Supplemental Data

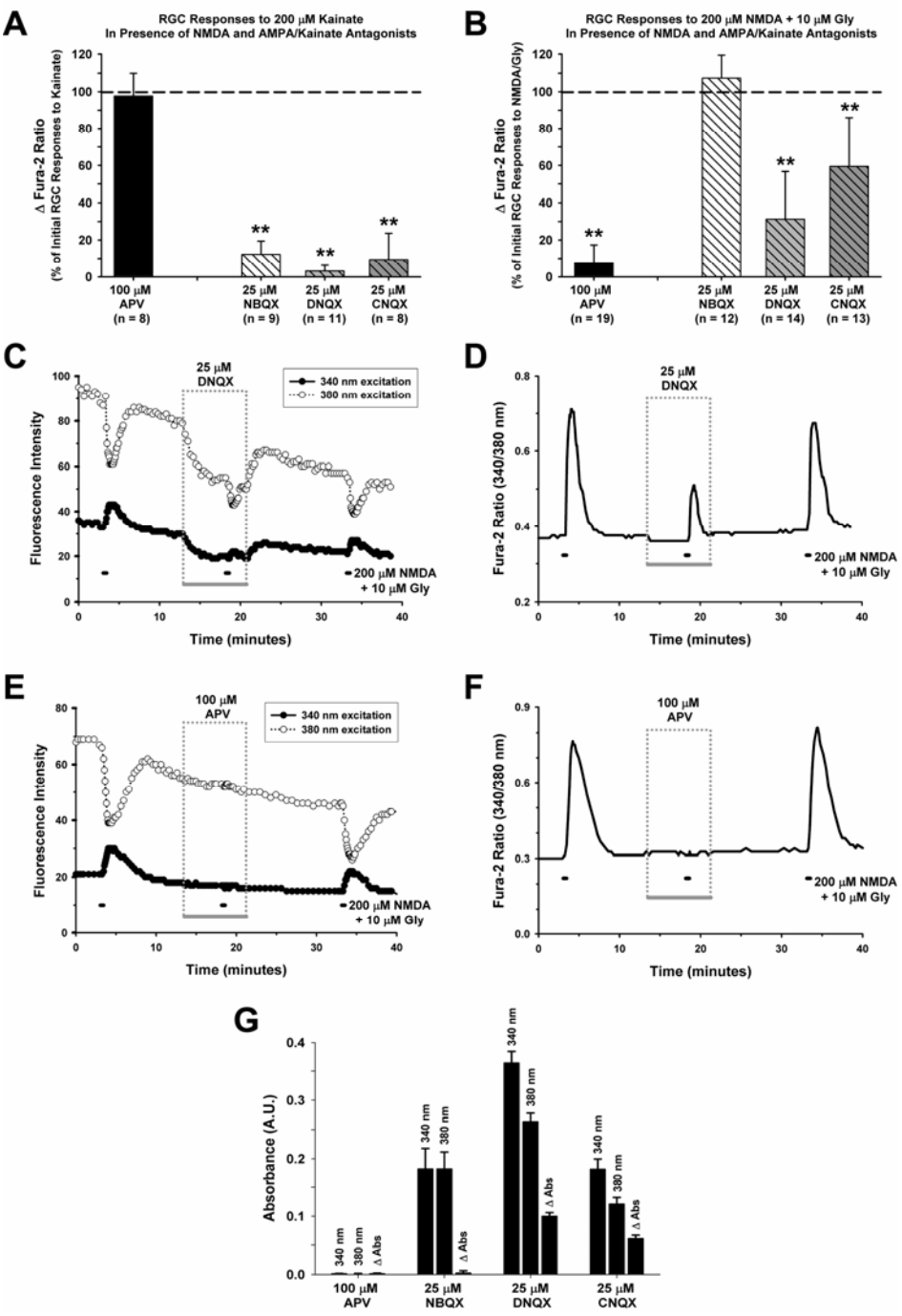
The quinoxalines DNQX, CNQX, and NBQX are commonly used AMPA/kainate-R antagonists, and DNQX was utilized in an earlier study that concluded the glutamate-evoked calcium signal in immunopanned RGCs occurs through an AMPA/kainate-R-mediated pathway (Otori *et al.*, 1998). Unexpectedly, in initial experiments, DNQX and CNQX appeared to inhibit both kainate and NMDA responses (partially, in the case of NMDA; summary of data in Supplemental Figure 1 A, B). Further analysis showed that the apparent inhibitory effect of DNQX and CNQX on NMDA-evoked signals was artefact due to the fluorescence quenching properties of these compounds. In contrast to the colourless 100 μM APV solution, the DNQX, CNQX, and NBQX solutions, dissolved at 25 μM in HBSS, all were yellow in colour. The addition of the yellow AMPA/kainate-R antagonists to the microscope chamber caused a decrease in fluorescence produced by either 340 or 380 nm excitation. The quenching effect of DNQX on raw fluorescence intensities is illustrated in Supplemental Figure 1 C, and the resulting trace of the fura-2 ratio (the quotient obtained after dividing the fluorescence intensity obtained with 340 nm excitation by that obtained with 380 nm excitation; 510 nm emission for both excitation wavelengths) for this same experiment is shown in Supplemental Figure 1 D. For comparison, the fluorescence intensities under 340 and 380 nm excitation and the resulting ratios for a RGC in which the NMDA-induced calcium signal was blocked by the colourless APV is shown in Supplemental Figures 1 E, F.

The quenching properties of the glutamate receptor antagonists (dissolved in HBSS at the same concentrations as for the imaging experiments) were further quantified by putting the compounds in quartz cuvettes (1 cm width) and measuring absorbances using an Ultrospec 2000 single-beam spectrophotometer (Amersham Biosciences, Piscataway NJ). The absorbance at 340 and 380 nm was determined and the absorbance of HBSS alone (no glutamate receptor antagonists added) was subtracted from each reading. The absorbance of NBQX (25 μM) at 340 and 380 nm was almost identical, while DNQX (25 μM) and CNQX (25 μM) solutions exhibited greater absorbance at 340 relative to 380 nm (Supplemental Figure 1 G). The differential absorbance at the two excitation wavelengths alters the relationship of the fura-2 ratio to $[Ca^{2+}]_i$ and therefore the apparent decrease in NMDA-evoked signals was artefact due to quenching effects of these compounds on fura-2 fluorescence. A

compound that quenches both excitation wavelengths equally, as occurred with 25 μM NBQX, does not appreciably alter the relationship of the fura-2 ratio to $[Ca^{2+}]_i$.

Therefore, 25 μM NBQX, which blocked kainate-induced calcium influx without affecting NMDA-related calcium signals (Supplemental Figure 1 A, B) was the AMPA/kainate-R antagonist utilized in subsequent experiments. This artefact is more pronounced when imaging with an upright microscope (as in the present study), because the excitation light travels a longer path through the absorbing solution than with an inverted microscope. Regardless, these results indicate that caution must be exercised in the interpretation of ratiometric data from imaging experiments involving fluorescence-quenching solutions such as quinoxaline AMPA/kainate-R antagonists.

Supplemental Figure 1. Fluorescence quenching by AMPA/kainate-R antagonists and its effect on ratiometric calcium imaging. **(A-B)** Mean data (± 1 SD) showing effect of NMDA-R (dark bar) and AMPA/kainate-R (hatched bars) antagonists on (A) kainate- and (B) NMDA-induced calcium influx (extracellular Mg^{2+} absent), normalized to initial kainate of NMDA responses (dashed line). ** $P < 0.01$, Friedman ANOVA, Tukey, as compared to both initial and recovery kainate/NMDA responses. **(C)** Fluorescence intensities following 340 and 380 nm excitation (510 emission) for an RGC treated with 200 μM NMDA plus 10 μM glycine in the presence or absence of 25 μM DNQX (AMPA/kainate-R antagonist), and **(D)** the resulting fura-2 ratio trace for the same experiment. Note the quenching effect of the yellow DNQX solution. **(E)** For comparison, the traces of raw fluorescence intensities and **(F)** ratios are shown for an imaged RGC in which the NMDA response is blocked by the colourless NMDA-R antagonist APV (100 μM). **(G)** Mean (± 1 SD) absorbance (in arbitrary units) of glutamate antagonist solutions (dissolved in HBSS, from 3 separate experiments) at 340 and 380 nm, as measured in a spectrophotometer (path length = 1 cm). The absorbance of HBSS alone was subtracted from each reading. The DNQX and CNQX solutions absorbed more at 340 nm relative to 380 nm (Δ ABS is difference between 340 and 380 nm absorbances), while the NBQX and APV solutions did not exhibit unequal absorbance.



Supplemental Figure 1.