Using dithiothreitol as a pharmacological tool to study the effect of extracellular disulfide bridges in serotonin 2_A receptor functionality

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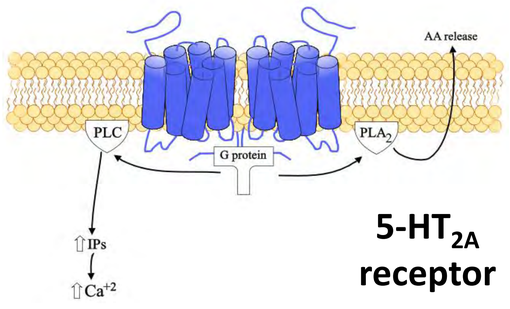
Introduction

G Protein Coupled Receptors (GPCRs) extracellular domains are emerging as a determining factor in receptor functionality, not only for orthosteric ligands but also as an allosteric modulation site[1]. A common feature among the GPCR extracellular domains is the presence of, at least, one disulfide bridge between ECL-2 and TM-3, and the presence of extra linkages depends on the receptor type. Therefore, the study of the implication of disulfide bridges in both ligand binding and its consequential intracellular signalling emerges as a necessity in new drug design. For studying this implication, dithiothreitol (DTT), a reducing agent that has been used to demonstrate GPCR dimerization[2], has been previously employed at serotonin 2_A (5-HT_{2A}) receptor, concluding that 5-HT_{2A} remains in its homodimeric form in the presence of 20 mM DTT, while with an impaired capacity of binding [^{3}H]LSD and IP signalling[3].



5-HT_{2A} receptor

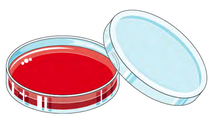
Hypothesis

Our hypothesis was that disulphide bridges formed among extracellular cysteines in 5-HT_{2A} receptor are capable of modulating its pharmacological response to drugs.

Objective

We aim to employ DTT as a pharmacological tool to study the implication of extracellular disulphide bridges in 5-HT_{2A} receptor IP and calcium functionality.

Materials and methods



CHO 5-HT_{2A} cells were expanded in P150 dishes

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Cells were pelleted at 1000 G

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DTT TREATMENT

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Cells were lysated and centrifuged at 45000 x *g* to precipitate the membrane fraction

↓

Cell membranes were incubated at 37 ºC with

- Different concentrations of [^{3}H]Ketanserin (Perkin Elmer) for saturation assays for 30 minutes
- 1 nM [^{3}H]Ketanserin (Perkin Elmer), at different times for association assays
- 1 nM [^{3}H]Ketanserin (Perkin Elmer), for 30 min for dissociation assays. Then, dissociation was initiated at different times by the addition of 1 µM methysergide
- 1 nM [^{3}H]Ketanserin (Perkin Elmer), for 30 minutes in the presence of different compounds in competition assays.

Non specific binding was measured in the presence of 1 µM methysergide.

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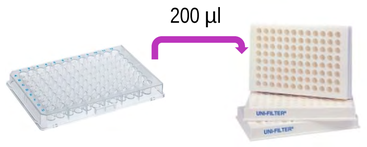
200 µl



Assay plate — GF/B filter plate (Millipore)

Filtration and addition of Universol™

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Response measurement in a Microbeta™ Trilux^2 (Perkin Elmer)

CHO 5-HT_{2A} cells were seeded at a density of 20000 cells/well in a 96 well plate

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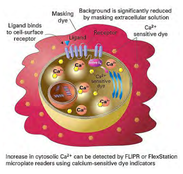
24 hours later, cells were incubated for 1 hour at 37 ºC in Fura-2 QBT™ (Molecular Devices)



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DTT TREATMENT

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Correspondent compounds were added

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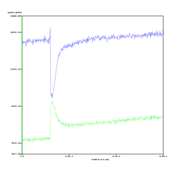
Response measurement in a FDSS 7000EX (Hammamatsu)



Results

1. The use of DTT reduces [^{3}H]Ketanserin binding capacity without altering neither kinetic parameters nor affinity of different compounds at h5-HT_{2A} receptor.

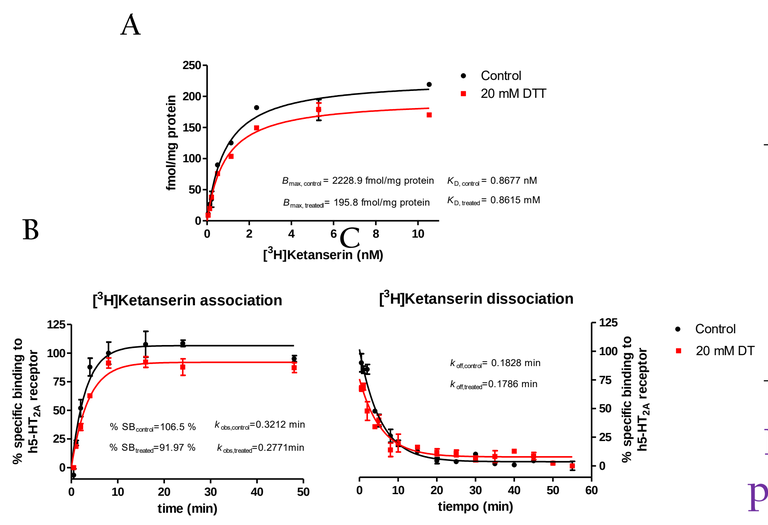
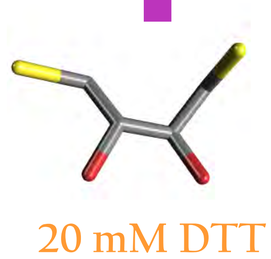


Figure 1. Effect of DTT on (A) saturation binding curve, (B) association and (C) and dissociation assays, for [^{3}H]Ketanserin. Values represent the mean ± SEM of one representative experiment of two independent experiments, carried out in triplicate.

Table 1. Effect of DTT on competition parameters of [^{3}H]Ketanserin on human 5-HT_{2A} receptor. Values represent the mean ± SEM of three independent experiments carried out in duplicate.

COMPETITION	pK_i		% SB	
	Control	20 mM DTT	Control	20 mM DTT
5-HT	6.00 ± 0.03	5.98 ± 0.03	98.00 ± 1.15	85.58 ± 1.03
(±)DOI	High 8.69 ± 0.30 Low 6.85 ± 0.03	6.94 ± 0.03	100.3 ± 1.82	82.55 ± 1.54
LSD	7.88 ± 0.04	7.85 ± 0.05	97.21 ± 1.61	79.00 ± 1.73
Clozapine	7.84 ± 0.03	7.89 ± 0.03	91.04 ± 1.38	76.31 ± 1.26
Haloperidol	6.78 ± 0.02	6.77 ± 0.03	99.41 ± 1.23	76.17 ± 1.12

Binding properties



20 mM DTT
10 min
37 ºC

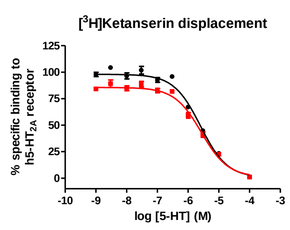


Figure 2. Representative curve of the effect of DTT on competition parameters of [^{3}H]Ketanserin, representing 5-HT displacement. Values represent the mean ± SEM of three independent experiment carried out in duplicate.

2. DTT pretreatment does not alter agonist and antagonist potencies when measuring IP accumulation, but it induces, at least, a 20 % reduction in compounds efficacy.

IP accumulation

Table 2. pEC_{50} values and %E_{max} exhibited after 5-HT, (±)DOI and LSD stimulation when measuring IP accumulation. Values represent the mean ± SEM of at least two experiments carried out in triplicate

AGONIST	pEC_{50}		% E_{max} (10 µM agonist)	
	Control	20 mM DTT	Control	20 mM DTT
5-HT	6.53 ± 0.07	6.08 ± 0.09	98.82 ± 2.76	73.46 ± 2.39
(±)DOI	7.29 ± 0.12	7.66 ± 0.22	105.00 ± 4.35	65.08 ± 4.58
LSD	8.69 ± 0.26	8.36 ± 0.44	98.30 ± 7.40	74.40 ± 8.35

Table 3. pIC_{50} values and %E_{max} elicited after 5-HT, (±)DOI and LSD stimulation in the presence of clozapine or haloperidol, when measuring IP accumulation. Values represent the mean ± SEM of at least two experiments carried out in triplicate

ANTAGONISTS		pIC_{50}		% E_{max} (max agonist)	
		Control	20 mM DTT	Control	20 mM DTT
Clozapine[3]	1 µM 5-HT	7.32 ± 0.10	7.00 ± 0.10	99.87 ± 4.07	41.88 ± 1.39
Haloperidol		7.01 ± 0.12	6.56 ± 0.15	92.40 ± 3.65	69.67 ± 3.19
Clozapine	0,1 µM (±)DOI	7.39 ± 0.08	6.80 ± 0.13	97.37 ± 2.82	60.32 ± 2.80
Haloperidol		5.95 ± 0.11	6.08 ± 0.11	100.50 ± 2.75	70.75 ± 2.38
Clozapine	0,1 µM LSD	7.10 ± 0.17	7.07 ± 0.26	90.44 ± 5.34	52.35 ± 3.94
Haloperidol		5.8 ± 0.21	5.89 ± 0.31	100.50 ± 4.40	88.41 ± 11.16

3. DTT pretreatment does not alter agonist and antagonist potencies when measuring calcium mobilization, but it induces, at least, a 30 % reduction in compounds efficacy.

Calcium mobilization

Table 4. pEC_{50} values and %E_{max} exhibited after 5-HT, (±)DOI and LSD stimulation when measuring calcium mobilization. Values represent the mean ± SEM of at least three experiments carried out in triplicate

AGONIST	pEC_{50}		% E_{max} (10 µM agonist)	
	Control	20 mM DTT	Control	20 mM DTT
5-HT	7.67 ± 0.07	7.24 ± 0.10	100.5 ± 2.61	68.33 ± 2.59
(±)DOI	7.98 ± 0.10	7.81 ± 0.14	99.43 ± 3.33	53.97 ± 0.14
LSD	7.52 ± 0.16	7.36 ± 0.38	102.9 ± 6.47	42.14 ± 6.51

Table 5. pIC_{50} values and %E_{max} elicited after 5-HT, (±)DOI and LSD stimulation in the presence of clozapine or haloperidol, when measuring calcium mobilization. Values represent the mean ± SEM of at least three experiments carried out in triplicate

ANTAGONISTS		pIC_{50}		% E_{max} (0,1 µM agonist)	
		Control	20 mM DTT	Control	20 mM DTT
Clozapine	0,1 µM 5-HT	7.24 ± 0.07	7.81 ± 0.11	101.40 ± 2.24	46.00 ± 1.42
Haloperidol		5.71 ± 0.09	6.06 ± 0.26	95.59 ± 1.41	41.70 ± 2.43
Clozapine	0,1 µM (±)DOI	7.40 ± 0.06	8.21 ± 0.24	98.09 ± 1.75	35,44 ± 3.17
Haloperidol		5.45 ± 0.13	5.91 ± 0.11	100.20 ± 3.10	46.88 ± 1.28
Clozapine	0,1 µM LSD	High 8.73 ± 0.38 Low 7.05 ± 0.08	8.30 ± 0.14	101.10 ± 2.39	36.41 ± 2.06
Haloperidol		5.85 ± 0.13	6.45 ± 0.35	89.09 ± 2.53	32.09 ± 2.37

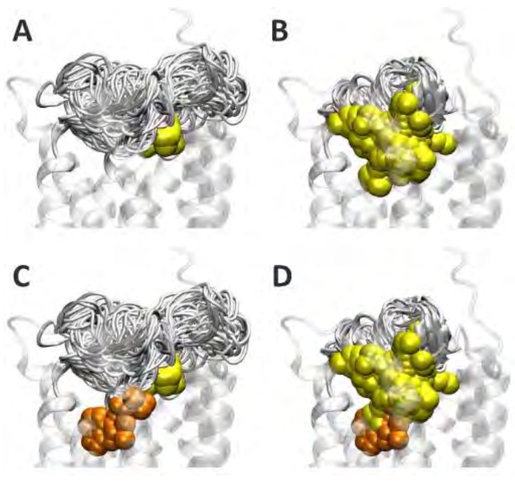


Figure 3. Breaking the linkage between ECL-2 and TM-3 in 5-HT_{2A} receptor results in the collapse of the orhosteric binding site (B, D), difficulting LSD (orange dots in C and D) to be properly accomodated[3].

Conclusions

1. DTT creates a reducing environment that cleaves extracellular disulphide bridges at 5-HT_{2A} receptor. Therefore, it adopts a conformation where the linkage between ECL-2 and TM-3 is broken, increasing the flexibility of this extracellular domain. As a result, fully binding of different compounds is impeded.
2. This impaired ability to proper accommodate ligands of different types is fully transferred into a truncated IP accumulation and calcium mobilization.
3. Together with previous data, we conclude that DTT is altering 5-HT_{2A} binding pocket, which is reflected into an impaired signalization. Thus, DTT emerges as a pharmacological tool to study the effect of extracellular disulphide bridges in GPCRs functionality, which will allow the designing of more selective drugs.

References

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Grants

Ministry of Economy and Competitiveness (MICINN (SAF2014-57138-C2-1-R & SAF2017-85225-C3-1-R)
Xunta de Galicia (GRC2014/011 & ED431C 2018/21) and European Regional Development Fund (ERDF)
Grants to support predoctoral stage in the SUG universities, in public research organizations in Galicia and other entities of the Galician system of R & D, co-funded partially by the ESF program Galicia 2014-2020 (ESF- investing in your future).

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