Psychiatry CEDD

Act One:
A Novel Real-time cAMP Assay using the FLIPR

Nisha Patel

Elena Ratti

Kate Cato

Jennie Heath

Martyn Wood

Christopher Langmead

Psychiatry CEDD

Psychiatry Centre of Excellence for Drug Discovery

"We will provide direction and light
to minds lost in the shadow
of Psychiatric illnesses"

gsk GlaxoSmithKline

Introduction

- Changes in the intracellular levels of cAMP is an important second messenger system

- Key for G protein coupled receptors which either stimulate adenylyl cyclase activity through G_s or which inhibit adenylyl cyclase through G_i/G_o

- Current assay technologies to measure changes in cAMP levels are end-point assays which usually involve lysing cells, sometimes an extraction step, followed by lengthy incubation and antibody detection

Introduction cont.

- FLIPR has become the screening platform of choice for many assays allowing real-time measurements in live cells

- However, for GPCRs this is limited to those which change intracellular Ca^{++} levels

- Although chimeric G proteins have been widely used to circumvent this restriction, there is always concern that this is an artificial coupling and may affect the pharmacological profile

Introduction cont.

- At a previous Molecular Devices users meeting, a novel method for measuring real-time changes in cAMP levels on the FLIPR was presented

- This is the ACT:One from ATTO Pharmaceuticals (http://www.atto.com/)

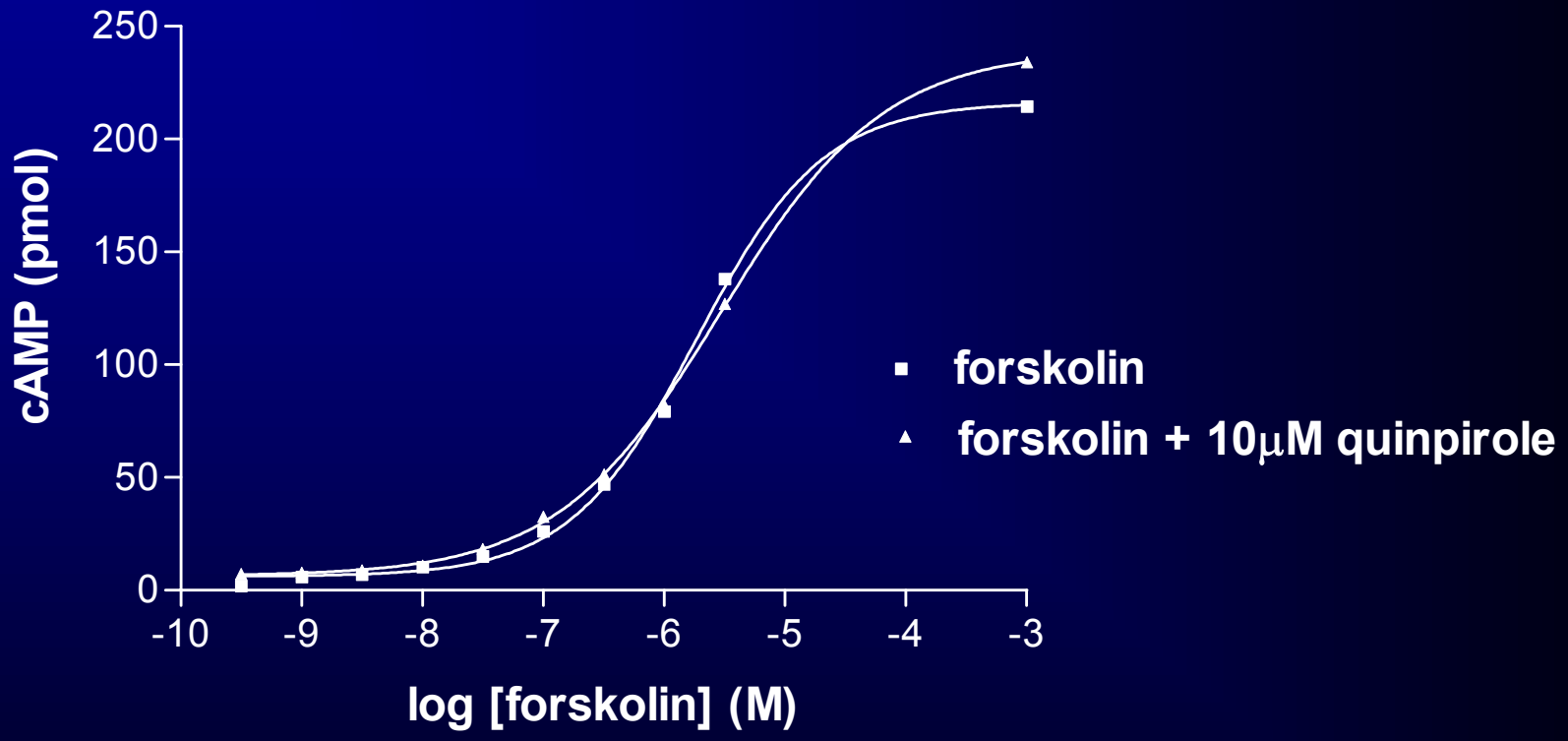
- We have now evaluated this technology and studied the pharmacological profile of the G_i coupled human dopamine D_2 receptor and evaluated endogenous and transfected G_s receptor coupled responses.

Introduction cont.

- cAMP assays
  - need to be sensitive over the physiological range of intracellular cAMP (basal level tends to be around 10 nM)
  - need to amplify signal as changes in intracellular cAMP tend to be small (may rise to 30-40 nM)
  - many assay formats suffer from compound interference

- ACT:One biosensor is sensitive to physiological levels and changes in cAMP
- Molecular Devices membrane potential dye as a reporting system

Dopamine D_2 - FlashPlate cAMP



- [^{125}I]cAMP RIA
- No shift of forskolin curve seen with quinpirole

Cell lines from Atto Bioscience

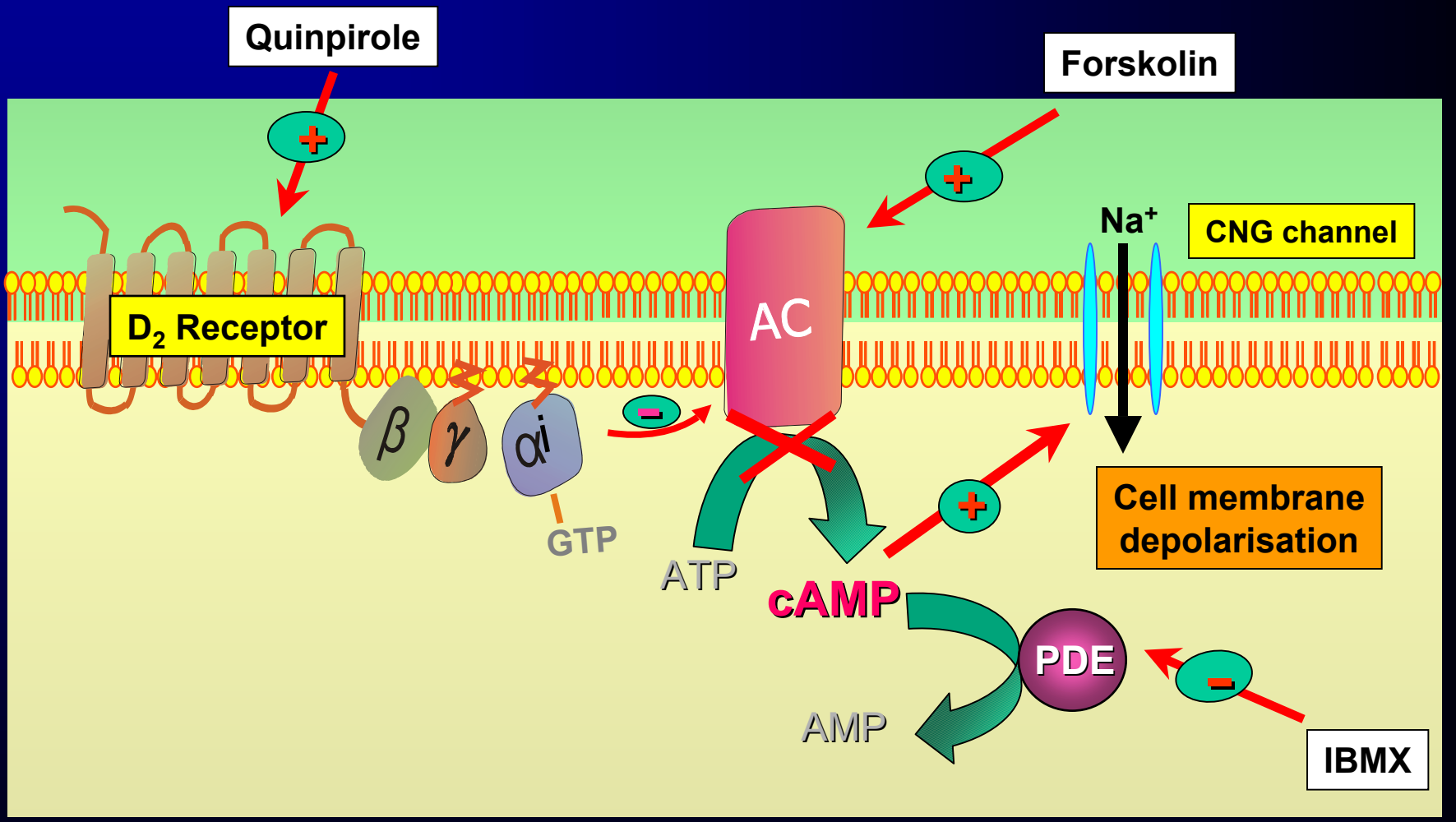
HEK_293_ASC0012

- Wild-type cell line stably expressing a cyclic nucleotide gated ion channel

HEK_293_ASC0083

- Stable cell line expressing both the dopamine D_2 receptor and a cyclic nucleotide gated ion channel

ACT:One cAMP Assay



Assay Optimisation

- Cell densities
  - 50K per well (96-well plate) - cell density appears critical
- Assay and reagent volumes
  - Need minimise reagent addition volumes and use slow settings to minimise dilution artefacts - 25 μl over 2 s
- Membrane potential dye
  - Reducing dye concentration significantly reduces signal; use recommended Molecular Devices dye concentration.
  - No response seen with Fluo-4
- Incubation and read times
  - 5 min forskolin, then 5 min quinpirole (for D_2)
  - Agonist response stable for 5-15 min

Assay Method

Cells seeded (50K) on 96-well poly-d-lysine coated plates
24h prior to assay

↓

Cells loaded for 2hrs @ 37^0C
with membrane potential dye
containing dye load buffer (Ca^{2+} free - HEPES/PBS)

↓

Antagonist/forskolin addition on FLIPR - 5 min
(Drugs diluted in dye load buffer + EGTA)

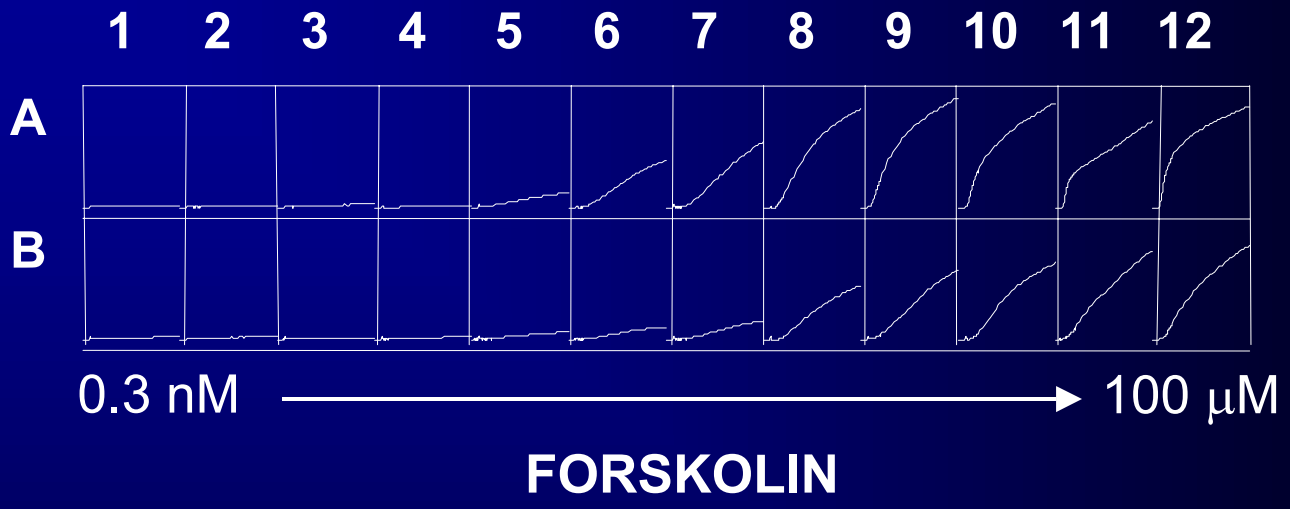
↓

Addition of agonist on FLIPR - 5 min
(Drugs diluted in dye load buffer + EGTA)

↓

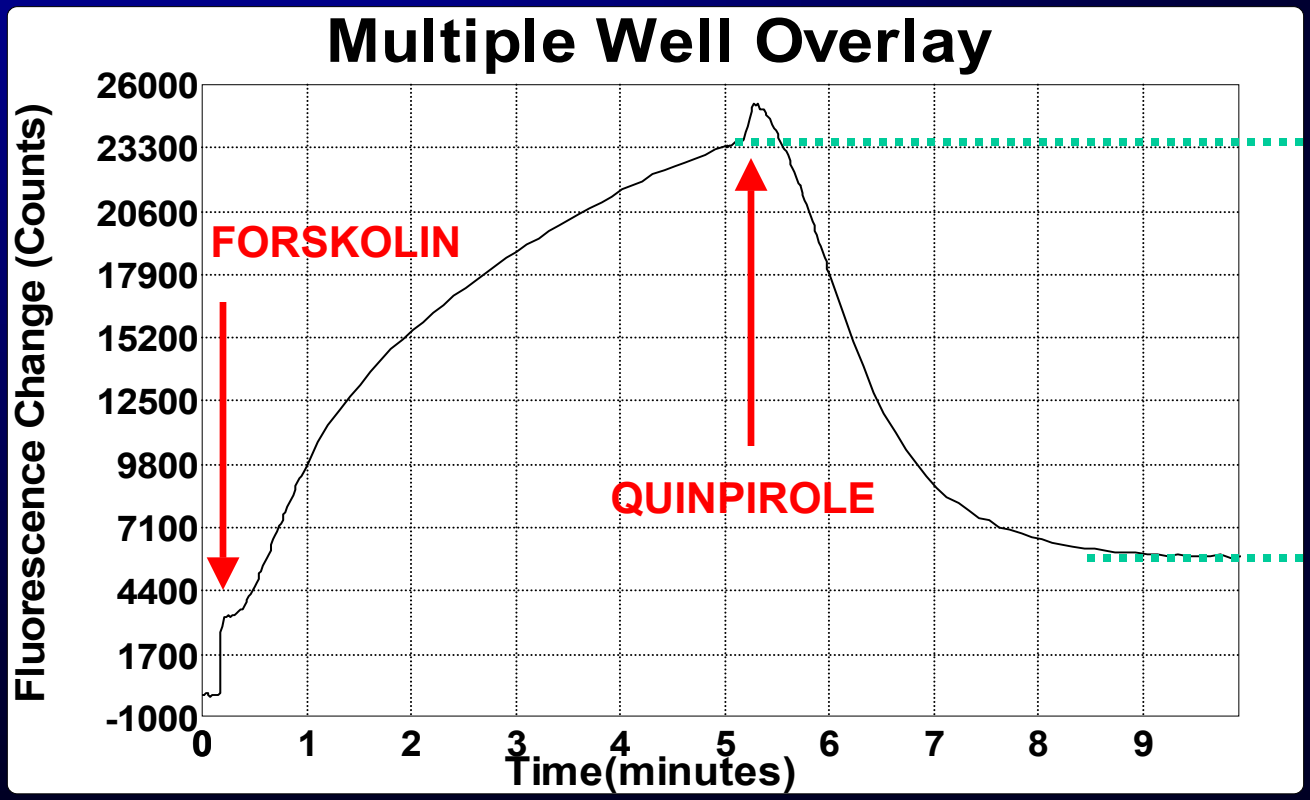
Read on FLIPR

Stimulation of adenylyl cyclase

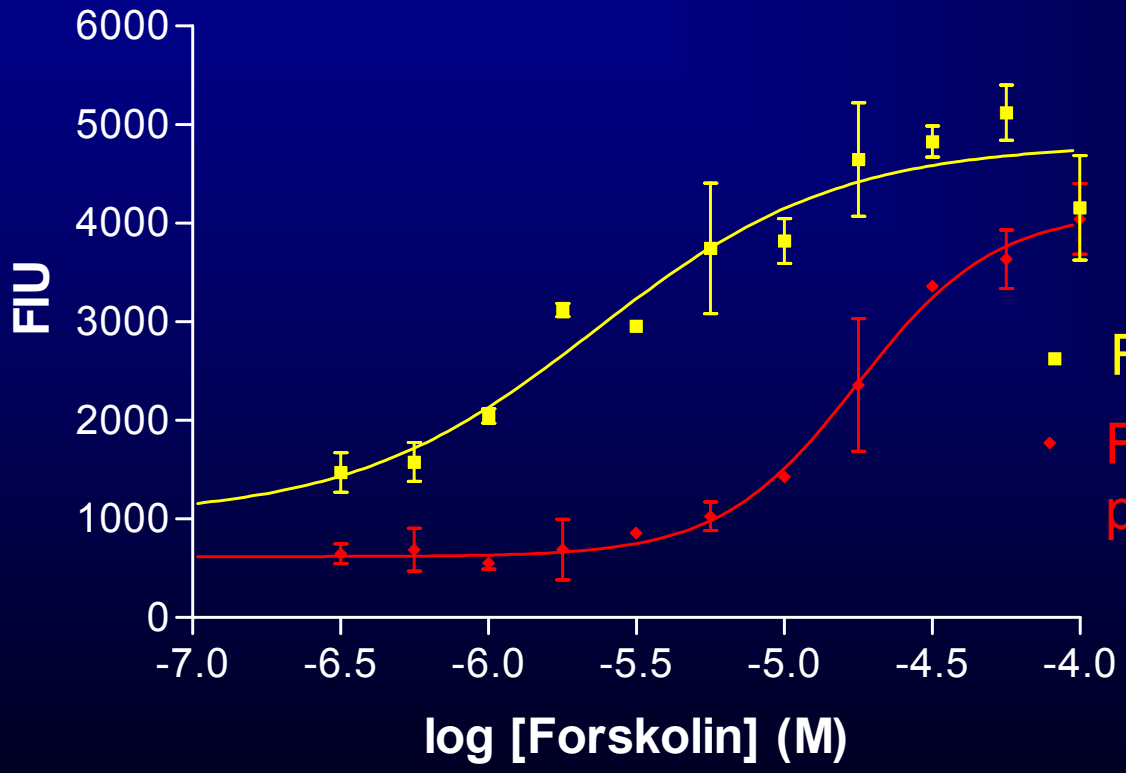


- Forskolin gives a concentration-dependent change in membrane potential
  - pEC_{50} = 4.4 in absence of IBMX
  - pEC_{50} = 6.4 in presence of IBMX
- IBMX potentiates a stimulation of cAMP levels but this appears to mask any inhibition via G_i pathway
  - IBMX was not used for subsequent D_2 studies

Inhibition of forskolin stimulated cAMP levels by quinpirole

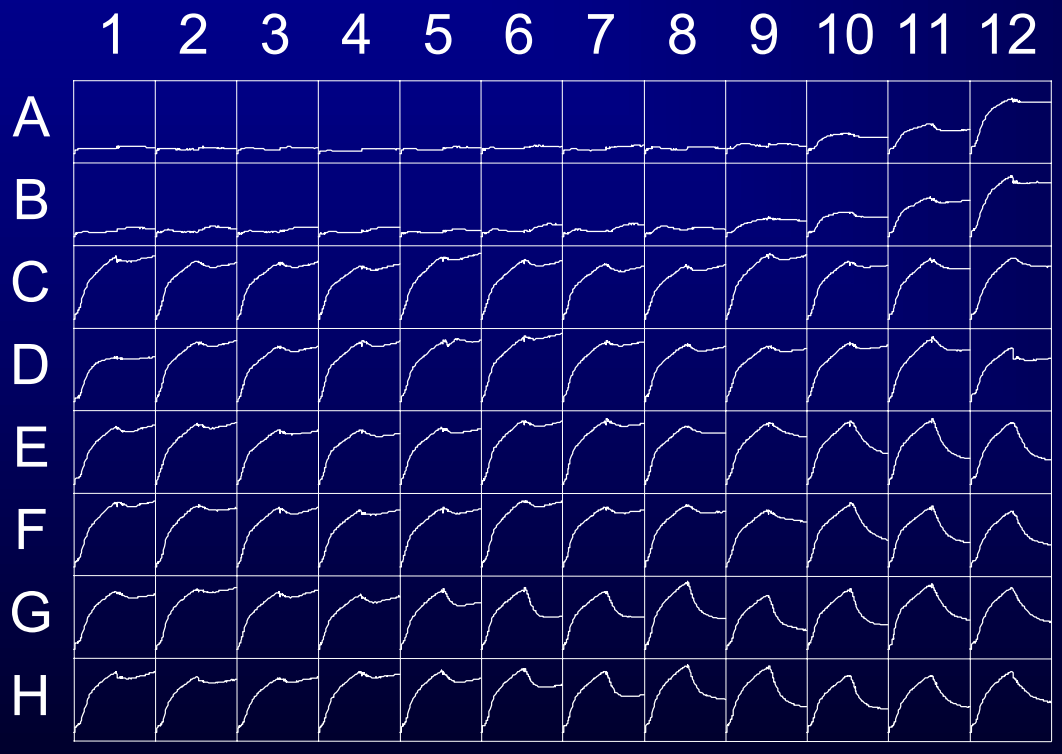


Inhibition of forskolin stimulated cAMP levels by quinpirole



- Forskolin, pEC_{50} = 5.6
- Forskolin + 100nM Quinpirole, pEC_{50} = 4.8

Inhibition of forskolin stimulated cAMP levels by D_2 agonists



Forskolin, 0.3nM - 100μM

Forskolin (30μM) + Aripiprazole (30pM - 10μM)

Forskolin (30μM) + Bromocriptine (30pM - 10μM)

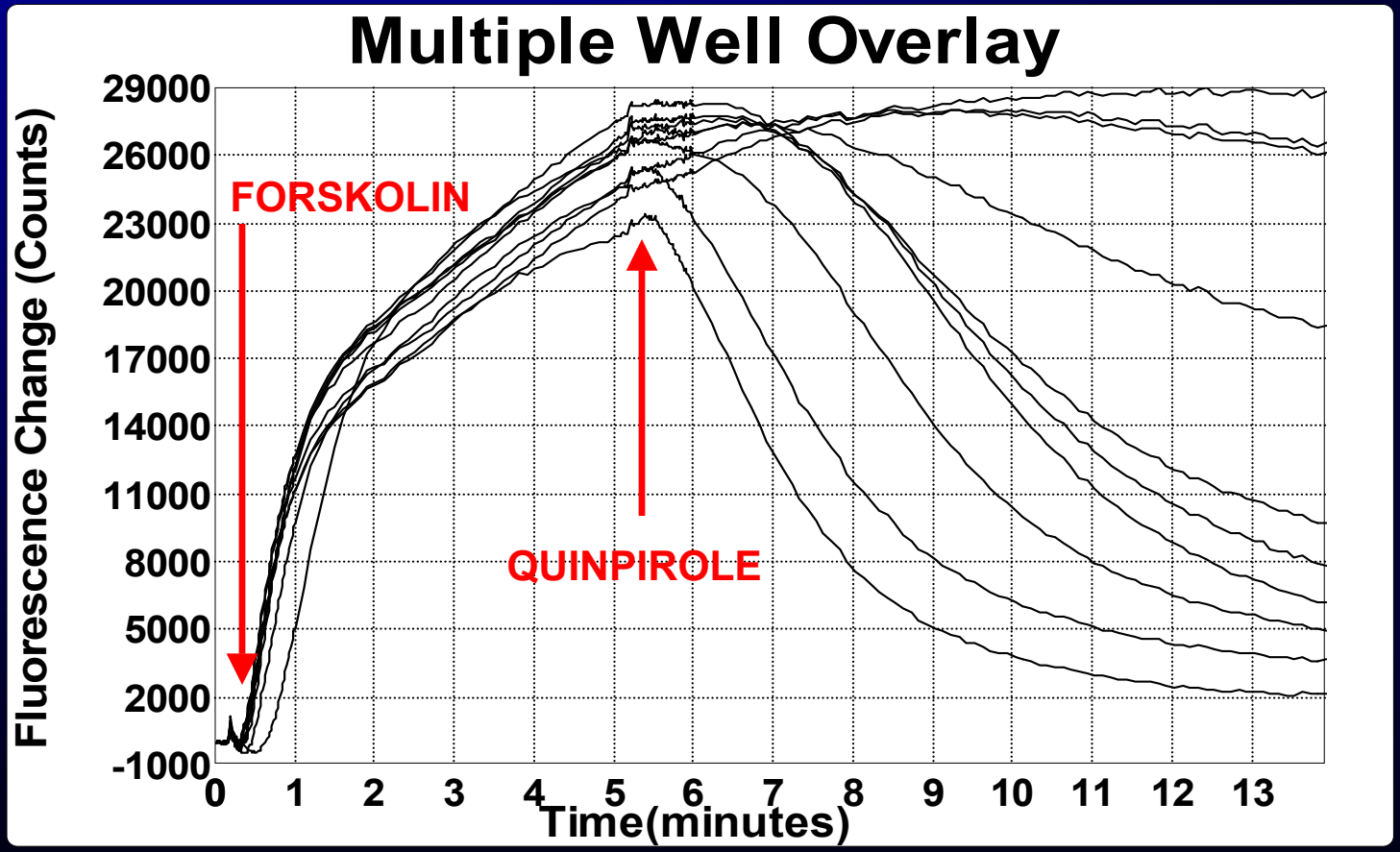
Forskolin (30μM) + Dopamine (30pM - 10μM)

Mini Graphs

Inhibition of forskolin stimulated cAMP levels by D_2 agonists

	cAMP			[^{35}S]GTPγS	
	pEC_{50}	sem	n	pEC_{50}	sem
Quinpirole	8.18	0.08	3	8.09	0.05
Dopamine	8.07	0.13	6	8.04	0.06
Bromocriptine	6.38	0.05	6	6.97	0.07
Forskolin	4.92	0.07	3	-	-

Reversal of quinpirole stimulated inhibition of cAMP levels by D_2 antagonists



HALOPERIDOL

1μM

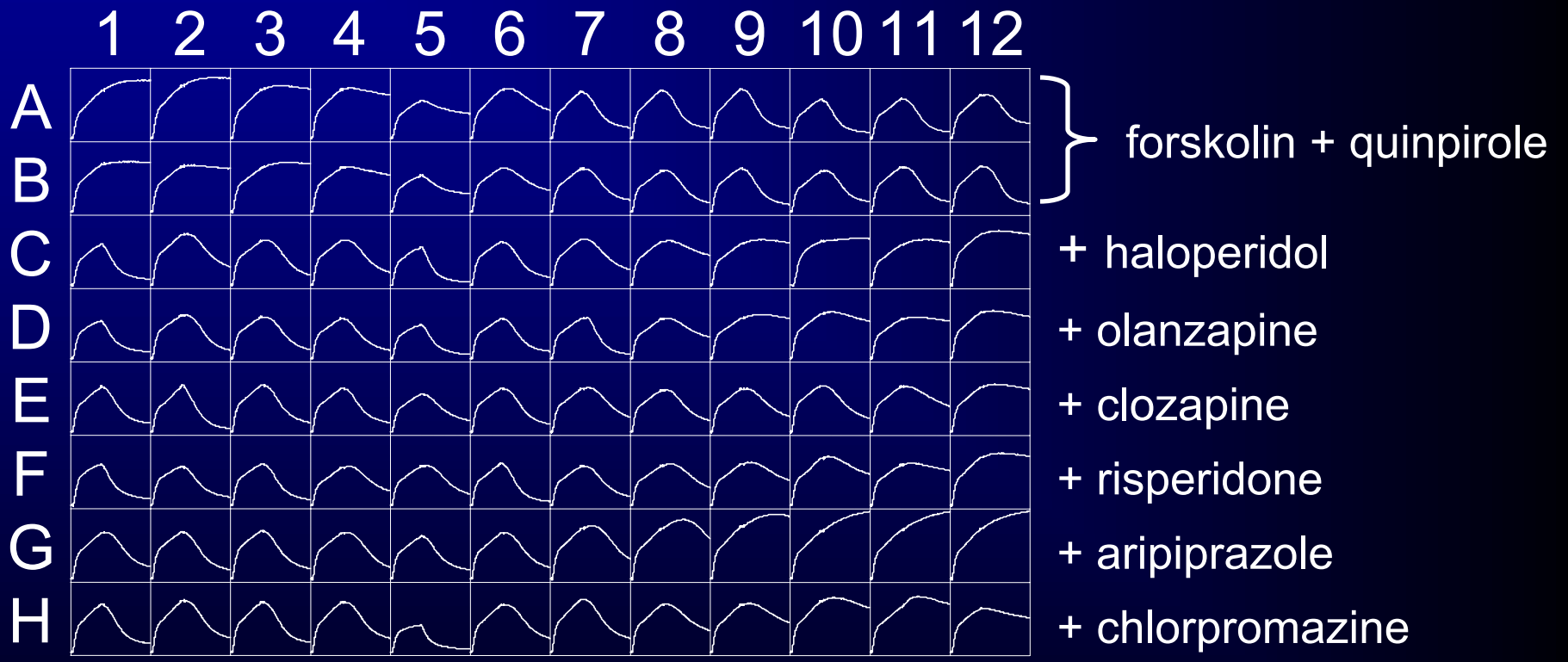
100nM

10nM

1nM

0.1nM

Reversal of quinpirole stimulated inhibition of cAMP levels by D_2 antagonists

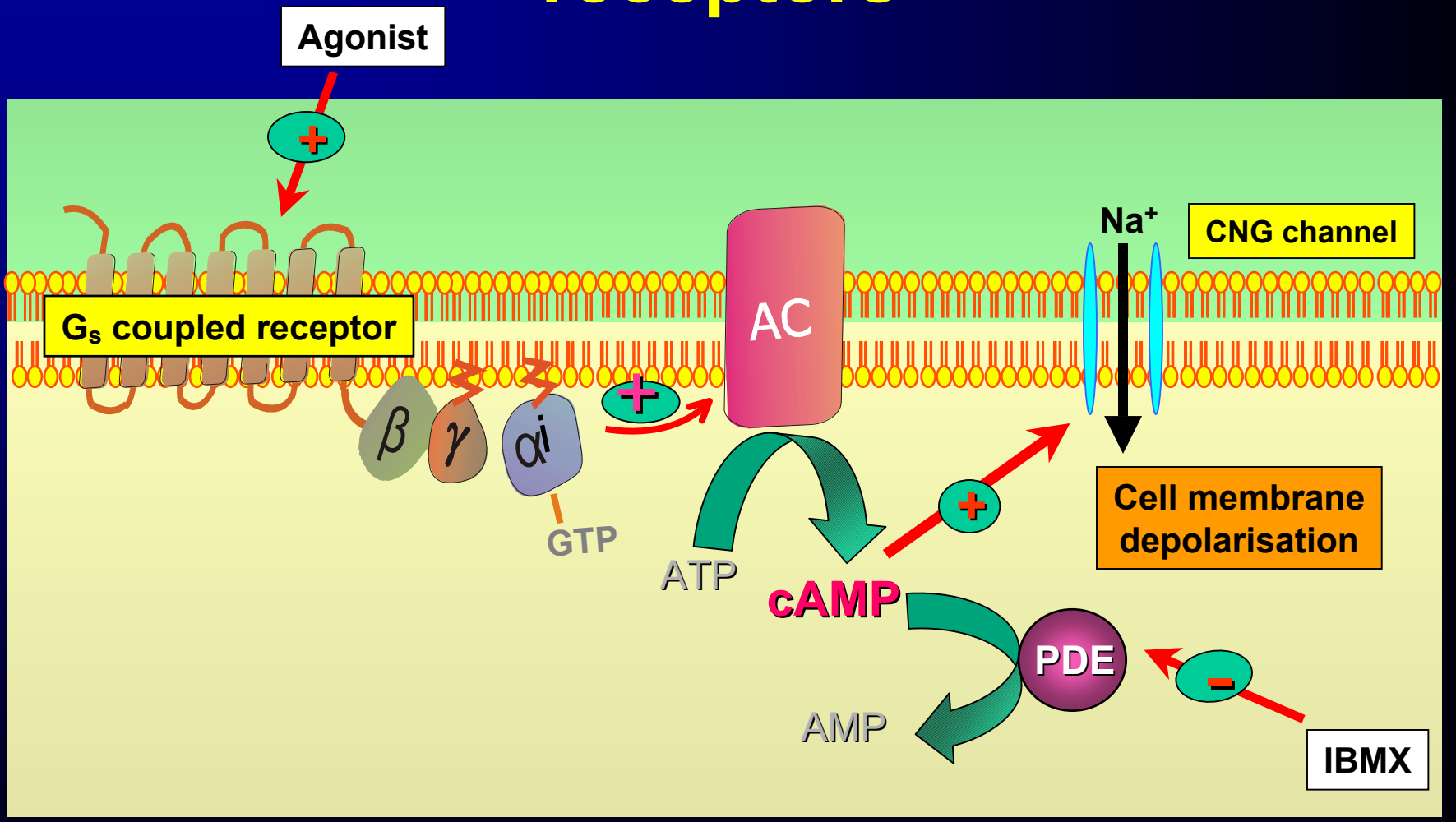


D_2 antagonist data - summary

	mean	sem	n	pK_i
Forskolin	4.63	0.02	4	
Quinpirole	7.90	0.14	4	
Haloperidol	8.13	0.18	3	8.8
Olanzapine	7.73	0.05	3	7.9
Clozapine 4	6.60	0.14	4	7.0
Risperidone	7.44	0.16	3	8.2
Aripiprazole	8.37	0.11	3	7.8
Chloropromazine	7.63	0.06	3	8.2

No partial agonist activity seen with aripiprazole

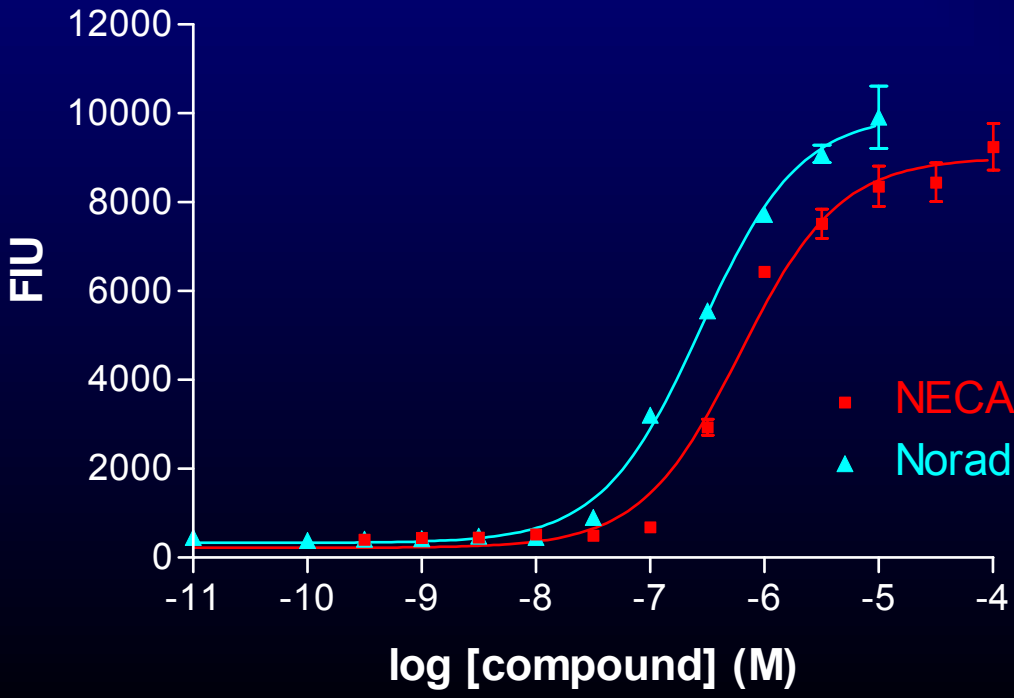
cAMP studies with G_s coupled receptors

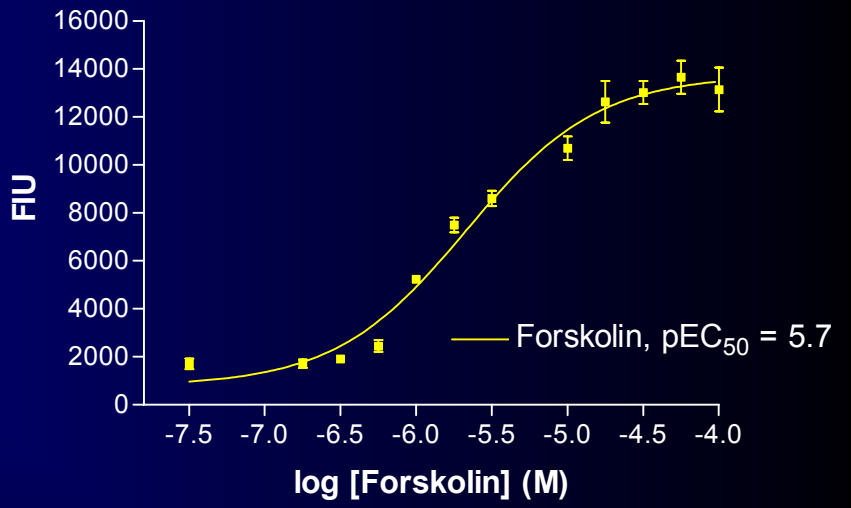


Measurement of G_s coupled receptor stimulated cAMP production

Stimulation of cAMP by endogenous Gs coupled receptors in HEK293 cells

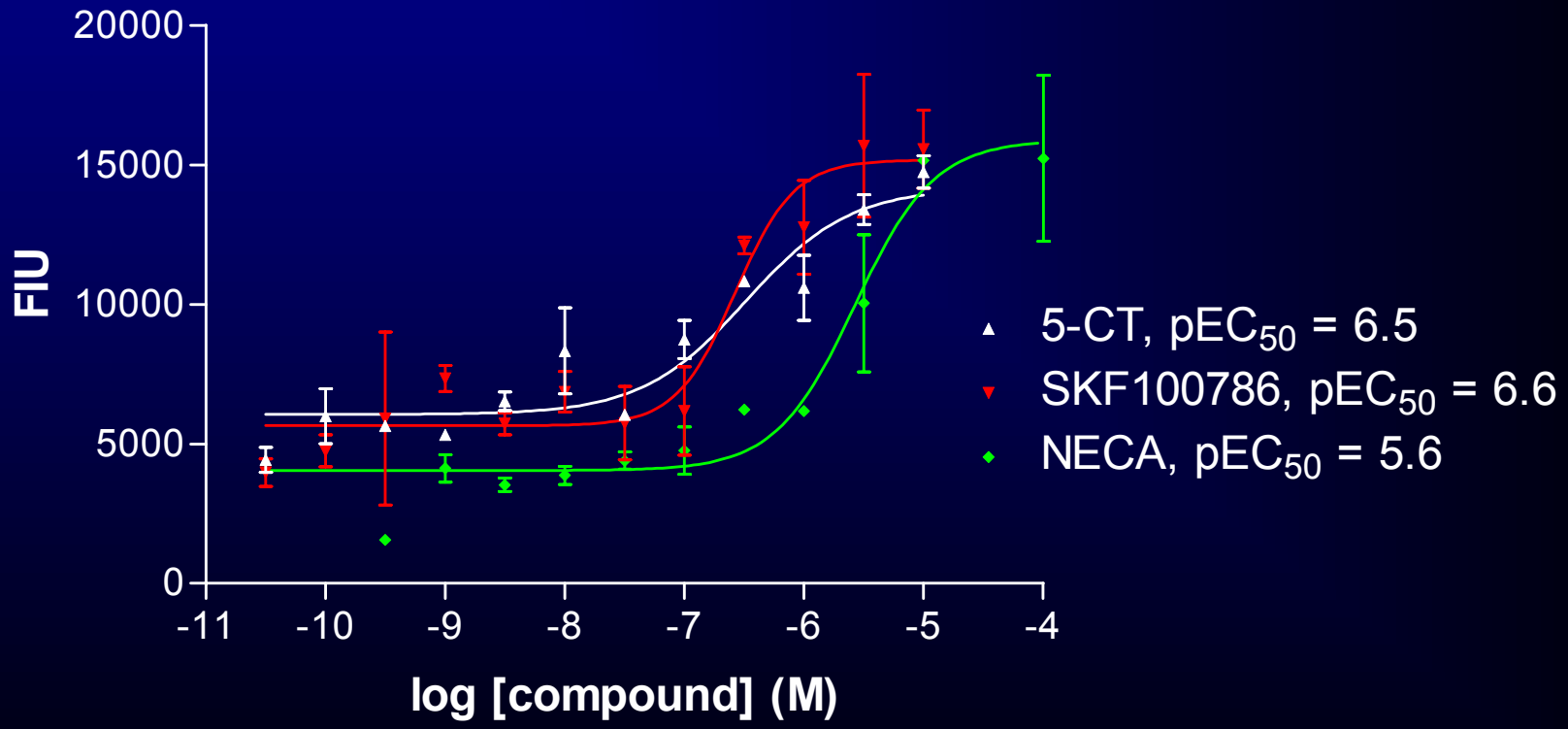
- Adenosine A2b
- β_1 adrenoceptor





Measurement of 5-HT_6 receptor stimulated cAMP production

- Stimulation of cAMP by transiently (Lipofectamine) expressed 5-HT_6 receptor by 5-CT and SKF100786
- Non detergent transfection protocols may give more robust pharmacology



SKF100786 = [2-(5-methoxy-2-methyl-1*H*-indol-3-yl)-ethyl]-dimethyl-amine

Summary

ACT One: Technology

- Ability to measure cAMP using FLIPR.
- Sensitive, robust, homogeneous assay.
- Good signal /noise ratio; kinetic or end point assay
- Real time, live cell assay completed in minutes after ligand addition
- Potentially amenable to HTS or SAR

Measurement of G_i coupled receptors eg D_2

- Robust assay
- Pharmacology correlates with published data

Measurement of G_s coupled receptors eg 5-HT_6

- Assay results from transient transfections are as good as the current Flashplate technology
- Use of non-detergent transfection technologies eg. viral transduction may be less disruptive to membrane and increase expression and therefore yield a more robust pharmacology.

Acknowledgements

- Atto Bioscience
  - Ling Cao
  - Yong Yao
  - Jim Mannion
- GlaxoSmithKline
  - Claire Scott
  - Affinity Profiling Team