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Pharmacological evaluation of clozapine n-oxide for its use in the *in vivo* chemical genetic investigation of M_1 muscarinic acetylcholine receptor function

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Introduction: Our laboratory has been working to define the pharmacological and signalling properties of muscarinic receptor models being used to validate muscarinic receptors as targets in neurological diseases. Rational mutations within the ligand binding pocket of muscarinic receptors (in TM3 and TM5) creates Designer Receptor Exclusively Activated by Designer Drug (DREADD), which are less sensitive to the endogenous ligand, acetylcholine (ACh), but instead are activated by an otherwise chemically inert synthetic ligand, clozapine *n*-oxide (CNO) (1). Furthermore, by mutating phosphorylation sites to alanine residues, we have created mutant receptors deficient in their phosphorylation status (phosphorylation-deficient; PD), therefore showing biased signalling (i.e. G protein bias). We have generated transgenic mouse lines whereby M_1-DREADD or M_1 DREADD-PD are expressed in the place of WT M_1 mAChRs. We anticipate these mice to behave like a knock-out until activated activated by CNO. PD mutations allow us to test the notion that by directing the signalling of receptors to one pathway (i.e. G proteins) over another (i.e. receptor phosphorylation/arrestin) might lead to beneficial therapeutic outcomes. Combining these technologies, it will be possible to gain a deeper understanding of the role of M_1 mAChRs *in vivo* and the therapeutic potential of these receptors in disease. Here we aim to fully characterize CNO at WT mAChRs.

Method: Using radioligand binding and cell signalling assays, we have characterised the properties of CNO at multiple mAChR subtypes, looking for competitive binding, agonism, and functional antagonism.

Results: At the M_1 mAChR, CNO was shown to be a partial agonist in calcium, ERK 1/2 activation and inositol phosphate accumulation assays. Competition binding assays have revealed a similar affinity of CNO to Ach (table 1.). Functional antagonism studies using an IP-one assay showed CNO also behaving as a functional antagonist at the mouse M_1-WT receptor, with a pA_2 of 5.35 ± 0.09. Investigations of M_2, M_3, and M_4 WT mAChR expressing cell lines found no activation by CNO, and competition binding has found CNO binds with a lower affinity.

Conclusion: Although CNO was seen to interact with WT receptors, the concentration of CNO required to activate the M_1-DREADD and M_1-DREADD-PD receptors was lower than the concentrations causing these potentially confounding effects. It will be important to evaluate dosages for the animal studies to successfully activate the M_1-DREADD and DREADD-PD receptors without antagonising or activating WT mAChRs in the DREADD or WT animals.

References: (1) Armbruster BN *et al.* (2007) PNAS **104:** 5163-5168