses (column 1) if different from Affymetrix gene symbols/names. -Fold change values are expressed as mean $\pm$ S.D.

Gene Symbol	Gene Title	Function	Accession No.	-Fold Change	p Value
<i>PAIHC3 (ITIH3)</i>	Inter-alpha trypsin inhibitor, heavy chain 3	Hyaluronan metabolism	BE106814	3.4 $\pm$ 0.4	0.042
<i>Dusp6 (MKP-3)</i>	Dual specificity phosphatase 6	Regulation of cell cycle progression	AI231350	3.3 $\pm$ 0.1	0.034
<i>Sgk1</i>	Serum/glucocorticoid regulated kinase	Protein kinase activity	NM_019232	2.5 $\pm$ 0.01	0.039
<i>Gpd1^a</i>	Glycerol-3-phosphate dehydrogenase 1	Carbohydrate metabolism	NM_022215	2.5 $\pm$ 0.02	0.009
<i>RGD1311086^{a,b} predicted</i>	Similar to RIKEN cDNA 2610029K21	Nucleic acid binding	BF410646	2.5 $\pm$ 0.1	0.003
	(predicted)	Nucleic acid binding	BI288424	2.4 $\pm$ 0.1	0.006
<i>Fkbp5^a predicted</i>	FK506 binding protein 5 (predicted)	Protein folding	BI284255	2.2 $\pm$ 0.1	0.004
<i>Abcg2</i>	ATP-binding cassette, subfamily G (WHITE), member 2	Efflux transporter	AB094089	2.1 $\pm$ 0.1	0.0004
<i>Gpd1</i>	Glycerol-3-phosphate dehydrogenase 1	Carbohydrate metabolism	NM_022215	2.1 $\pm$ 0.5	0.001
<i>Sult1a1</i>	Sulfotransferase family 1A, phenol-preferring, member 1	Catecholamine metabolism	AF394783	2.1 $\pm$ 0.02	0.009
<i>Rpe65</i>	Retinal pigment epithelium 65	Eye development, vitamin A metabolism, visual perception	NM_053562	2.0 $\pm$ 0.2	0.003
<i>Fkbp5 predicted</i>	FK506 binding protein 5 (predicted)	Protein folding	BI284255	2.0 $\pm$ 0.03	0.003
	Similar to Coronin, actin binding protein 1C	Cell adhesion	AI598550	2.0 $\pm$ 0.1	0.023
<i>Mt1a (metallothionein-1X)</i>	Metallothionein 2610029K21 (predicted)	Metal ion homeostasis	AF411318	2.0 $\pm$ 0.01	0.028
<i>RGD1311086^b predicted</i>	Similar to RIKEN cDNA,	Nucleic acid binding	BI288424	1.9 $\pm$ 0.1	0.001
<i>Per2</i>	Period homolog 2 (<i>Drosophila</i>) 2610029K21 (predicted)	Rhythmic process regulator	NM_031678	1.8 $\pm$ 0.02	0.026
<i>RGD1311086 predicted (CD163)^b</i>	Similar to RIKEN cDNA, CD163 antigen (predicted)	Nucleic acid binding	BI288424	1.8 $\pm$ 0.1	0.007
		Scavenger receptor activity	AA956555	1.6 $\pm$ 0.4	0.002
<i>Smpd13b predicted</i>	Sphingomyelin phosphodiesterase acid-like 3B (predicted)	Carbohydrate metabolism	AW251315	1.6 $\pm$ 0.1	0.016
<i>Dio2</i>	Deiodinase, iodothyronine, type II	Thyroid hormone generation	AW526991	1.5 $\pm$ 0.2	0.049
<i>Lims2 predicted (PINCH-2)</i>	LIM and senescent cell antigen-like domains 2 (predicted)	Zinc ion binding	BI275904	1.5 $\pm$ 0.3	0.022
<i>Klf15 (KLF15)</i>	Kruppel-like factor 15	Nucleic acid binding	NM_053536	1.5 $\pm$ 0.02	0.006
<i>Cryab (Alpha crystalline B)</i>	Crystallin, alpha B	Eye development	NM_012935	1.5 $\pm$ 0.01	0.043
<i>Thrsp (S14 protein)</i>	Thyroid hormone responsive protein	Lipid metabolism	NM_012703	1.5 $\pm$ 0.04	0.009
<i>Dsipi [DSIP1 (GILZ)]</i>	Delta sleep-inducing peptide, immunoreactor	Fluid secretion	NM_031345	1.5 $\pm$ 0.01	0.006
<i>Net1 predicted</i>	Neuroepithelial cell transforming gene 1 (predicted)	Guanyl-nucleotide exchange factor activity	AI180187	1.5 $\pm$ 0.02	0.020
<i>(SRX1)^b</i>	Neoplastic progression 3	DNA binding	AI172302	1.5 $\pm$ 0.04	0.011
<i>Nt5 (5'-NTD)</i>	5 Nucleotidase	DNA metabolism	NM_021576	1.5 $\pm$ 0.2	0.003
<i>Usp2 (UBP41)</i>	Ubiquitin-specific protease 2	Ubiquitin-dependent protein catabolism	AF106659	1.5 $\pm$ 0.01	0.008
<i>RGD1309044 predicted</i>	Similar to RIKEN cDNA 0610039C21 (predicted)	Nucleic acid binding	BI278288	1.5 $\pm$ 0.02	0.036
<i>Tac2 (Nk3)</i>	Tachykinin 2	Neuropeptide signaling synaptic transmission	NM_019162	1.5 $\pm$ 0.2	0.041

^a Gene represented more than once.^b Gene symbol not available.

Many algorithms are integrated in MetaCore to enable construction and analysis of gene networks (Ekins et al., 2006). The Auto expand algorithm was used for building the gene network. The Auto expand algorithm starts with a number of uploaded prespecified root nodes (Tables 2 and 3) and builds networks around objects (genes) from the uploaded set consisting of the nearest neighbors. Nearest neighbors may not be a gene on the initial uploaded list but rather, an object incorporated automatically by the Auto expand algorithm from the MetaCore database. Each connection between objects in the network represents a direct, experimentally confirmed, physical interaction between the objects (Ekins et al., 2006). The Auto expand algorithm has the advantage of providing a means to look up one or more genes and identify regulatory cascades that lead to or from the gene(s) of interest (Ekins et al., 2006).

Determination of the Expression of Abcg2 (Bcrp) Protein by Western Blot Analysis in Brain Tissues of Oxycodone- and Saline-Treated Rats. Two groups ($n = 6$ /group) of Sprague-Dawley rats were treated as described above with saline or oxycodone for 8 days. Rats were then sacrificed by decapitation, and brain tissues were isolated and homogenized according to the procedure described elsewhere (Aquilante et al., 2000). In brief, tissues were removed, rinsed in 4°C isotonic phosphate-buffered saline and homogenized in hypotonic lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4). Tissue homogenates were centrifuged at 3000g (4°C) for 15 min to remove nuclear

debris and particulate matter. The supernatants were collected and centrifuged at 40,000g (4°C) for 30 min, and the resulting pellets were resuspended in 1 ml of lysis buffer each. The protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories). Bovine serum albumin was used as the standard and Abcg2 protein levels were quantitated by Western blot analysis.

SDS-PAGE was performed in 10% polyacrylamide gels using the discontinuous buffer system. Each well was loaded with 60 μ g of membrane fraction protein isolated from whole brain tissues from oxycodone- and saline-treated rats. Resolved proteins were transferred electrophoretically to Bio-Rad Immun-Blot polyvinylidene difluoride (PVDF) membranes (0.2 μ M). After transfer, the SDS-PAGE gel was stained with Coomassie Blue to confirm successful transfer of proteins. PVDF membranes were washed several times with TBS-T washing buffer consisting of 1× Tris-buffered saline (TBS) and 0.05% Tween 20 (T), and incubated for 1 h at 25°C in a solution of 5% nonfat dry milk in TBS-T to block protein-binding sites. Membranes were incubated overnight at 4°C with Abcg2 antibody (M-70) raised in rabbits (1:650 dilution) that was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PVDF membrane was exposed to goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology, Inc.) at a dilution of 1:10,000. Immunoreactive protein was detected using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Chemical, Rockford, IL). Band density was quantified by densitometry using Quantity One 4.4.1 software (Bio-Rad Laboratories). For the control, the

TABLE 3

Down-regulated genes in brain tissues of oxycodone-treated rats

MetaCore assigned gene symbols/names are indicated in parentheses (column 1) if different from Affymetrix gene symbols/names. -Fold change values are expressed as mean $\pm$ S.D.

Gene Symbol	Gene Title	Function	Accession No.	-Fold Change	p Value
<i>Slc16a1</i> (<i>MCT1</i>)	Solute carrier family 16 (monocarboxylic acid transporter 1), member, 1	Organic anion transport	NM_012716	-1.5 $\pm$ 0.1	0.001
<i>Nkx2-2</i> predicted	NK2 transcription factor related (<i>Drosophila</i>) (predicted)	Brain development	AA925143	-1.5 $\pm$ 0.04	0.011
<i>Cklfsf6</i> predicted	Chemokine-like factor - super family 6 (predicted)	Chemotaxis	AI406687	-1.5 $\pm$ 0.02	0.012
<i>LOC498276</i> (<i>FcγRIIα</i>)	Fc gamma receptor II alpha	Immune response	NM_053843	-1.5 $\pm$ 0.1	0.013
<i>Col4a1</i> predicted	Procollagen, type IV alpha 1 (predicted)	Phosphate transport, cell adhesion	AI176393	-1.5 $\pm$ 0.06	0.028
<i>RGD1307925</i> predicted (<i>TBC1D23</i>)	Similar to RIKEN cDNA 4930451A13 (predicted)	Nucleic acid binding	BM390350	-1.5 $\pm$ 0.2	0.044
<i>Akap1</i>	A kinase (PRKA) anchor protein 1	Protein kinase binding	NM_053665	-1.5 $\pm$ 0.03	0.013
<i>ErbB3</i>	V-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	Regulation of cell cycle progression	BI284288	-1.5 $\pm$ 0.2	0.016
<i>Tmem27</i> (<i>Collectrin</i>)	kidney-specific membrane protein	Renal organogenesis	NM_020976	-1.5 $\pm$ 0.04	0.033
<i>Serpinh1</i> (<i>HSP47</i>)	Serine/cysteine proteinase inhibitor, clade H, member 1	Protein folding	BI285495	-1.5 $\pm$ 0.2	0.026
<i>Dpyd</i>	Dihydropyrimidine dehydrogenase	Electron transport	NM_031027	-1.5 $\pm$ 0.1	0.033
<i>Gna12</i> (<i>G-protein alpha-12</i>)	Guanine nucleotide binding protein, alpha 12	G-protein-coupled receptor binding	NM_031034	-1.5 $\pm$ 0.2	0.047
<i>Rffl</i>	Fring	Protein ubiquitination	AW524664	-1.6 $\pm$ 0.1	0.009
<i>Prss35</i>	Protease, serine, 35	Proteolysis	AA866443	-1.6 $\pm$ 0.2	0.017
<i>Scap2</i>	Src family associated phosphoprotein 2	Negative regulation of cell proliferation	AA891732	-1.6 $\pm$ 0.1	0.048
<i>Rt1.Da</i> (<i>HLADRA</i>)	Rt1 class II, locus Da	Immune response	Y00480	-1.6 $\pm$ 0.03	0.004
<i>Map1b</i>	Microtubule-associated protein 1b	Microtubule bundle formation	BE096402	-1.6 $\pm$ 0.02	0.033
<i>Kdr</i> (<i>VEGFR-2</i>)	Kinase insert domain protein receptor	Angiogenesis	U93307	-1.7 $\pm$ 0.1	0.038
<i>Tnfrsf11b</i> (<i>Osteoprotegerin</i>)	Tumor necrosis factor receptor superfamily, member 11b	Apoptosis	NM_012870	-1.7 $\pm$ 0.1	0.020
<i>Tgm2</i>	Transglutaminase 2, C polypeptide	G protein-coupled receptor protein signaling pathway	NM_019386	-1.9 $\pm$ 0.1	0.028
(<i>STAM2</i>) ^a	Signal transducing adaptor molecule, (SH3 domain and ITAM motif) 2	Protein binding	BF397766	-1.9 $\pm$ 0.2	0.010
<i>Adamts1</i> (<i>ADAM-TS1</i>)	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	Metalloendopeptidase activity	NM_024400	-2.1 $\pm$ 0.2	0.019
<i>Tm4sf1</i> predicted [<i>TM4SF1</i> (<i>TAAI6</i>)]	Transmembrane 4 superfamily member 1 (predicted)	Signal transduction	AW435343	-2.1 $\pm$ 0.2	0.039
<i>S100a8</i> (<i>Calgranulin A</i>)	S100 calcium binding protein A8	Chemotaxis, calcium ion binding	NM_053822	-5.5 $\pm$ 0.6	0.018
<i>S100a9</i> (<i>Calgranulin B</i>)	S100 calcium binding protein A9	Chemotaxis, calcium ion binding	NM_053587	-8.2 $\pm$ 0.5	0.027

^a Gene symbol not available.

same membrane was probed in an analogous fashion for immunoreactive β -actin using monoclonal anti- β -actin (A5316) produced in mice (1:5000) (Sigma-Aldrich, St. Louis, MO) and a 1:10,000 dilution of goat anti-mouse IgG horseradish peroxidase (KPL, Gaithersburg, MD).

Oxycodone-Stimulated Abcg2 (Bcrp) ATPase Activity. Oxycodone-stimulated Abcg2 (Bcrp) ATPase activity was estimated by the Glo (Promega, Madison, WI) assay system. This method relies on the ATP dependence of the light-generating reaction of firefly luciferase. ATP consumption is detected as a decrease in luminescence. In a 96-well plate, recombinant rat Abcg2 (25 μ g) (BD Biosciences, San Jose, CA) was incubated with Glo assay buffer (20 μ l) (control, $n = 4$), mitoxantrone (500 μ M) ($n = 4$), sodium orthovanadate (500 μ M) ($n = 4$), and oxycodone (4–4000 μ M) ($n = 3$ /concentration). Mitoxantrone served as a positive control, whereas sodium orthovanadate was used as an ATPase inhibitor. In the presence of sodium orthovanadate, ATP consumption by Abcg2 is negligible and without sodium orthovanadate, Abcg2 consumes ATP to a greater or lesser extent than the control, dependent on the effect of the test compound. The reaction was initiated by addition of MgATP (10 mM) and stopped 40 min later by addition of 50 μ l of firefly luciferase reaction mixture (ATP detection reagent) that initiated an ATP-dependent luminescence reaction. Signals were measured 60 min later, integrated for 10 s using an LMax luminometer (Molecular Devices, Sunnyvale, CA) and converted to ATP concentrations by interpolation from a luminescent ATP standard curve. Rates of ATP consumption (picomoles of ATP consumed per microgram of Abcg2 per minute) and -fold stimulation of the basal Abcg2 activity were calculated as described below (eqs. 1–3). K_m and V_{max} values were estimated using Enzyme Kinetics software (Module 1.1, SigmaPlot 2001, version 7.0; Systat Software Inc.).

ATPase Assay Data Analysis. Basal Abcg2 activity, test compound-stimulated Abcg2 activity, and -fold stimulation by a test compound were calculated according to the following equations:

Basal Abcg2 activity (pmol ATP consumed/ μ g of Abcg2/min)

$$= \frac{\text{ATP}_{\text{vanadate}} - \text{ATP}_{\text{control}}}{25_{\mu\text{g Abcg2}} \times 40_{\text{min}}} \quad (1)$$

Test compound stimulated Abcg2 activity (pmol ATP consumed/ μ g of Abcg2/min)

$$= \frac{\text{ATP}_{\text{vanadate}} - \text{ATP}_{\text{compound}}}{25_{\mu\text{g Abcg2}} \times 40_{\text{min}}} \quad (2)$$

-Fold stimulation by a test compound

$$= \frac{\text{Test compound stimulated Abcg2 activity}}{\text{Basal Abcg2 activity}} \quad (3)$$

where $\text{ATP}_{\text{vanadate}}$ is the number of nonconsumed (total) picomoles of ATP in the presence of sodium orthovanadate, $\text{ATP}_{\text{control}}$ is the number of nonconsumed picomoles of ATP in presence of the assay buffer, and $\text{ATP}_{\text{compound}}$ is the number of nonconsumed picomoles of ATP in the presence of a test compound. ANOVA followed by Dunnett's post hoc test (SigmaStat 2.03 statistical package) was used to determine the statistical significance of the difference between groups. The 0.05 level of probability was used as the criterion of significance.

Brain Uptake of a Model Abcg2 (Bcrp) Substrate, Mitoxantrone, in Oxycodone- and Saline-Treated Rats. To further validate our microarray findings we conducted two independent functional-brain uptake studies. Functional studies were adopted because they can give a better idea regarding the adaptive physiological changes in the activity (stimulation or suppression) of a protein. We used mitoxantrone to determine adaptive functional changes in the efflux activity of the induced Abcg2 (Table 5), which was chosen over other genes for two reasons. First, to the best of our knowledge no studies have

ever been conducted to determine Abcg2-mediated interactions in any opioid-treated rodents or humans. Second, Abcg2 was significantly ($p < 0.0001$) up-regulated by >2 -fold (Table 5), and oxycodone is commonly administered with chemotherapeutic agents that are Abcg2 substrates (e.g., mitoxantrone, daunorubicin, and doxorubicin) (Mao and Unadkat, 2005). Thus, it was intriguing to determine whether the Abcg2 induction would result in restriction of the brain uptake of any of these Abcg2 substrates. On the other hand, mitoxantrone was selected because its brain uptake was previously demonstrated to be Abcg2-dependent (Cisternino et al., 2004). The distribution of [3 H]mitoxantrone (model Abcg2 substrate) (Moravek, Brea, CA) (Imai et al., 2003; Wang et al., 2003; Cisternino et al., 2004; Zhang et al., 2004; Ejendal and Hrycyna, 2005) was determined in the brain and plasma samples of both oxycodone- and saline-treated rats. Sprague-Dawley rats were assigned to one of two groups ($n = 9$ –10/group) for one of the following regimens: oxycodone 15 mg/kg i.p. dose twice daily for 8 days or saline 1 ml/kg i.p. twice daily for 8 days. On day 9, all rats in both groups received a single intraperitoneal dose of [3 H]mitoxantrone (120 μ Ci/kg) (0.013 mg/kg). Rats were sacrificed by CO₂ asphyxiation, 30 and 60 min after mitoxantrone dosing ($n = 4$ –5/group/time point). The brain and plasma samples were collected and analyzed for [3 H]mi-

toxantrone using a Beckman Coulter LS 6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

Analysis of Radiolabeled Mitoxantrone. Brain tissues were weighed and homogenized with an equal volume (1g/1 ml) of phosphate-buffered saline. Tissue solubilizer (Solvable, 1 ml; PerkinElmer Life and Analytical Sciences, Waltham, MA) was added to the tissue homogenate (200 μ l) then incubated at 50°C for 3 h. Glacial acetic acid (50 μ l) was added to the homogenate, and samples were incubated at 50°C for 1 h for decolorization. Samples were left to cool down to room temperature. Then Ultima Gold scintillation cocktail (10 ml) (PerkinElmer Life and Analytical Sciences) was added to each sample, and samples were kept at room temperature for 1 h before counting. For plasma samples, 200 μ l of plasma was added directly to 10 ml of Ultima Gold scintillation cocktail. Brain and plasma samples were analyzed at the same time using a Beckman Coulter LS 6500 multipurpose scintillation counter.

Results

Oxycodone-Induced Gene Expression Changes in Brain Tissues of Sprague-Dawley Rats. For the oxycodone-treated group, no behavioral or withdrawal effects were noticed during or after oxycodone treatment except for the common opioid-induced Straub tail effects. After 8 days of repeated oxycodone or saline administration, RNA was isolated from brain tissues and used for microarray hybridization. We used biological as well as technical replicates to minimize individual (biological) and technical variabilities and, more importantly, to increase the statistical power of the conducted experiments (Peng et al., 2003). Considering all 31,000 probe sets that correspond to the 28,000 genes represented on each microarray slide, oxycodone treatment significantly ($p < 0.05$) induced changes in 641 probe sets ($\sim 2\%$ of probe sets); of those, 56 probe sets (corresponding to 52 genes) were up- or down-regulated by ≥ 1.5 -fold (Fig. 1). Thirty-one probe sets were up-regulated by ≥ 1.5 -fold (Table 2), whereas 25 probe sets were down-regulated by ≥ 1.5 -fold (Table 3). From the significantly ($p < 0.05$) regulated probe sets ($n = 641$) 15 probe sets encoding for 15 transporters were significantly regulated (10 up-regulated and 5 down-regulated) (Table 4). Among the regulated transporters, only two transporters belong to the ATP-binding cassette superfamily [Abcg2 (Bcrp) and Abcc4 (Mrp4)], whereas the rest belong to the solute-linked carrier transporter superfamily.

Validation of the Microarray Data by Q-PCR. To validate the data generated from the microarray studies, the expression level of 13 significantly ($p < 0.05$) and 9 nonsignificantly ($p > 0.05$) regulated genes (as determined by the microarray analysis) were analyzed using Q-PCR analysis. After ascertaining the amplicon specificity by first-

FIG. 1. Gene array profiles of brain tissues of saline- and oxycodone-treated rats. The x- and y-axes represent average normalized signal intensities ($\log_2$) of the significantly ($p < 0.05$) expressed probe sets from saline- and oxycodone-treated rats, respectively. Lines indicate the points where the -fold difference in expression between the transcripts was at least 1.5-fold. -Fold changes and statistical analysis were calculated using the Spotfire DecisionSite 8.2.1 algorithm.

TABLE 4

Differentially expressed transporters in brain tissues of oxycodone-treated rats

-Fold change values are expressed as mean $\pm$ S.D.

Gene Symbol	Gene Title	Accession No.	-Fold Change	p Value
Up-regulated transporters				
<i>Abcg2</i>	ATP-binding cassette, subfamily G (WHITE), member 2	AB094089	2.1 $\pm$ 0.1	0.0004
<i>Abcc4</i>	ATP-binding cassette, subfamily C (CFTR/MRP), member 4	BE100533	1.3 $\pm$ 0.2	0.013
<i>Slc2a1</i>	Solute carrier family 2 (facilitated glucose transporter), member 1	BI284218	1.3 $\pm$ 0.02	0.020
<i>Slc25a25</i>	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	A1177358	1.3 $\pm$ 0.01	0.008
<i>Slc29a3</i>	Solute carrier family 29 (nucleoside transporters), member 3	BM392280	1.3 $\pm$ 0.1	0.001
<i>Slc37a4</i>	Solute carrier family 37 (glycerol-6-phosphate transporter), member 4	NM_031589	1.2 $\pm$ 0.03	0.020
<i>Slc30a5</i> predicted	Solute carrier family 30 (zinc transporter), member 5 (predicted)	BF418332	1.2 $\pm$ 0.02	0.017
<i>Slc4a3</i>	Solute carrier family 4 (anion exchange protein), member 3	NM_017049	1.1 $\pm$ 0.01	0.018
<i>Slc1a2</i>	Solute carrier family 1 (glial high-affinity glutamate transporter), member 2	NM_017215	1.1 $\pm$ 0.1	0.045
<i>Slc6a15</i>	Solute carrier family 6 (neurotransmitter transporter), member 15	L22022	1.1 $\pm$ 0.02	0.039
Down-regulated transporters				
<i>Slc6a6</i>	Solute carrier family 6 (neurotransmitter transporter, taurine), member 6	NM_017206	-1.2 $\pm$ 0.03	0.023
<i>Slco1a4</i>	Solute carrier organic anion transporter family 1, member 4	NM_131906	-1.3 $\pm$ 0.01	0.037
<i>Slc38a1</i>	Solute carrier family 38 (neuronal glutamine transporter), member 1	AF075704	-1.3 $\pm$ 0.02	0.022
<i>Slc22a8</i>	Solute carrier family 22 (organic anion transporter), member 8	NM_031332	-1.3 $\pm$ 0.1	0.034
<i>Slc16a1</i>	Solute carrier family 16 (monocarboxylic acid transporter 1), member 1	NM_012716	-1.5 $\pm$ 0.1	0.001

TABLE 5

Validation of the microarray data using Q-PCR

-Fold changes of 13 significantly regulated genes (as determined by the microarray analysis in Table 1) were determined by Q-PCR. Consistent with the microarray data, all -fold changes determined by the Q-PCR analysis were significantly different ($p < 0.05$) from the control (saline treatment). There was a strong significant correlation between -fold changes obtained by the microarray analysis and those obtained by the Q-PCR analysis ($r = 0.979$, $p < 0.0000001$). Results are expressed as mean $\pm$ S.D. ($n = 3$). An additional group of genes ($n = 9$) that were not significantly ($p > 0.05$) regulated in the arrays (Table 1) were also tested to confirm the reliability of the statistical approach used for the microarray data analysis; none of these genes was significantly different in the Q-PCR analysis ($p > 0.05$). For these nine nonsignificantly regulated genes, there was also a strong significant correlation between -fold changes obtained from the microarray analysis and those obtained by the Q-PCR analysis ($r = 0.958$, $p < 0.001$) (data not shown).

Gene Symbol	Gene Title	-Fold Change (Microarray)	-Fold Change (Q-PCR)
<i>Sgk1</i>	Serum/glucocorticoid regulated kinase	2.5 ± 0.01	2.4 ± 0.1
<i>Gpd1^a</i>	Glycerol-3-phosphate dehydrogenase 1	2.5 ± 0.02	
		2.1 ± 0.5	2.4 ± 0.3
<i>Fkbp5^a predicted</i>	FK506 binding protein 5 (predicted)	2.2 ± 0.1	
		2.0 ± 0.03	2.2 ± 0.3
<i>Abcg2</i>	ATP-binding cassette, subfamily G (WHITE), member 2	2.1 ± 0.1	2.1 ± 0.6
<i>Sult1a1</i>	Sulfotransferase family 1A, phenol-preferring, member 1	2.1 ± 0.02	1.7 ± 0.1
<i>Mt1a</i> (Metallothionein-IX)	Metallothionein 2610029K21 (predicted)	2.0 ± 0.01	2.0 ± 0.5
<i>Per2</i>	Period homolog 2 (<i>Drosophila</i>) 2610029K21 (predicted)	1.8 ± 0.02	2.0 ± 0.2
<i>Slc16a1</i> (MCT1)	Solute carrier family 16 (monocarboxylic acid transporter 1), member 1	-1.5 ± 0.1	-1.4 ± 0.2
<i>Tnfrsf11b</i> (Osteoprotegerin)	Tumor necrosis factor receptor superfamily, member 11b	-1.7 ± 0.1	-1.7 ± 0.02
<i>Adamts1</i> (ADAM-TS1)	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	-2.1 ± 0.2	-1.5 ± 0.1
<i>Tm4sf1</i> predicted [TM4SF1 (TAAI6)]	Transmembrane 4 superfamily, member 1 (predicted)	-2.1 ± 0.2	-1.8 ± 0.1
<i>S100a8</i> (Calgranulin A)	S100 calcium binding protein A8	-5.5 ± 0.6	-5.0 ± 0.2
<i>S100a9</i> (Calgranulin A)	S100 calcium binding protein A9	-8.2 ± 0.5	-4.5 ± 0.2

^a Gene represented twice on the microarray chips.

derivative melting curve analysis, the C_t values obtained for each gene were normalized to that of the corresponding *Gapdh*. All significantly ($n = 13$) (Table 5) and nonsignificantly ($n = 9$) (data not shown) regulated genes as indicated by the microarray analysis, showed similar patterns (comparable -fold changes and p values) upon evaluation of their expression levels in the Q-PCR analysis. Computation of the Pearson correlation coefficient (for the 13 significantly regulated genes; Table 1) indicated that there was a significantly strong correlation ($r = 0.979$, $p < 0.0000001$) between the data generated from the Q-PCR analysis and that generated from the microarray analysis. Likewise, for the 9 nonsignificantly regulated genes (Table 1) and consistent with the microarray analysis, none of these genes was significantly different in the Q-PCR analysis ($p > 0.05$) (data not shown). For these nine nonsignificantly regulated genes, there was also a significant strong correlation between -fold changes obtained from the microarray analysis and those obtained by the Q-PCR analysis ($r = 0.958$, $p < 0.001$). These results confirm the validity of the microarray data and give confidence in the normalization and the statistical method used herein for the microarray data analysis.

Gene Network Visualization of the Microarray Data in MetaCore. High throughput data, derived from microarrays, have been visualized mainly by clustering approaches. Gene clustering has the limitation of driving associations among genes only available on the microarray and provides no information on their interactions. By using a curated gene database as the one incorporated in MetaCore, a network building tool, one can predict pathways and build interconnected gene networks with genes available on the microarray and those available from the curated database. The genes that were significantly ($p < 0.05$) up- or down-regulated by at least 1.5-fold (Tables 2 and 3) were uploaded in MetaCore. A predetermined algorithm within MetaCore, the Auto expand algorithm, was used to generate a gene network highly saturated with differentially expressed genes and nondifferentially expressed genes (e.g., *SP1* or *CRY1*) to show inter-regulatory cascades among oxycodone-regulated and oxycodone-nonregulated genes. The network illustrated in Fig. 2 was assembled by the Auto expand algorithm around the transcription factor *SP1*. Although *SP1* was not regulated by oxycodone, it was incorporated in the network as a function of the Auto expand algorithm. *SP1* is a transcriptional regulator of many genes and appears to

act as a focal point in oxycodone-induced regulations of many genes such as *VEGFR-2* and *DPYD*, as indicated in the network. In other words, the generated network highlights the possible role of *SP1* regulatory cascades in mediating oxycodone-induced regulations of many genes such as *VEGFR-2*, *DPYD*, and *SULT1A1* (Fig. 2). In the generated network, up- and down-regulated genes can be visualized simultaneously, and they were identified by expression signs at the top right of each node (red and blue small circles for up- and down-regulation, respectively). In addition, positive interactions (green hexagons) (e.g., interaction between *SP1* and *SULT1A1*), unspecified interactions (black hexagons) (e.g., interaction between *SP1* and *COL4A1*), and logic relationships (blue hexagons) [e.g., interaction between monocarboxylate transporter (MCT1) and MCT] were identified (Fig. 2). Each of these interactions/relationships represent validated, experimentally confirmed interactions (Ekins et al., 2006). The most statistically significant biological processes (based on p value, in parentheses) associated with genes in the generated network were determined by the MetaCore algorithm and they were the following: mevalonate transport (1.151×10^{-5}), regulation of integrin biosynthesis (1.151×10^5), drug metabolism (3.446×10^{-5}), response to metal ion (1.144×10^{-4}), actin cytoskeleton reorganization (1.144×10^{-4}), positive regulation of immune response (5.090×10^{-4}), immune cell chemotaxis (5.090×10^{-4}), organic anion transport (7.251×10^{-4}), antigen presentation via MHC class II (7.433×10^{-4}), dopamine receptor signaling pathway (1.175×10^{-3}), transmembrane receptor tyrosine kinase signaling pathway (1.364×10^{-3}), and humoral defense mechanism (1.515×10^{-3}).

Expression of Abcg2 Protein in Brain Tissues of Oxycodone- and Saline-Treated Rats. Using SDS-PAGE and Western blotting techniques, the expression of Abcg2 protein (~ 72 kDa) was determined in the brain tissues of both saline (Fig. 3A, lanes 1–3)- and oxycodone (Fig. 3A, lanes 4–6)-treated rats. We observed a significant increase (1.8-fold, $p < 0.01$) in Abcg2 protein expression in the oxycodone group versus the saline group; however, no significant change ($p > 0.10$) in β -actin protein (~ 43 kDa) expression was observed between the two groups. Optical density calculations (mean $\pm$ S.D., $n = 3$) of Abcg2 bands normalized to the β -actin control for saline and oxycodone groups (Fig. 3B) were 0.45 ± 0.08 and 0.82 ± 0.07 , respectively ($p < 0.01$).

FIG. 2. Gene network illustrates genes and interactions of the differentially expressed genes due to oxycodone treatment versus saline treatment in brain tissues of Sprague-Dawley rats. The gene network was obtained using MetaCore software after applying the Auto expand algorithm. Colored highlighted symbols (nodes) represent genes [enzymes (yellow arrows), transporters (purple shapes), receptor ligands (green shapes), transfectors (red shapes), and other proteins (blue shapes)]. Red and blue small solid circles (top right of each node) correspond to genes with significant ($p < 0.05$) up- and down-regulation of ≥ 1.5 -fold, respectively. Of 56 gene identifiers (probe sets) (Tables 2 and 3), 45 genes were mapped into the MetaCore database. Six genes (indicated by blue vectors) were automatically incorporated by the MetaCore Auto expand algorithm to indicate logic relationships. Three genes (not highlighted) were also automatically incorporated by the Auto expand algorithm to show interactions with other genes. The small colored hexagons on vectors between nodes describe positive interaction (green), unspecified interactions (black), or logical relationships (blue). Each connection (vector) represents a direct, experimentally confirmed, physical interaction between genes (Ekins et al., 2006).

Effect of Oxycodone on Abcg2 (Bcrp) ATPase Activity. Different oxycodone concentrations were examined to determine their effects on the Abcg2 ATPase activity. The -fold stimulation of the basal Abcg2 activity by different oxycodone concentrations ranged from 0.7- to 4.6-fold over drug-free membranes, whereas the rates of ATP consumption ranged from 43.9 ± 8.1 to 284 ± 0.2 pmol ATP/ μ g of Abcg2/min (Fig. 4). The rates of ATP consumption were significantly different ($p < 0.01$) from those of the drug-free control only at higher oxycodone concentrations (≥ 500 μ M), whereas at concentrations ≤ 400 μ M no significant differences were observed ($p > 0.05$). These results indicate that oxycodone requires a threshold concentration (≥ 500 μ M) for measurable Abcg2 ATPase activation. Using the Enzyme Kinetics software, the K_m and V_{max} values were estimated to be 1160 ± 442 μ M and 344 ± 53 pmol ATP/ μ g of Abcg2/min, respectively. The known Abcg2 substrate, mitoxantrone (500 μ M), stimulated the rate of ATP consumption by 283 ± 1.4 pmol ATP/ μ g of Abcg2/min, and enhanced the Abcg2 ATPase activity by 4.6-fold.

These results suggest that oxycodone is an Abcg2 substrate but with a very low affinity.

Brain Uptake of a Model Abcg2 (Bcrp) Substrate, Mitoxantrone, in Oxycodone- and Saline-Treated Rats. To determine the functional relevance of the microarray, Q-PCR, and Western blot data, we investigated whether the increase in Abcg2 expression observed in oxycodone-treated rats would translate into modulation of the brain uptake of mitoxantrone, the well known Abcg2 substrate (Cisternino et al., 2004). Both brain and plasma levels of mitoxantrone were evaluated in oxycodone- and saline-treated rats. Up-regulation of Abcg2 in the brain tissues of oxycodone-treated rats enhanced the brain efflux of mitoxantrone and significantly ($p < 0.05$) hindered its accumulation in the CNS. The brain/plasma levels of mitoxantrone were decreased significantly ($p < 0.05$) in oxycodone-treated rats by 1.99- and 2.32-fold after 30 and 60 min of mitoxantrone dosing, respectively (Fig. 5). These results indicate that the up-regulation of Abcg2 upon repeated oxycodone administration reduces the distribu-

FIG. 3. Effect of repeated oxycodone treatment on the protein expression of Abcg2 in the brain. A, representative immunodetection of Abcg2 expression (72 kDa) and β -actin expression (43 kDa) in membrane protein fractions isolated from brain tissues of oxycodone- and saline-treated rats. B, densitometric analysis of Abcg2 protein expression normalized to the β -actin control. Optical density was computed for each band according to the procedure outlined under *Materials and Methods*. Bars represent the mean $\pm$ S.D. ($n = 3$) of normalized band densities. **, significant difference at $p < 0.01$.

FIG. 4. Influence of different oxycodone concentrations and mitoxantrone (MX) on the rates of ATP consumption by recombinant rat Abcg2. Recombinant rat Abcg2 (25 μ g) samples were incubated with 20 μ l of Glo assay buffer (control, $n = 4$), 500 μ M mitoxantrone (positive control, $n = 4$), and 4 to 4000 μ M oxycodone ($n = 3$ /conc.). Data are expressed as the mean $\pm$ S.D. ANOVA followed by Dunnett's post hoc test (SigmaStat 2.03 statistical package) was used to determine the statistical significance of the difference between groups. **, significant difference from the nontreated group at $p < 0.01$.

tion of mitoxantrone in the brain. It can be contended that mitoxantrone metabolites may have contributed to the total radioactivity measured in the brain tissues. However, in vivo metabolism studies with mitoxantrone in rats indicated that mitoxantrone is metabolized mainly by hepatic and biliary pathways, and none of the metabolites was detected in the brain or any other tissue (Batra et al., 1986). Based on these studies it is believed that the radiolabeled mitoxantrone quantified in the brain represents the parent compound.

Discussion

We used microarray profiling and gene mapping analysis to identify target genes and biological processes that are regulated in brain tissues of rats repeatedly treated with oxycodone. The results of this study demonstrate that oxycodone indeed modulates gene expression in the brain and regulates many important biological processes in-

FIG. 5. Mitoxantrone (MX) brain/plasma ratio 30 and 60 min after a single intraperitoneal injection of 120 μ Ci/kg [3 H]mitoxantrone to Sprague-Dawley rats. Rats received pretreatment with either oxycodone (15 mg/kg) or saline (1 ml/kg) twice daily for 8 days. Data are expressed as the mean $\pm$ S.D. ($n = 4-5$). *, significant difference at $p < 0.05$ as determined by Student's t test using the SigmaStat 2.03 statistical package.

cluding drug metabolism, immune response, organic anion transport, antigen presentation via MHC class II molecule, the dopamine receptor signaling pathway, and the transmembrane receptor tyrosine kinase signaling pathway. We have observed in this study that many oxycodone-regulated genes have been reported previously to be modulated by morphine (e.g., *Fkbp5*, *Per2*, *Sgk*, *Mt1a*, *Dusp6*, *Gna*, *Tnfrsf*, *Hsp*, and MHC II molecules) (Loguinov et al., 2001; Ammon et al., 2003; Beagles et al., 2004; McClung et al., 2005; Stefano et al., 2005). This finding is not surprising because both oxycodone and morphine are μ -opioid receptor agonists (Beardsley et al., 2004).

FK506 binding protein 5 (*Fkbp5*) was one of the important up-regulated genes (≥ 2 -fold) (Table 2; Fig. 2). *Fkbp5* functions as a cochaperone of heat shock protein 90, regulates glucocorticoid receptor sensitivity (Sinars et al., 2003), and plays a significant role in immunoregulation and other basic cellular processes (e.g., protein folding and trafficking) (www.ncbi.nlm.nih.gov). More importantly, *Fkbp5* is believed to mediate the abuse potential of opioids and to be a key regulator of the development of opioid tolerance and dependence (Homayoun et al., 2003; McClung et al., 2005). Likewise, repeated morphine treatment in rats resulted in a comparable up-regulation (1.8-fold) of *Fkbp5* and inhibition of *Fkbp5* with tacrolimus attenuated the development of morphine dependence (McClung et al., 2005). Up-regulation of *Fkbp5* by repeated administration of both oxycodone and morphine supports the hypothesis that *Fkbp5* plays a role in modulating tolerance and dependence to opioids.

Periodic homolog 2 (*Per2*) was another gene up-regulated by oxycodone (~ 2 -fold) (Table 2; Fig. 2). *Per2* is widely expressed in the suprachiasmatic nucleus and is believed to be involved in regulating the circadian rhythm. *Per1* and *Per2* genes modulate cocaine sensitization as demonstrated by *Per1* and *Per2* knockout mice (Abarca et al., 2002). *Per2* was also up-regulated by 4.2-fold after repeated morphine treatment (Ammon et al., 2003). Up-regulation of *Per2* by oxycodone and morphine may be involved in modulating the sensitization phenomenon and the phase-shift of the circadian rhythm, the pattern that is commonly observed upon repeated opioid administration (Moriya et al., 2000; Nikaïdo et al., 2001; Ammon et al., 2003).

MetaCore analysis indicated that oxycodone administration significantly regulated the drug metabolism process ($p = 3.446 \times 10^{-5}$). This regulation is believed to be mediated through up-regulation of *Sult1a1* (Table 2; Fig. 2). *Sult1a1* is an important phase II metabolizing enzyme that is involved in the sulfation of many exogenous and endogenous compounds (e.g., drugs, neurotransmitters, carcinogens, and steroids) (Nagar et al., 2006). Figure 2 illustrates that up-regulation of *Sult1a1* is mediated by Sp1 through positive transcriptional regulation, a mechanism that was recently discovered using HepG2

cells (Hempel et al., 2004). Up-regulation of Sult1a1 by oxycodone may play a significant role in enhancing the metabolism of concomitantly administered drugs that undergo phase II sulfation.

Drugs of abuse such as morphine and heroin have been shown to modulate the immune response through suppression of T lymphocyte activity (involved in innate immunity) and B lymphocyte activity (involved in acquired immunity) (Beagles et al., 2004). In the present study, we suggest that oxycodone may also modulate the immune response. MetaCore indicated that repeated oxycodone administration regulated the antigen presentation process ($p = 7.433 \times 10^{-4}$) via regulation of the MHC class II molecules. These molecules play a significant role in regulating both acquired and innate immune responses. They are antigen-presenting molecules that activate T lymphocytes. This activation leads to self-proliferation in the case of the innate immune response or cytokine production and B-lymphocyte proliferation in the case of the acquired immune response. Down-regulation of *Rt1.D α* (*HLADRA*), the MHC class II molecule (Table 3; Fig. 2) by repeated oxycodone administration may affect the antigen presentation process, leading to a decrease in T lymphocyte activity and hence development of immunosuppression. Likewise, systemic morphine administration in Sprague-Dawley rats resulted in down-regulation of many MHC II molecules such as *Rt1.D β* , *Rt1.B α* , and *Rt1.B β* (Beagles et al., 2004). These results suggest that, increased susceptibility to infectious diseases among drug abusers and patients receiving opioids may be due to down-regulation of certain MHC II molecules.

Fifteen transporters were significantly regulated by oxycodone (Table 4). Two of these transporters, Abcg2 and Abcc4, are efflux transporters, whereas the rest are solute-linked carrier transporters (e.g., Slc16a1 and Slc22a8). MetaCore analysis indicated that oxycodone significantly regulated both the mevalonate transport ($p = 1.151 \times 10^{-5}$) and the organic anion transport ($p = 7.251 \times 10^{-4}$) processes through down-regulation of Slc16a1 (Table 4; Fig. 2). Slc16a1 belongs to the MCT family. Members of the MCT family are involved in lactate, pyruvate, mevalonate, and monocarboxylate transport. In addition, they regulate the intracellular pH by transporting protons along with the monocarboxylates. Maintaining optimum intracellular physiological pH is important for many vital processes (e.g., enzyme activity, metabolism, protein folding, and ligand binding). Down-regulation of Slc16a1 may result in reduction of intracellular pH, affect the aforementioned processes, and lead to cell death (Fang et al., 2006). Neurotoxicity studies indicated that repeated opioid administration resulted in scattered apoptotic neurons and astrocytes in rodents (Emeterio et al., 2006). Down-regulation of Slc16a1 may be one of the mechanisms by which apoptosis takes place.

The blood-brain-barrier (BBB) acts as a regulator of xenobiotics flux between the systemic circulation and the CNS. The permeability of drugs across the BBB has been traditionally considered to be a factor of molecular weight, protein binding, degree of ionization, and lipid solubility. However, many studies indicated that drug efflux transporters present in the brain may also play a role in regulating drug permeability across the BBB (Letrent et al., 1999; Lin, 2003). Drug efflux transporters such as P-glycoprotein (Abcb1), multidrug resistance-associated proteins (Abcc), and breast cancer-related proteins (Abcg2) are integral components of the BBB, they regulate drug disposition in the CNS, extrude drugs out of the brain, and mediate drug-drug interactions (Lin, 2003; Endres et al., 2006). We previously reported that oxycodone is a P-gp substrate that, upon repeated administration, induces P-gp protein levels by 4.0-, 2.0-, 1.6-, and 1.3-fold in liver, intestine, kidney, and brain tissues, respectively, which hinder the tissue distribution of paclitaxel (Hassan et al., 2007).

In this study, P-gp mRNA levels were not significantly changed ($p > 0.05$), suggesting that oxycodone mediates P-gp protein induction in the brain through a post-transcriptional mechanism. It is interesting to note that our microarray data indicated that oxycodone induced the expression of Abcg2, the half P-gp transporter (Table 2; Fig. 2). This observation was further confirmed by both Q-PCR and Western blot analysis (Table 5; Fig. 3). Although many opioids including oxycodone are P-gp substrates (Hassan et al., 2007, 2009a,b; Mercer et al., 2007; Cunningham et al., 2008), nothing is known about the Abcg2 substrate activity of oxycodone or any other opioid. As a result, we expanded our work to determine the Abcg2 substrate activity of oxycodone to 1) predict whether coadministered Abcg2 substrates or inhibitors can affect the pharmacokinetics (PK) and/or pharmacodynamics (PD) of oxycodone and 2) predict whether oxycodone can affect the PK/PD of the coadministered Abcg2 substrates (e.g., mitoxantrone). The assay demonstrated that oxycodone stimulated ATP consumption by Abcg2 only at very high concentrations ($\geq 500 \mu\text{M}$), suggesting that oxycodone is a very weak Abcg2 substrate (Fig. 4). In addition, oxycodone exhibited a very low affinity for Abcg2 ($K_m = 1160 \pm 442 \mu\text{M}$) as estimated by Enzyme Kinetics software. These results indicate that at physiologically relevant concentrations (110–908 nM) (Huang et al., 2005; Boström et al., 2006) Abcg2 is unlikely to affect oxycodone CNS permeability; however, in vivo studies are needed to validate this assumption.

To the best of our knowledge no studies have ever been conducted to determine Abcg2-mediated interactions in any opioid-treated rodents or humans. Therefore, we conducted a series of brain uptake studies using mitoxantrone (Fig. 5) to determine the physiological relevance of the up-regulated Abcg2 (Fig. 3). Mitoxantrone was selected for the following reasons: 1) mitoxantrone is a well known Abcg2 substrate (Imai et al., 2003; Wang et al., 2003; Zhang et al., 2004; Ejendal and Hrycyna, 2005), and induction of Abcg2 may have an impact on its brain distribution; and 2) oxycodone is concomitantly administered with mitoxantrone (antineoplastic agent) for alleviating pain in patients with cancer. There were significant ($p < 0.05$) decreases in brain/plasma ratios of mitoxantrone in oxycodone-treated rats. The levels were decreased by 1.99- and 2.32-fold after 30 and 60 min of mitoxantrone dosing, respectively (Fig. 5). These results are in full agreement with our microarray, Q-PCR, and Western blot data, which showed Abcg2 induction by (~ 2.1 -fold) and demonstrate that repeated oxycodone administration can indirectly enhance the brain efflux of mitoxantrone mainly through up-regulation of Abcg2. Down-regulation of the uptake solute-linked carrier transporters may have contributed to these results; however, this putative contribution, if any, is unlikely because mitoxantrone brain uptake was demonstrated to be mainly Abcg2-dependent (Cisternino et al., 2004) and the magnitudes of solute-linked carrier transporter down-regulation are relatively low (1.2- to 1.50-fold) in comparison with that of Abcg2 (~ 2 -fold).

In conclusion, we used microarray technology to identify genes that were significantly changed upon repeated oxycodone administration; then, we used these genes to generate novel functional gene networks. This combined approach elucidated significant biological processes modulated upon repeated oxycodone administration and provided insight into regulatory pathways that otherwise evade detection. Furthermore, our results indicated that oxycodone induced the expression of Abcg2 in a manner that enhanced the brain efflux of mitoxantrone. Extrapolation of these results to other Abcg2 substrates such as daunorubicin, doxorubicin, epirubicin, methotrexate, and rhodamine 123 (Mao and Unadkat, 2005) indicates that their brain uptake may be affected when they are administered concomitantly with oxycodone. Evaluation of the Abcg2 affinity status of other opioids

may be necessary to determine the influence of Abcg2 on the PK/PD of their concomitantly administered Abcg2 substrates. Taken together, oxycodone is one of the most extensively used yet understudied opioids, and the results reported herein provide additional information regarding its pharmacological activity and identify target genes for future studies. These studies can encompass distinct brain regions (e.g., hypothalamus, hippocampus, or striatum) to unveil the contribution of each regulated gene to the pharmacological action of oxycodone.

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