Proceedings of the British Pharmacological Society at http://www.pA2online.org/abstracts/Vol19Issue1abst029P.pdf

Agonist activity of clozapine at muscarinic DREADD receptors

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Introduction Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are a chemogenetic tool widely used to dissect signalling *in vitro* and *in vivo*[1], of which muscarinic DREADDs - hM1Dq, hM3Dq, hM4Di - are most widely used[2]. Transmembrane mutations render these receptors largely unresponsive to endogenous acetylcholine, but sensitive to the otherwise inert clozapine-*N*-oxide (CNO). Recent reports suggest back-metabolised clozapine, rather than CNO, is the muscarinic DREADD activator *in vivo*[1]. We therefore investigated clozapine agonism at hM1Dq and hM4Di *in vitro*, and whether back-metabolised clozapine could be detected in CNO-injected mice.

Methods *[^{3}H]-NMS Displacement*: Confluent monolayers of FLP-in CHO cells expressing hM1WT, hM4WT, hM1Dq, or hM4Di were incubated in Kreb's buffer with [^{3}H]-NMS at K_d concentration and increasing ligand concentrations for 2 hr at 37°C. Cells were washed then lysed. Liquid scintillation counting determined bound radioactivity. *Functional assays:* Assays were carried out according to IP-One-Gq and phosphoERK1/2 (Thr^{202}/Tyr^{204}) Kits (CisBio, France). For IP_1, washed and detached cells were resuspended in Stimulation Buffer and stimulated for 1 hr at 37°C. For Thr^{202}/Tyr^{204} phosphorylation, serum-starved cells were stimulated for 5 min at 37°C then lysed. Resulting IP_1 accumulation or Thr^{202}/Tyr^{204} phosphorylation were determined with cryptate/D2 antibodies and HTRF. *Pharmacokinetics*: C57bl/6J mice were injected with varying CNO concentrations. After 30 min mice were exsanguinated and brains removed to assess plasma and brain drug exposure (in accordance with ASPA 2012). *Data Analysis*: All data analysis used GraphPad Prism 7.

Results Clozapine and CNO displaced [^{3}H]-NMS in hM1WT, hM1Dq, hM4WT, and hM4Di receptor cell lines (Table 1; n=3) but demonstrated no wild type receptor agonism in functional assays (n=3). In contrast, clozapine stimulated Thr^{202}/Tyr^{204} phosphorylation at hM4Di receptors (Table 1; n=3) and IP_1 accumulation in hM1Dq receptors (Table 1; n=3) more potently than CNO. C57bl/6J mice exhibited a dose-dependent increase in plasma CNO following 0.3, 1, or 1.5 mg/kg CNO injection (50.1 nM ± 0.16; 575.44 nM ± 0.03; 467.7 nM ± 0.09; n=3), yet CNO brain exposure was not detected. However, clozapine was detected in plasma and brains of these mice, indicating CNO back-metabolism.

Conclusions Clozapine has greater affinity and potency than CNO at muscarinic DREADDs *in vitro*. Furthermore, CNO was back-metabolism to brain-penetrating clozapine *in vivo*. Collectively, this suggests clozapine may be a muscarinic DREADD agonist *in vivo*.

Log (M) pKi and EC50 values at hM1WT, hM1Dq, hM4WT and hM4Di receptors

	[^{3}H]-NMS Displacement Log_{pKi} (M) (S.E.M.)			**IP_1 Accumulation or Thr^{202}/Tyr^{204} phosphorylation Log_{EC50} (M) (S.E.M.)**		
	ACh	**CNO**	**Clozapine**	**ACh**	**CNO**	**Clozapine**
hM1WT	-5.02 (± 0.04)	-5.09 (± 0.09)	-7.4 (± 0.04)	-7.85 (± 0.14)		
hM1Dq	-2.95 (± 0.08)	-6.91 (± 0.06)	-8.84 (± 0.07)	-3.24 (± 0.13)	-8.18 (± 0.08)	-10.65 (+/- 0.2)
hM4WT	-4.55 (± 0.33)	-4.85 (± 0.05)	-7.08 (± 0.28)	-6.92 (± 0.13)		
hM4Di	-2.66 (± 0.11)	-6.24 (± 0.03)	-8.03 (± 0.432)	-2.76 (± 0.09)	-7.26 (± 0.14)	-9.53 (+/- 0.26)

References

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2. Armbruster, B. N. et al. (2007). *Proc Natl Acad Sci USA, 104* (12), 5163-8. 4.