ORIGINAL ARTICLE

Neurotransmitter Gene Microarray Analysis in Human White Blood Cells and Human Stem Cells Following Morphine Exposure

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Abstract

Human white blood cells (WBC) and stem cells were treated with morphine and total RNA from these cells was analyzed using the Human Genome Survey microarray (Applied Biosystems). WBC nicotinic receptor gene expression, including variants, were down regulated. In general, of the 57 pre-selected neurotransmitter-related genes, only 11 changed significantly. Only 2 genes were up regulated, dopamine receptor 3 and cholinergic receptor, nicotinic, gamma polypeptide. The remaining 9 genes were down regulated, 5 in WBC and 4 in stem cells. The dopamine receptor D4 was the only gene that was commonly modulated in both cell types. It was down regulated by approx. 200% in stem cells and by 50% in WBC. The genes that exhibited the greatest extent of down regulation were 5-hydroxytryptamine receptor 3A in WBC and prepronociceptin in stem cells. Acetylcholinesterase, tyrosine hydroxylase, proenkephalin and dopamine receptor 3 were among the genes only found in WBC, whereas collagen-like tail subunit -single strand of homotrimer- of asymmetric acetylcholinesterase, dopamine receptor 1 and the serotonin transporter SLC6A4, were among those genes expressed only in stem cells. The genes for multiple sub-types of cholinergic receptors were lacking in stem cells. Thus, differential expression of genes was evident as well as specific morphine effects on gene expression, suggesting that morphine effects other neurotransmitter systems.

Introduction

Recently, it was demonstrated that normal human white blood cells (WBC) contain and have the ability to synthesize morphine and release it into the microenvironment (Zhu *et al* 2005a). Similar findings were reported in invertebrate ganglia (Zhu *et al* 2005b). Additionally, human stem cells were found to contain a opiate subtype receptor mu-3 like or mu-4, which only responds to morphine by releasing constitutive nitric oxide as previously described for mu-3 (Cadet *et al* 2003; 2007). Taken together, these studies provide evidence that the synthesis of morphine by various tissues and diverse animals is more widespread than previously thought and now includes human immune cells and potentially human stem cells.

Importantly, morphine biosynthesis uses elements of the catecholamine pathway as precursor molecules

Abbreviations:
white blood cells (WBC); L-3,4-dihydroxyphenylalanine (L-DOPA); α bungarotoxin (α-BuTx); multi-lineage progenitor cells (MLPC); cholinergic receptor, nicotinic, gamma polypeptide (CHRNG); acetylcholinesterase (ACHE); tyrosine hydroxylase (TH); proenkephalin (PENK); dopamine receptor 1 (DRD1); dopamine receptor 3 (DRD3); dopamine receptor D4 (DRD4); 5-hydroxytryptamine (serotonin) receptor 3A (HTR3A); prepronociceptin (PNOC); 5-hydroxytryptamine receptor 1D (HTR1D); collagen-like tail subunit -single strand of homotrimer- of asymmetric acetylcholinesterase (COLQ); polymorphonuclear cells (PMN); Catechol-O-methyl transferase (COMT);

in its synthesis, such as tyrosine, L-DOPA and dopamine, suggesting that their modulation is critical for endogenous morphinergic processes (Kream & Stefano 2006; Zhu *et al* 2005b; 2005c). Furthermore, cholinergic nicotinic processes has also been shown to be involved with endogenous morphine processes via novel nicotinic receptors, which can modulate cellular morphine release into its microenvironment (Zhu *et al* 2006b; 2006c; 2006d; 2006e; 2007; 2008). These reports show that both nicotine and epibatidine, a nicotine agonist, promoted evoked release of ^{125}I-trace labeled morphine that is selectively linked to activation of invertebrate nicotinic receptors based on pharmacological inhibition by α bungarotoxin (α-BuTx) not other antagonists In an even earlier report using mRNA microarray expression we also noted that chemical messenger processes were altered following acute morphine exposure (Stefano *et al* 2005). In the present report, we extend the examination of morphine effects to include human WBC and human stem cells given the parallels associated with morphine cellular influences.

Material and Methods

Heparinized whole blood, from individuals without pre-existing pathophysiological conditions, was obtained from the Long Island Blood Services (20 ml per treatment). The cells were allowed to recover in 75-cm^2 tissue culture at 37°C in RPMI-1640 media supplemented with 10% fetal bovine serum (Invitrogen) for 1 hour prior to incubation with 10^{-6}M morphine for 2 hours. Morphine sulfate was purchased from Sigma (St. Louis, Mo). Total cellular RNA was isolated using the Qiagen RNeasy mini kit (Qiagen, Stanford, CA, USA). Pelleted cells were resuspended in buffer RLT and homogenized by passing the lysate 5 times through a 20-gauge needle fitted to a syringe. The samples were then processed following the manufacturer's instructions. In the final step, the RNA was eluted with 50 µl of RNase-free water by centrifugation for 1 min at 10,000 rpm. Quality of the RNA was analyzed on a 1% agarose gel and the concentration was determined by an RNA/DNA spectrophotometer (Amersham). As described below, Applied Biosystems Human Genome Survey Arrays were used to analyze the transcriptional profiles of two leukocyte RNA samples (control and treated), following acute morphine exposure.

Frozen multi-lineage progenitor cell (MLPC) preparations were obtained from BioE, Inc., quickly thawed in a 37°C water bath and transferred to 75cm^2 tissue culture flasks containing 15 ml of Mesenchymal Stem Cell Growth Medium Bullet Kit (MSCGM, Lonza, PT-3001). MLPC were incubated overnight in a 5% CO_2 incubator at 37°C followed by a change of medium. At 40% confluency, cells were detached using 15 ml of PBS containing 0.1 % EGTA, pH 7.3. Detached MLPC were pelleted by centrifugation (500 x g for 7 minutes) and re-seeded.

Differentiation of MLPC into neural progenitor cells was accomplished in Neural Progenitor Maintenance Medium (NPMM) containing human recombinant basic fibroblast growth factor, human recombinant epidermal growth factor, neural survival factor-1 (Lonza, CC-3209) supplemented with fibroblast growth factor-4 (SIGMA), Glutamax I Supplement (Invitrogen) and penicillin and streptomycin (Invitrogen). After the development of neurospheres (10 days), the neurospheres were further differentiated into neurons by supplementing the NPMM media with 10 ng/ml brain-derived neurotrophic factor (SIGMA) and 10 ng/ml neurotrophin-3 (Sigma) for 21 days. Medium was changed every 3-4 days. Separate treatments of cells were differentiated as described above except that the media was supplemented with morphine at a final concentration of 1µM.

Human Genome Survey Arrays were also used to analyze the transcriptional profiles of neuronally differentiated and undifferentiated MLPC. Cells were washed with phosphate buffered saline solution detached from their flasks with a cell scraper and pelleted by centrifugation at 400g for 10 min. Total RNA was isolated with the RNeasy Protect Mini Kit (Qiagen, Valencia, CA). Pelleted cells were resuspended in 600 µL buffer RLT and homogenized by passing the lysate 20 times through a 1 mL pipette tip. The samples were then processed following the manufacturer's detailed instructions. In the final step, the RNA was eluted with 50 µl of RNase-free water by centrifugation for 1 min at 10,000 rpm. The RNA was analyzed on a model 2100 bioanalyzer (Agilent, Santa Clara, CA) using a total RNA nanochip according to the manufacturer's protocol. Digoxigenin-UTP labeled cRNA was generated and linearly amplified from 1 µg of total RNA using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit v 2.0 and manufacturer's protocol. Array hybridization, chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol. Two chips per sample each used 15 µg of labeled cRNA targets and were hybridized at 55°C for 19h.

Algorithm

AB1700 Expression System software was used to extract Assay Signal, and Assay Signal to Noise ratio values from the microarray images. Bad spots flagged by the software were removed from the analysis. For all genes scored, the fold change was calculated by dividing the morphine-treated value by the control assay value using the Spotfire Decisionsite software. If this number was less than one, the reciprocal (negative) was listed. A change was deemed significant and reported in the lists containing genes > 1.5-fold down (or up) based on the following criteria: the gene was scored, the fold change was more than 1.5-fold in at least one of the two independent experiments, and the change in the values was above background values in both comparisons. Hence, genes with fold change values ≤ 1.5 and ≥ -1.5 were removed from the selected list of differentially expressed genes. Further gene filtering was accomplished through the application of a portfolio list containing a selection of 57 neurotransmitter-related genes by the use of the Spotfire Decisionsite software.

Results

Human WBC isolated from whole blood were treated with morphine sulfate (1 μg/ml) for 2 hrs, and total RNA from these cells was analyzed using the Human Genome Survey microarray (Applied Biosystems). ***Figure 1*** demonstrates that WBC nicotinic receptor gene expression contains numerous variants, resulting in many being down (9 out of 10) regulated following acute morphine exposure compared to control experiments, which were normalized to 100%. The number of variants suggests that indeed novel nicotinic receptors may be present, while simultaneously demonstrating the significance of the cholinergic receptor expression in these cells, especially since the majority is subject to morphine modulation.

In general, of the 57 pre-selected neurotransmitter-related genes, only 11 changed significantly (***Tables 1-3***). Interestingly, only 2 genes were up regulated after morphine treatment as noted only in WBC. These up regulated genes were dopamine receptor 3 (DRD3), with a fold change of 2.0, and cholinergic receptor, nicotinic, gamma polypeptide (CHRNG) with a fold change of 1.6 (***Table 3***). The remaining 9 genes were down regulated, 5 in WBC and 4 in stem cells. The dopamine receptor D4 (DRD4) was the only gene that was commonly modulated in both cell types. It was down regulated by approx. 200% in stem cells and by 50% in WBC, with fold changes of 3.2 and 1.5 respectively (***Table 1***). The genes that exhibited the greatest extent of down regulation were 5-hydroxytryptamine (serotonin) receptor 3A (HTR3A) in WBC and prepronociceptin (PNOC) in stem cells, with negative fold changes of 15.4 and 8.4, respectively. Moreover, the former was the only serotonin receptor significantly modulated by morphine

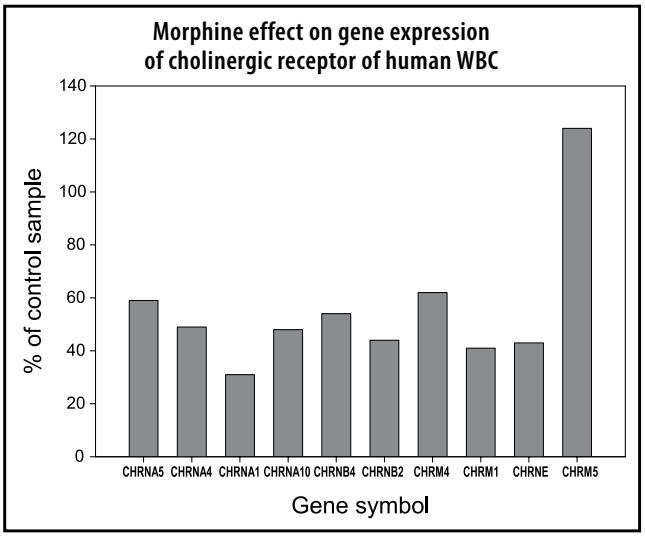

Figure 1.
CHRNA5 = cholinergic receptor, nicotinic, alpha polypeptide 5
CHRNA4 = cholinergic receptor, nicotinic, alpha polypeptide 4
CHRNA1 = cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)
CHRNA10 = cholinergic receptor, nicotinic, alpha polypeptide 10
CHRNB4 = cholinergic receptor, nicotinic, beta polypeptide 4
CHRNB2 = cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
CHRM4 = cholinergic receptor, muscarinic 4
CHRM1 = cholinergic receptor, muscarinic 1
CHRNE = cholinergic receptor, nicotinic, epsilon polypeptide
CHRM5 = cholinergic receptor, muscarinic 5

Table 1. Genes Down Regulated by Morphine Exposure

WBC

Gene Symbol	*Gene Name*	*Fold Change*
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	-15.4
CHRNE	cholinergic receptor, nicotinic, epsilon polypeptide	-2.7
DRD2	dopamine receptor D2	-1.7
DRD4	dopamine receptor D4	-1.5
CHRNB4	cholinergic receptor, nicotinic, beta polypeptide 4	-1.5

Stem Cells

Gene Symbol	*Gene Name*	*Fold Change*
PNOC	Prepronociceptin	-8.4
CHRM1	cholinergic receptor, muscarinic 1	-3.2
DRD4	dopamine receptor D4	-3.2
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D	-3.1

WBC= human white blood cells

Table 2. Function of Genes Down Regulated by Morphine Exposure

WBC

Gene Symbol	*Gene Name*	*Molecular Function*
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	serotonin-activated cation-selective channel activity
CHRNE	cholinergic receptor, nicotinic, epsilon polypeptide	cholinergic receptor, nicotinic, epsilon polypeptide
DRD2	dopamine receptor D2	dopamine receptor D2
DRD4	dopamine receptor D4	dopamine receptor D4
CHRNB4	cholinergic receptor, nicotinic, beta polypeptide 4	cholinergic receptor, nicotinic, beta polypeptide 4

Stem Cells

Gene Symbol	*Gene Name*	*Molecular Function*
CHRM1	cholinergic receptor, muscarinic 1	muscarinic acetylcholine receptor activity; phosphoinositide phospholipase C activity
DRD4	dopamine receptor D4	dopamine receptor activity
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D	serotonin receptor activity
PNOC	Prepronociceptin	opioid peptide activity

WBC= human white blood cells

Table 3. Genes Up Regulated by Acute Morphine Exposure

WBC

Gene Symbol	*Gene Name*	*Fold Change*
CHRNG	cholinergic receptor, nicotinic, gamma polypeptide	1.6
DRD3	dopamine receptor D3	2.0

WBC= human white blood cells

Table 4. Genes found in WBC but not in Stem Cells

Gene Symbol	*Gene Name*
ACE	acetylcholinesterase (YT blood group)
CHRNA1	cholinergic receptor, nicotinic, alpha polypeptide 1 (muscle)
CHRNA10	cholinergic receptor, nicotinic, alpha polypeptide 10
CHRNA4	cholinergic receptor, nicotinic, alpha polypeptide 4
CHRNA5	cholinergic receptor, nicotinic, alpha polypeptide 5
CHRNB2	cholinergic receptor, nicotinic, beta polypeptide 2 (neuronal)
CHRND	cholinergic receptor, nicotinic, delta polypeptide
CHRNE	cholinergic receptor, nicotinic, epsilon polypeptide
CHRNG	cholinergic receptor, nicotinic, gamma polypeptide
DRD3	dopamine receptor D3
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A
HTR4	5-hydroxytryptamine (serotonin) receptor 4
PENK	Proenkephalin
SLC5A7	solute carrier family 5 (choline transporter), member 7
SLC6A3	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
TH	tyrosine hydroxylase

WBC= human white blood cells

Table 5. Genes found in Stem Cells but not in WBC

Gene Symbol	*Gene Name*
CHRNB3	cholinergic receptor, nicotinic, beta polypeptide 3
COLQ	collagen-like tail subunit (single strand of homotrimer) of asymmetric acetylcholinesterase
DRD1	dopamine receptor D1
DRD1IP	dopamine receptor D1 interacting protein
HTR1B	5-hydroxytryptamine (serotonin) receptor 1B
SLC18A3	solute carrier family 18 (vesicular acetylcholine), member 3
SLC6A4	solute carrier family 6 (neurotransmitter transporter, serotonin), member 4

WBC= human white blood cells

in WBC, whereas HTR1D (i.e., 5-hydroxytryptamine receptor 1D) was the only serotonin receptor significantly modulated in stem cells, with a negative fold change of 3.1.

Among the neurotransmitter-related genes expressed but not significantly modulated by morphine treatment, there were some noticeable differential expressions between WBC and stem cells. For example, acetylcholinesterase (ACHE), tyrosine hydroxylase (TH), proenkephalin (PENK) and dopamine receptor 3 (DRD3) were among the genes only found in WBC, whereas COLQ (collagen-like tail subunit -single strand of homotrimer- of asymmetric acetylcholinesterase), dopamine receptor 1 (DRD1) and the serotonin transporter SLC6A4, were among those genes expressed in stem cells but not in WBC (***Tables 4, 5***).

It is also of interest to note that many of the genes detected in WBC were not present in the stem cells.

While both cell types had dopamine receptors they did not share the same sub-types (***Table 4-5***). The genes for multiple sub-types of cholinergic receptors were lacking in stem cells as was TH (***Table 5***).

Discussion

Overall, our results show that morphine has a predominantly down regulatory effect on catecholamine-related genes as well as others in both, stem cells and in WBC. This includes cholinergic, dopaminergic and serotonergic receptors, interacting elements and transporters. In this regard, Figure 1 further shows this opiate alkaloid down regulatory effect on both, muscarinic and nicotinic cholinergic receptors in WBC. This is in agreement with a study by Zhu and colleagues (2007) where similar down regulatory effects were demonstrated in isolated polymorphonuclear cells (PMN), where one of the cholinergic genes, CHRNB4, was further validated by *Taqman* assay. In this study, PMN contained all of the cholinergic receptors expressed in WBC, except for CHRNE and CHRM4 (Zhu *et al* 2007).

These results also strongly suggest that these neurotransmitter/neuroimmune signaling chemical messengers exhibit relationships indicative of autocrine and paracrine signaling (Kream *et al* 2007b). Endogenous morphine is synthesized via established and predictable components of the catecholamine pathway (Zhu *et al* 2005a; 2005b). Its synthesis and release results in nitric oxide release via diverse tissue sources, which serves as a negative feedback modulator, effecting key enzymes in the pathway, such as Catechol-O-methyl transferase (COMT), TH and CYP2D6 (Atmanene *et al* 2009; Ghelardini *et al* 2008; Kream *et al* 2007a; 2007b; Kream & Stefano 2008; Mantione *et al* 2008; Neri *et al* 2008; Stefano *et al* 2007; 2008; 2009; Stefano & Kream 2007; 2008; 2009; Zhu *et al* 2006a; 2006b; 2006c; 2006e; 2007; 2008) . These reports also note the interaction of nicotinic, cocaine and alcohol related processes coupling with endogenous morphine processes.

Interestingly, early on a relationship was found with dopamine, a morphine precursor, and serotonin (Hiripi & Stefano 1980; Stefano *et al* 1976; 1981). It appears that gene expression microarray has captured these relationships, which have been validated over the years in diverse animals and tissues.

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