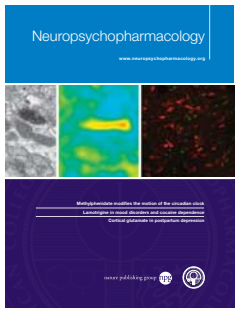

CNO Evil? Considerations for the Use of DREADDs in Behavioral Neuroscience

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CNO Evil? Considerations for the Use of DREADDs in Behavioral Neuroscience

Response to Gomez et al. (2017) "Chemogenetics revealed: DREADD occupancy and activation via converted clozapine." Science, 357 (6350), 503-7.

Running Title: Using DREADDs in behavioral neuroscience

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Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are an increasingly popular approach for "remotely controlling" selected neuronal populations and pathways (*1*). Gomez et al (*2*) provides important new details on an underappreciated mechanism by which DREADDs can produce CNS effects following peripheral administration of clozapine-n-oxide (CNO). A small proportion of systemically-administered CNO is metabolized to clozapine (*3, 4*), an antipsychotic drug with activity at numerous endogenous receptors (*5*, *6*), and they show that clozapine both much more readily penetrates the blood brain barrier (BBB), and more potently binds DREADDs than CNO (as previously reported (*1*, *7*)). They conclude that clozapine is therefore likely to be a major contributing factor activating DREADDs after systemic administration of CNO.

This report has given pause to hundreds of labs using DREADDs to control neural circuits in vivo. If present at high enough concentrations to affect endogenous receptors,

clozapine could cause effects beyond those mediated by CNO acting at DREADDs. We agree with the authors that these findings do not discount conclusions drawn from well-controlled DREADD experiments, but they highlight several important issues regarding interpretation of data from DREADD experiments, and choice of DREADD agonist for use with designer receptors going forth.

First, this paper shows that clozapine back-metabolized from CNO may contribute to DREADD activation after peripheral CNO injection. Gomez et al report that clozapine metabolized from CNO accumulates over time (though see (*3*)), such that effects of clozapine may be strongest long after CNO injection (>2hrs). Therefore, it is important to consider whether clozapine accumulates after CNO injection to concentrations sufficient to activate endogenous receptors classically associated with clozapine (e.g., 5-HT, dopamine, or histamine receptors). In addition, unwanted effects of back-metabolized clozapine may also depend on the behavior in question, and the presence or absence of other pharmacological agents (e.g., self-administered cocaine) that could interact with clozapine's endogenous (non-DREADD) receptor targets (*2, 3, 8*). It is also possible that low doses of clozapine could cause complex effects via concurrent actions at DREADDs and at endogenous receptors present in the same neurons or circuits. This means that even if the CNO/clozapine concentration is low enough to cause no observable effects in non-DREADD-expressing animals, its actions at endogenous receptors could interact with DREADD effects in unknown ways. Therefore, knowledge of clozapine blood and brain levels over time after CNO application is important, and caution is especially warranted for studies examining prolonged testing periods, repeated CNO administrations, and especially chronic CNO dosing. In general, effects of any DREADD agonist should be compared in DREADD-expressing versus non-DREADD-expressing animals, allowing identification of

DREADD-specific effects. The potential for long-lasting effects of DREADD agonists on outcomes occurring outside the ~2 hour testing window after acute dosing should also be examined.

The authors suggest that the best path forward for DREADD users is to switch to low-dose clozapine instead of CNO, thus removing potential variability in CNO metabolism and therefore clozapine dosing – however, there are potential drawbacks to this approach. One important and useful feature of the CNO/DREADD system is the extended duration of action of CNO after systemic injection. This is desirable for lengthy behavioral experiments, where commonly used optogenetic tools would require extended light application which can cause heating or other artifacts(*8*). If as implied by Gomez et al CNO is essentially a pro-drug for the true DREADD agonist clozapine, ongoing metabolism might be expected to extend the duration of activity at DREADDs relative to an acute injection of very low-dose clozapine (as required to avoid nonselective effects). In support of this, clozapine and CNO can activate DREADDs at very low systemic dosages to cause behavioral effects (e.g. (*9*)), but in our experience, higher CNO doses are required to maintain efficacy for 2 hours—a window that is commonly used in certain behavioral experiments. We and others have failed to find significant effects of up to 10mg/kg CNO on various motivated behaviors in non-DREADD expressing animals, at least within a 30-150 min timeframe after i.p. injection. This underscores the fact that if clozapine levels remain in the range of specificity for DREADDs, but below the threshold for altering signaling at endogenous receptors during the allotted testing period, CNO can be a suitable agonist for use in such experiments.

An uncertain point touched upon by Gomez et al. regards the mechanism by which CNO acts when washed onto brain slices, or when injected directly into the brain in vivo. The main

mechanism of metabolism of CNO into clozapine in vivo is via cytochrome P450 enzymes, primarily in liver (*10, 11*). Notably, these enzymes metabolize a wide range of drugs, so the presence of other compounds can inhibit CNO/clozapine metabolism significantly (*12*). Cytochrome P450s are also present at low levels in brain (*13-16*), and the authors speculate that CNO may be metabolized to clozapine directly in brain. However, CNO has also been found effective in reduced systems including mammalian and drosophila neuronal (*17-20*) and non-neuronal cell cultures (*1, 21*). In addition, conversion of CNO is altered by pH and temperature (*22-24*), so these factors could also affect the stability and metabolism of CNO in CNS tissue. Clearly, identification of the enzymatic substrates or other mechanisms by which CNO converts to clozapine in brain or other reduced systems is required, as is further characterization of the binding, intracellular signaling, and behavioral effects of CNS-applied CNO and its metabolites.

Of note, numerous reports have failed to find DREADD-independent behavioral effects of CNO microinjection in vivo in ventral tegmental area (*25*), lateral septum (*26*), dorsal hippocampus (*27*) or orbital cortex (*28*) at a concentration of 1 mM — far in excess of the concentration found by Gomez et al to be capable of binding endogenous receptors in rat (10μM). Our own study (*25*) found that intra-VTA CNO microinjections attenuated cued reinstatement of cocaine seeking when DREADDs were expressed in afferents from the rostral ventral pallidum (VP), but identical CNO microinjections had no effect on that behavior when DREADDs were instead expressed in caudal VP. This is one example of the remarkable specificity achievable with DREADD technology using local intraparenchymal injections of CNO. These, and other results showing no effect on local CNO injections, indicate that the presence of receptors susceptible to nonspecific CNO/clozapine binding may be brain region-dependent, or that CNO/clozapine activity at these receptors fails to affect behaviors that have

been examined to date (reinstatement of operant cocaine or heroin seeking, or cue-induced food seeking (*25-28*)). It is also noteworthy that drug injected directly into CNS yields much lower concentrations at local receptors than the injected liquid, because of substantial diffusion and dilution that occurs after intraparaenchymal injection. Therefore, based on currently available data, intracranial CNO may sidestep some potential issues resulting from systemic CNO administration, and resulting liver metabolism to clozapine.

Another way around potential off-target effects of CNO/clozapine is to employ a DREADD agonist other than CNO that does not have active metabolites, but that penetrates the BBB and selectively activates DREADDs. Alternative DREADD agonists include the FDA-approved hypnotic compound perlapine, and the newly developed "compound 21," both of which have significant functional effects at DREADDs in vitro (*29*). However, neither of these compounds have to date been screened for use with DREADDs in vivo, and key pharmacokinetic/ pharmacodynamic profiling, and characterization of potentially active metabolites, are not available. Numerous labs including ours are currently testing these compounds, but the jury is still out regarding their efficacy, specificity, and time-courses of action, especially in any given brain system.

The high affinity of clozapine for DREADDs has been known since the first description of these designer receptors[1]. Although the Gomez et al paper makes several interesting observations, the major claim that CNO should be abandoned as a DREADD agonist seems premature. DREADD users may be well advised to employ the relatively well-characterized CNO until a more selective agonist is fully characterized. Although there are possible caveats to take into account with the use of CNO, as laid out in Gomez et al. and above, we note that most potential off-target effects of CNO/clozapine are well controlled by administration of CNO to

non-DREADD-expressing animals. As always, it is prudent for investigators to be mindful of the limitations of the methods they use. That said, DREADD technology is a major advance forward in neuroscience regardless of the agonist employed, and we urge readers not to throw out the baby with the bathwater when it comes to experimental use of this powerful, but evolving, neuroscience method.

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