Ligand Bias on GPCR Signaling Pathways in μ Opioid Receptors

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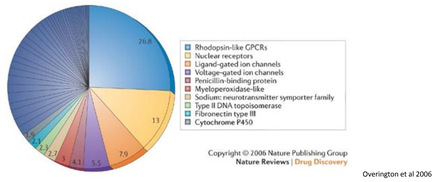
Introduction

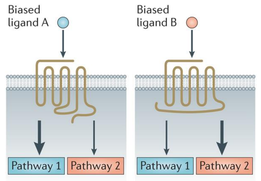
G-protein coupled receptors (GPCR) currently provide a widely used drug target, despite incomplete understanding of their mechanisms of action.



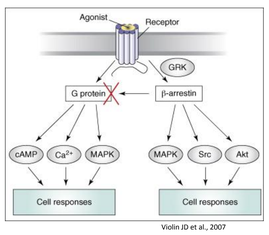
Overington et al 2006

In the clinic, opioids are used to relieve pain, despite highly addictive and detrimental systemic effects. Opioids bind to opioid receptors, a GPCR sub-group widely distributed in the central nervous system (CNS).

An intriguing feature of GPCRs is their 'ligand-biased signaling': when GPCRs bind with different ligands, they may activate different downstream signaling pathways, resulting in different physiological consequences.



Nature Reviews | Drug Discovery
Kenakin T et al., 2013



Violin JD et al., 2007

μ opioid receptors, of interest for their role in opioid-related behaviors, exhibit a high affinity for binding with natural peptides, such as beta-endorphins, as well as opiates, such as morphine and fentanyl.

However, as compared with G-protein and ß-arrestin pathways, the downstream pathways of μ opioid receptors, are less well studied.

Mutations in μ opioid receptors pose another set of factors to consider. These mutations may lead to further differentiation in downstream μ opioid signaling pathways.

Of particular concern here, the influence of μ opioid receptor polymorphisms on signaling pathways is also not well known.

The present study thus seeks to establish a dataset of GPCR signaling pathways for μ receptor polymorphisms under the binding of different agonists.

Materials

Cell Line

CHO: Chinese Hamster Ovary
HEK-293: Human embryonic kidney cells
SK-N-MC: Human neuroblastoma cells.

Receptor

Human Origin:
A118:17C, G118:17C, A118:17T

Rhesus Origin:
C77, G77

Human	A118:17C
Human	G118:17C
Human	A118:17T
Non-Human Primates	C77
Non-Human Primates	G77

Agonists:

Morphine, Fentanyl, B-endorphin, DAMGO

Step 1: Identifying the GPCR signaling pathways in μ receptors

Methods

GPCR Signaling 10-pathway Reporter Array(QIAGEN)

10 commonly existing GPCR pathways were measured from the reporter array, each containing a unique transcriptional factor inducible firefly luciferase reporter.

These firefly luciferase signals are expressed only under the specific pathway transcription factor.

Tube	Pathway	Transcription Factor
1	ATF2/ATF3/ATF4	ATF2/ATF3/ATF4
2	cAMP/PKA	CREB
3	MAPK/ERK	ELK1/SRF
4	MAPK/JNK	FOS/JUN
5	MEF2	MEF2
6	Hedgehog	GLI
7	PI3K/AKT	FOXO
8	IL-6	STAT3
9	PKC/Ca++	NFAT
10	NFkB	NFkB
11	Negative Control	
12	Positive Control	

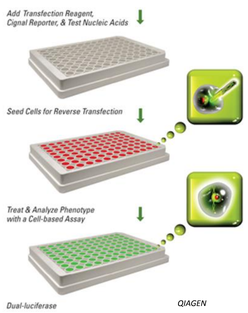
QIAGEN

We then transfected the 10 pathway arrays with CHO cells.

After transfection, we applied high dose agonists at 10μM to each well.

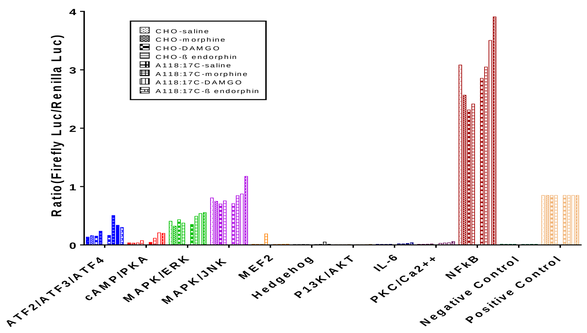
After incubating the agonists, we lysated the cells and measured the luciferase activities through a PerkinElmer Victor X5 Light Plate Reader system.

The luciferase signal ratios of Firefly/Renilla were then calculated for each group.



Data

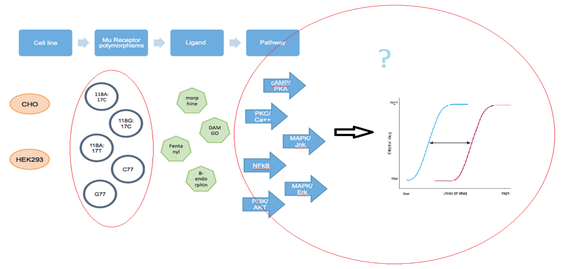
- **Five Pathways were notably expressed in A118:17C μ receptors.**



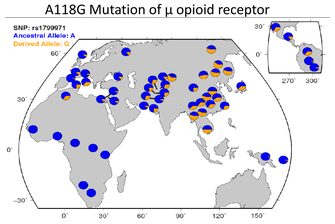
Identify the GPCR signaling pathways in Mu opioid receptors. *The x-axis are 10 GPCR signaling pathways, negative control and positive control. For each pathway/control, there are eight bars represent eight different groups in order as indicated in legend. The y-axis indicates the ratio of Firefly luciferase/Renilla luciferase.*

- **These five pathways were: ATF/2/3/4, cAMP/PKA, MAPK/ERK, MAPK/JNK and NFkB. In these pathways, signaling was higher when the agonists were binding with μ .**

Step 2: Detecting the effects of μ polymorphisms on the ligand bias.



A118G Mutation of μ opioid receptor



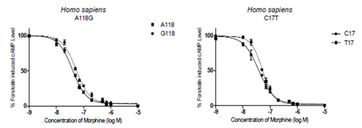
A118G and C17T are two common μ polymorphisms that induce the structural change of the receptor protein, which may alter the binding affinity of the ligands.

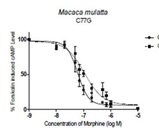
The mutations occurred across the populations, to understand GPCR signaling mutation might help to understand the changes in functional differences of μ receptors.

Previous studies have shown a shifting of dose response curves in the HEK cell line.

Here we further measured cAMP and four other pathways in CHO and other cell lines.

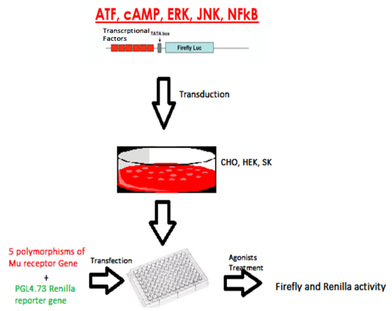
cAMP signaling in A118G and C17T polymorphisms in HEK cell line





Methods

Pathway transcriptional factor responsive luciferase reporter genes were stably transduced into our cell lines.



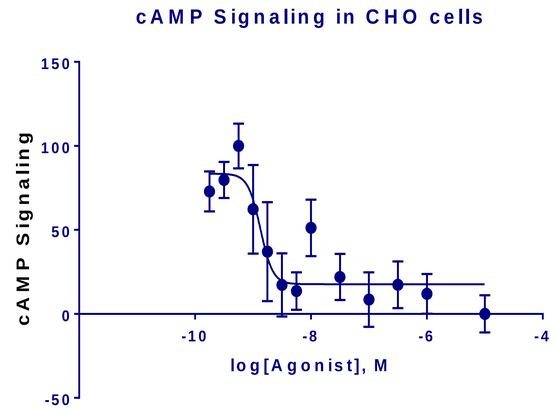
The cells were next incubated in a 96-well plate overnight, and then co-transfected with the μ-receptor gene and PGL4.73.

Concentrations of the agonists, ranging from 10^{-4} to 10^{-12} mol, will be added to the wells. (Treat the cAMP pathway with 10nM forskolin at the same time.)

After incubation, we lysated the cells, measured the Firefly and Renilla luciferase signals, and calculated their ratio.

Data

- **cAMP signaling pathway response to morphine.**



- **The graph above shows the dose response curve for cAMP that has now been created for the μA118:17C polymorphism. The cAMP in this receptor was Gi-linked. EC50 was approximately 10.5^{-8}.**
- **We will now repeat the experiment so as to measure other doses between the plateaus shown in this figure.**

Future Studies

- We will complete the tests described above with all other identified pathways in CHO and the other two cell lines with other μ polymorphisms so as to observe changes in the dose-response curves.
- In vivo studies of pathway functions will be conducted.
- The effects of dimerization of μ heterodimers on the ligand bias will be investigated.

Contributions

- Our studies will further understanding of the cellular and genetic mechanisms by which opioids initiate their physiological consequences
- This work will also provide important information with which to understand evolutionary differences in μ receptors that exist between human and non-human primates, of use in assessing experimental models of addiction.

Acknowledgement

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