TARP Auxiliary Subunits Switch AMPA Receptor Antagonists into Partial Agonists

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Science **318**, 815 (2007);
DOI: 10.1126/science.1146317

TARP Auxiliary Subunits Switch AMPA Receptor Antagonists into Partial Agonists

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Quinoxalinedione compounds such as 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) are the most commonly used α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists. However, we find that in the presence of transmembrane AMPA receptor regulatory proteins (TARPs), which are AMPA receptor auxiliary subunits, CNQX acts as a partial agonist. CNQX induced small depolarizing currents in neurons of the central nervous system, and reconstitution of this agonist activity required coexpression of TARPs. A crystal structure of CNQX bound to the TARP-less AMPA receptor ligand-binding domain showed that, although CNQX induces partial domain closure, this movement is not transduced into linker separation, suggesting that TARPs may increase agonist efficacy by strengthening the coupling between domain closure and channel opening. Our results demonstrate that the presence of an auxiliary subunit can determine whether a compound functions as an agonist or antagonist.

Excitatory synaptic transmission in the brain is mediated by glutamate acting on two classes of ionotropic receptors: AMPA and *N*-methyl-D-aspartate (NMDA) receptors. A major breakthrough in the field of excitatory synaptic transmission came with the discovery of the quinoxalinedione series of competitive AMPA receptor antagonists in 1988 (*1*–*3*). These drugs—CNQX, 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX)—potently and selectively block AMPA receptors and have been indispensable in characterizing excitatory synaptic transmission in the central nervous system (CNS).

As competitive antagonists, quinoxalinediones interact with AMPA receptors in the same binding pocket as agonists, thereby occluding agonist binding. Unlike agonists, competitive antagonists have no efficacy, meaning that binding does not lead to opening of the ion channel (i.e., gating). Despite the current widespread use of quinoxalinediones as AMPA receptor antagonists to study synaptic transmission, a few reports indicate that these compounds can have excitatory actions on a subset of interneuron populations, though not on excitatory neurons (*4*–*7*). The mechanism for this effect remains unexplained.

While recording cerebellar granule cells, we also observed that application of CNQX (10 μM) increased spontaneous inhibitory postsynaptic current (sIPSC) frequency (0.22 ± 0.07 Hz to 1.58 ± 0.56 Hz, $n = 5$ cells, $P < 0.05$) without a change in amplitude (21.1 ± 6.1 pA to 19.3 ± 3.1 pA, $n = 5$ cells) (Fig. 1, A and B) (*5*), implying increased excitability of the presynaptic interneurons: cerebellar Golgi cells (*8*). Application of CNQX to Golgi cells reliably evoked an inward current in voltage-clamp recordings (–37.9 ± 4.4 pA, $n = 11$ cells), indicating a non-canonical depolarizing effect of CNQX on these cells (Fig. 1C). However, we found that antagonizing AMPA receptors does not simply lead to depolarization because application of GYKI 53655 (10 μM), which is a noncompetitive AMPA receptor antagonist (*9*), did not induce an inward current in Golgi cells (fig. S1). Given that the CNQX-induced depolarization was recorded in γ-aminobutyric acid type A ($GABA_A$), NMDA, and glycine receptor antagonists as well as in tetrodotoxin (TTX) to block network activity, our data suggested that CNQX was not simply acting through another neuronal receptor but instead that CNQX may act as an AMPA receptor agonist on these cells.

We therefore tested whether a noncompetitive AMPA receptor antagonist could block the CNQX-induced depolarization and whether a positive allosteric modulator could potentiate the response. Preincubation of cerebellar slices with GYKI 53655 blocked the CNQX-induced current (CNQX-induced current in GYKI = 0.4 ± 2.5 pA, $n = 5$ cells, $P < 0.001$, as compared with CNQX alone) (Fig. 1C). Furthermore, trichloromethiazide (TCM) (500 μM), a positive modulator structurally similar to cyclothiazide (*10*, *11*), increased the response to CNQX (–88.7 ± 21.6 pA, $n = 5$ cells, $P < 0.01$, as compared with CNQX alone) (Fig. 1D). Thus, CNQX appears to act as an agonist on these AMPA receptors, despite its previous characterization as a competitive antagonist.

Although AMPA receptors are expressed on most, if not all, neurons in the brain, a depolarizing action of CNQX was not previously reported for

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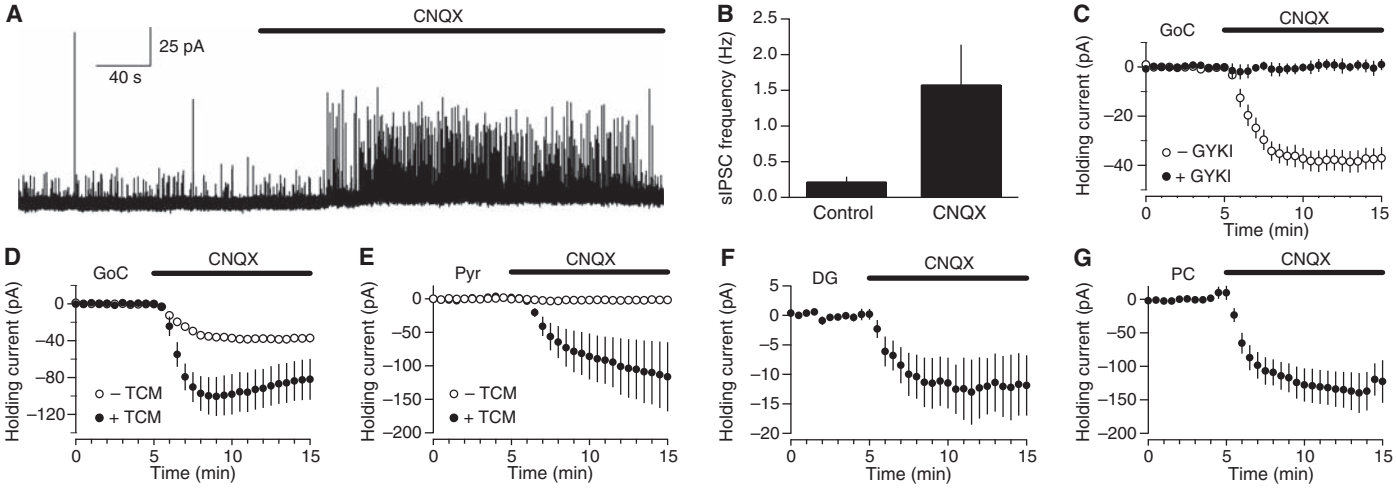

Fig. 1. Depolarizing current elicited by CNQX in neurons. (**A** and **B**) The frequency of sIPSCs in cerebellar granule cells voltage-clamped at 0 mV [excitatory postsynaptic current (EPSC) reversal potential] was measured before and after bath application of CNQX ($n = 5$ cells, $P < 0.05$). (**C** and **D**) The holding current needed to voltage-clamp cerebellar Golgi cells (GoC) to –70 mV was measured as CNQX was applied in the absence or presence of either 10 μM GYKI 53655 ($n = 11$ and 5 cells, respectively, $P < 0.001$) (C) or 500 μM TCM ($n = 11$ and 5 cells, respectively, $P < 0.01$) (D). (**E**) Similarly, CNQX was applied to hippocampal CA1 pyramidal cells (Pyr) in the presence and absence of TCM ($n = 5$ cells for each treatment). (**F** and **G**) CNQX was also applied to hippocampal dentate granule cells (DG) ($n = 5$ cells) and Purkinje cells (PC) ($n = 5$ cells) in the presence of TCM. Hippocampal neurons were voltage-clamped at –70 mV; Purkinje neurons were held at –10 mV to prevent voltage escape. Error bars in (B) to (G) indicate SEM.

excitatory neurons (*5*, *6*). This raised the possibility that AMPA receptors on interneurons somehow differ from those on other types of neurons. We therefore tested whether CNQX can act as an agonist on AMPA receptors expressed by hippocampal CA1 pyramidal cells. In the absence of TCM, CNQX did not evoke an inward current (–1.9 ± 2.6 pA, $n = 5$ cells) (Fig. 1E), as was reported previously (*6*, *12*). However, a CNQX-induced current was observed in the presence of TCM (–103.5 ± 43.3 pA, $n = 5$ cells) (Fig. 1E). A CNQX-induced current was also detected in the presence of TCM in dentate granule cells (–12.2 ± 5.1 pA, $n = 5$ cells) (Fig. 1F) and in cerebellar Purkinje cells (–131.7 ± 27.2 pA, $n = 5$ cells) (Fig. 1G). Given that CNQX could induce depolarizing currents in all neuron types tested, the agonist activity of CNQX is most likely a general property of CNS neurons and AMPA receptors. The previously reported lack of detection of CNQX-induced currents was most likely because CNQX was not tested in the presence of TCM.

To conclusively attribute the depolarizing current to AMPA receptor activation, we next attempted to reconstitute the agonist activity of CNQX on AMPA receptors expressed in cultured human embryonic kidney (HEK) 293 cells. A brief application of CNQX evoked an inward current on HEK293 cells transfected with the AMPA receptor pore-forming subunit GluR1Q) flop and γ-2, a member of the TARP family of AMPA receptor auxiliary subunits (*13*–*15*) (–12.7 ± 5.7 pA, $n = 6$ cells) (Fig. 2A). Furthermore, the CNQX-induced current in HEK293 cells had many properties consistent with AMPA receptor activation. The response to CNQX was significantly enhanced in the presence of TCM (–287.0 ± 104.6 pA, $n = 6$ cells, $P < 0.04$) (Fig. 2A). For ease of measurement, all further experiments in HEK293 cells were carried out in the presence of TCM. Comparison of the currents evoked by CNQX and glutamate (1 mM) indicated that CNQX is acting as a partial agonist (Fig. 2A). Partial agonists, such as kainate, bind AMPA receptors but only induce a fraction of the activation induced by full agonists, such as glutamate. As in Golgi cells, GYKI 53655 blocked the CNQX-induced inward current in HEK293 cells (inhibition: 99.3 ± 0.7%, $n = 4$ cells) (Fig. 2B), and the effect of GYKI was reversible. Furthermore, the current elicited by CNQX had the current-voltage (*I-V*) relationship expected for GluR1(Q) AMPA receptors ($n = 4$ cells) (Fig. 1C) (*16*). Together, our data indicated that CNQX acts as a partial agonist in a heterologous expression system.

We sought to determine whether other members of the quinoxalinedione family also have agonist activity. A brief application of DNQX elicited an inward current in transfected HEK293 cells (–284.0 ± 99.4 pA, $n = 13$ cells) (Fig. 2D), which was not statistically different from that seen with CNQX (–211.1 ± 69.9 pA, $n = 11$ cells, $P = 0.57$). In contrast, NBQX did not elicit an inward current in HEK293 cells (23.2 ± 7.0 pA, $n = 9$ cells) (Fig. 2D). We observed similar effects in cerebellar Golgi cells in the absence of TCM (DNQX: –10.0 ± 3.0 pA, $n = 6$ cells; NBQX: 2.1 ± 2.1 pA, $n = 5$ cells) (Fig. 2E). Therefore, NBQX acts purely as a competitive AMPA receptor antagonist, whereas both CNQX and DNQX act as partial agonists.

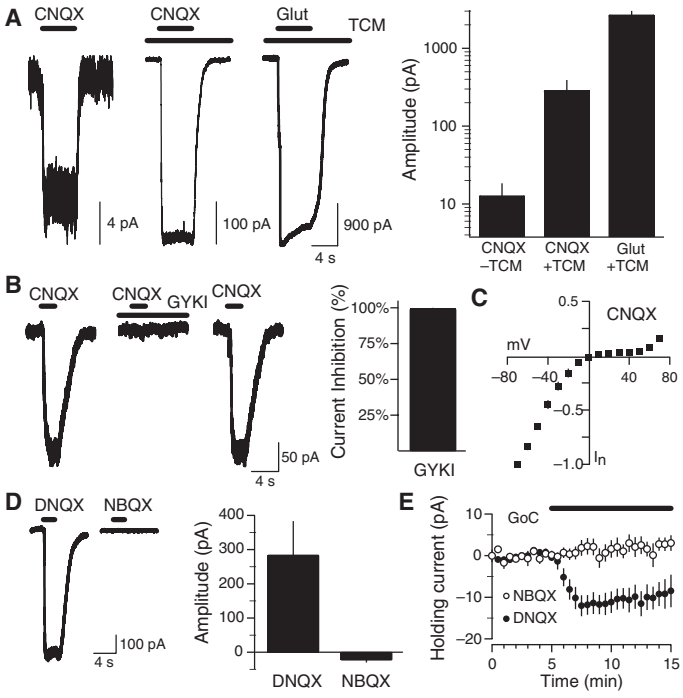

Fig. 2. AMPA receptor activation by CNQX and DNQX, but not NBQX, in a heterologous system. (**A**) The change in holding current, elicited by a brief application of 10 μM CNQX to voltage-damped (–70 mV) HEK293 cells expressing GluR1 flop and γ-2, was measured in the absence and presence of 500 μM TCM. A brief application of 1 mM glutamate (Glut) in TCM was later applied ($n = 6$ cells). (**B**) The CNQX-induced change in holding current was measured before, during, and after the application of 100 μM GYKI 53655 ($n = 4$ cells). (**C**) The *I-V* relationship of the CNQX-induced current in TCM is shown ($n = 4$ cells). (**D** and **E**) The effects of 10 μM DNQX and 10 μM NBQX were also measured in HEK293 cells cotransfected with GluR1 and γ-2 (DNQX: $n = 11$ cells; NBQX: $n = 9$ cells) (D) and in Golgi cells in the absence of TCM (DNQX: $n = 6$ cells; NBQX: $n = 5$ cells) (E). Error bars in (A) to (E) indicate SEM.

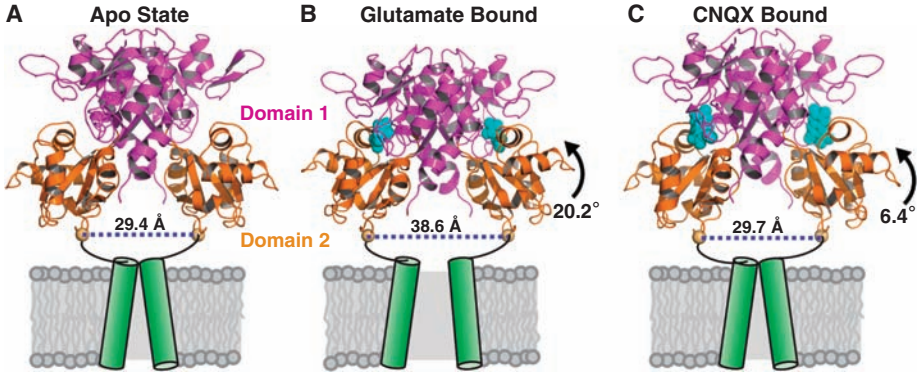

Fig. 3. Crystal structure of CNQX bound to the AMPA receptor LBD. iGluR monomers are composed of three transmembrane helices and one reentrant loop, an N-terminal domain, and a LBD. Shown are two pore-forming helices (rendered as green cylinders) within a membrane bilayer and the LBD [rendered as a ribbon diagram and colored in magenta (domain 1) and orange (domain 2)]. The LBD is a back-to-back dimer of two protomers, each of which contains a domain 1 and domain 2. (**A** and **B**) GluR2 LBD crystal structure in the *apo* and glutamate-bound states [Protein Data Bank codes 1FT0 and 1FTJ, respectively (*32*)]. Glutamate is rendered as a Corey-Pauling-Koltun (CPK) model and is colored in cyan in (B). Domain closure denotes the degree of domain 2 movement toward domain 1 relative to the *apo* state (20.2°). Linker distance is measured between the Cα atoms of Pro^{632} in each protomer. In the full-length receptor, the artificial linker (orange sphere) is connected to the pore-forming helices. Glutamate binding induces domain closure, increases the linker distance, and opens the channel pore. (**C**) GluR2 LBD crystal structure in the CNQX-bound state (rendered as a CPK model and colored in cyan).

Models of channel activation and desensitization have been developed through structural studies of isolated ionotropic glutamate receptor ligand-binding domains (LBDs). These LBDs consist of two domains arranged in a clamshell-like manner that undergo a conformational rearrangement, typically movement of domain 1 toward domain 2 (domain closure), upon ligand binding (Fig. 3). Binding of full agonists, such as glutamate, induces maximal domain closure (~21°), whereas partial agonists, such as kainate, induce partial closure (12°) relative to the unbound *apo* state (*17*). Therefore, channel activation is correlated with the degree of domain closure upon ligand binding (*17*, *18*). Agonist efficacy also correlates with the length of separation between the linker regions of each subunit, which connect the LBDs to the pore-forming transmembrane segments in the full-length receptor (Fig. 3) (*17*–*20*).

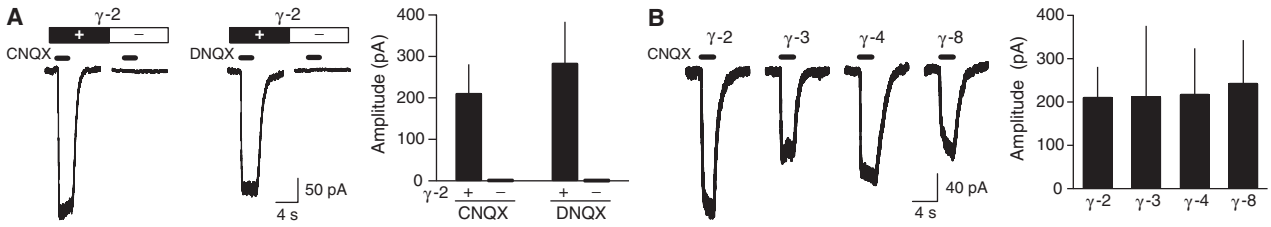

Fig. 4. Effects of CNQX and DNQX in the absence and presence of TARPs. (**A**) Either CNQX or DNQX was applied in the presence of TCM to HEK293 cells cotransfected with GluR1 and γ-2 or with GluR1 alone [CNQX: GluR1 + γ-2 (n = 11 cells) and GluR1 alone (n = 8 cells); DNQX: GluR1 + γ-2 (n = 13 cells) and GluR1 alone (n = 12 cells)]. (**B**) CNQX-induced holding current changes in cells transfected GluR1 and either TARP γ-2, γ-3, γ-4, or γ-8 (n = 11, 4, 5, and 4 cells, respectively). Error bars in (A) and (B) indicate SEM.

To test whether CNQX induces domain closure and linker separation consistent with agonist activity, we obtained the crystal structure of CNQX bound to the GluR2 LBD, also referred to as "S1S2" (Fig. 3C, table S1, and fig. S2). The CNQX-bound structure was ~6.4° closed relative to the *apo* state (~7.7° and ~5.1° for the two protomers), which is consistent with partial agonist activity. However, the linker separation in the CNQX-bound structure (29.7 Å) was not different from that of the *apo* state (29.4 Å), suggesting that CNQX may not transduce its domain closure into channel opening, and is therefore inconsistent with the partial agonist activity that we observed. Our CNQX-bound structure was similar to the structure of DNQX bound to the GluR2 LBD (Cα root mean square deviation = 0.71 Å) (*18*).

A key difference between the CNQX-bound LBD structure and native receptors is that the latter are coexpressed with TARPs, which include γ-2, γ-3, γ-4, and γ-8. To determine whether coexpression of TARPs is required for CNQX-induced receptor activation, we compared the CNQX-induced currents in HEK293 cells transfected with GluR1 and γ-2 to those transfected with GluR1 alone. Neither CNQX nor DNQX activated AMPA receptors in the absence of γ-2, even in the presence of TCM (CNQX: –1.63 ± 1.10 pA, n = 8 cells; DNQX: –1.08 ± 0.56 pA, n = 12 cells) (Fig. 4A). This was not due to a lack of AMPA receptor expression because the average glutamate-evoked currents in cells transfected with GluR1 alone were similar in the two conditions (GluR1 alone: 2941 ± 485 pA, n = 20 cells; γ-2 + GluR1: 3386 ± 409 pA, n = 24 cells; P = 0.48). Furthermore, the use of TCM to both slow and block AMPA receptor desensitization (*10*, *11*) suggests that the lack of CNQX-induced currents in the absence of γ-2 is not simply due to a detection difficulty resulting from rapid desensitization. This indicates that CNQX and DNQX act as pure competitive antagonists on AMPA receptors in the absence of γ-2 but as agonists in the presence of γ-2. Other members of the TARP family produced similar changes in CNQX activity (Fig. 4B). Therefore, inclusion of any TARP family member in an AMPA receptor complex switches the nature of CNQX and DNQX from competitive antagonists to partial agonists.

In light of our results, the structurally similar quinoxalinediones—CNQX and DNQX—are perhaps best considered to be partial agonists rather than competitive antagonists, given that most neurons in the CNS express TARPs (*21*, *22*). The lack of CNQX agonist activity in the absence of TCM in hippocampal CA1 pyramidal cells (Fig. 1E) (*6*) and other excitatory neurons may be due to rapid desensitization of CNQX-induced currents in these cells as a result of the expression of the flip splice variant of AMPA receptor subunits (*23*, *24*). The current induced by CNQX on GluR1 flip receptors was only ~3% of the current induced by CNQX in the presence of TCM (Fig. 2A and fig. S3B). If hippocampal CA1 pyramidal cells primarily express flip receptors, we would expect only 3 pA of CNQX-induced current in the absence of TCM, which is similar to the trend that we observed (–1.9 ± 2.6 pA). In contrast, the weaker TCM potentiation in Golgi cells may be due to expression of the flop variants of AMPA receptors, because the CNQX-induced currents from GluR1 flop receptors were only moderately potentiated by TCM (fig. S3). This moderate potentiation agrees with the known weaker potency of cyclothiazide, a congener of TCM, on flop receptors (*25*).

Although CNQX acts as a weak partial agonist, its occupancy of the AMPA receptor ligand-binding site would prevent the binding of other agonists and further activation. Thus, interpretations of previous experiments that used CNQX to antagonize AMPA receptors are valid, except perhaps in the case when neurons were incubated for extended periods with CNQX, which may lead to cell death and/or receptor internalization resulting from chronic AMPA receptor activity. Given that quinoxalinedione analogs, as well as other competitive antagonists of AMPA receptors, have undergone clinical testing for the treatment of numerous diseases, including epilepsy and stroke (*26*–*28*), better predictive activity of in vivo function may benefit from the inclusion of TARPs in in vitro drug screening assays.

Although previous work had shown that TARPs increase the efficacy of AMPA receptor agonists (*29*–*31*), their relationship to our structure- and function-based understanding of AMPA receptor gating had remained entirely unknown. Based on our current findings, we put forward a model in which TARPs either strengthen the coupling between agonist-induced domain closure and channel opening, perhaps by promoting linker separation, or directly enhance the degree of domain closure induced by CNQX. Further structural studies are required to distinguish between these two alternatives.

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33. We thank P. Apostolides, J. O'Brien, R. Robbins, and L. Martin for technical assistance, and A. Tzingounis, C. Jahr, and all members of the Nicoll lab for helpful discussions. This work was supported by NIH grants to R.A.N., L'Oreal UNESCO (United Nations Educational, Scientific, and Cultural Organization) and Epilepsy Foundation predoctoral fellowships to K.M., a National Research Service Award postdoctoral fellowship (GM078754) to F.A.H., and the Center for Innovation in Membrane Protein Production (P50 GM73210).

Supporting Online Material

www.sciencemag.org/cgi/content/full/318/5851/815/DC1
Materials and Methods
Figs. S1 to S3
Table S1
References

11 June 2007; accepted 1 October 2007
10.1126/science.1146317