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TCN 201 selectively blocks GluN2A-containing NMDARs in a GluN1 co-agonist dependent but non-competitive manner

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1. Introduction

NMDARs are a subtype of the ionotropic glutamate receptor family and are tetrameric assemblies, the majority of which contain two GluN1 subunits and two GluN2 subunits (Collingridge et al., 2009; Traynelis et al., 2010). It is thought that these subunits assemble in a ‘dimer of dimers’ configuration (Schorge and Colquhoun, 2003) forming a central non-selective cation-conducting ion channel pore that is permeable to Na⁺, K⁺ and Ca²⁺ ions but which is blocked by Mg²⁺ ions in a voltage-dependent manner (Traynelis et al., 2010). Alternative splicing of exons 5, 21 and 22 gives rise to eight GluN1 subunit isoforms (Sugihara et al., 1992) while 4 separate genes encode GluN2A-D subunits. These GluN2 subunits are spatially and temporally regulated (Monyer et al., 1994, 1992) and give rise to the majority of the distinct pharmacological and biophysical properties exhibited by NMDAR subtypes (Chen and Wyllie, 2006; Erreger et al., 2004; Traynelis et al., 2010). In vivo glutamate binds to the ligand-binding domain (LBD) of GluN2 subunits, while glycine (or D-serine) binds to the homologous NMD1 site (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Conversely, antagonism of NMDARs can be achieved by blocking agonist function at either GluN1 or GluN2 LBDs.

Relatively few NMDAR subtype-selective antagonists exist (Ogden and Traynelis, 2011) and until recently only the non-competitive allosteric inhibitors ifenprodil (Williams, 1993), CP 101,606 (Mott et al., 1998) and Ro 256981 (Fischer et al., 1997) are sufficiently selective for GluN2B-containing NMDARs to be identified unambiguously. For GluN2A-containing NMDARs the competitive antagonist NVP-AAM077 (Auberson et al., 2002) can be

Antagonists that are sufficiently selective to preferentially block GluN2A-containing NMDARs are few in number. In this study we describe a pharmacological characterization of 3-chloro-4-fluoro-N-[4-[[2-(phenylcarboxy)hydrazinocarbonyl]benzyl]benzenesulphonamide (TCN 201), a sulphonamide derivative, that was recently identified from a high-throughput screen as a potential GluN2A-selective antagonist. Using two-electrode voltage-clamp (TEVC) recordings of NMDAR currents from Xenopus laevis oocytes expressing either GluN1/GluN2A or GluN1/GluN2B NMDARs we demonstrate the selective antagonism by TCN 201 of GluN2A-containing NMDARs. The degree of inhibition produced by TCN 201 is dependent on the concentration of the GluN1-site co-agonist, glycine (or D-serine), and is independent of the glutamate concentration. This GluN1 agonist-dependency is similar to that observed for a related GluN2A-selective antagonist, N-(cyclohexylmethyl)-2-[[5-[(phenylmethyl)amino]-1,3,4-thiadiazol-2-y]thio]acetamide (TCN 213). Schild analysis of TCN 201 antagonism indicates that it acts in a non-competitive manner but its equilibrium constant at GluN1/GluN2A NMDARs indicates TCN 201 is around 30-times more potent than TCN 213. In cortical neurones TCN 201 shows only modest antagonism of NMDAR-mediated currents recorded from young (DIV 9–10) neurones where GluN2 expression predominates. In older cultures (DIV 15–18) or in cultures where GluN2A subunits have been over-expressed TCN 201 gives a strong block that is negatively correlated with the degree of block produced by the GluN2B-selective antagonist, ifenprodil. Nevertheless, while TCN 201 is a potent antagonist it must be borne in mind that its ability to block GluN2A-containing NMDARs is dependent on the GluN1-agonist concentration and is limited by its low solubility.
used to isolate GluN2A-containing NMDAR-mediated responses but care must be taken to use this antagonist at concentration that has minimal action at GluN2B-containing NMDARs since it only weakly discriminates between these two NMDAR subtypes (Frizzelle et al., 2006; Neyton and Paolelli, 2006; Wyllie and Chen, 2007).

More promisingly, two compounds have recently been identified (Bettini et al., 2010), now referred to as TCN 201 (originally called Compound 1) and TCN 213 (originally Compound 13), that appear to be selective for GluN1/GluN2A over GluN1/GluN2B NMDARs. In a recent study we have characterized the nature of TCN 213 antagonism and have demonstrated that this compound displays a high selectivity for GluN2A-containing NMDARs and can be used to monitor, pharmacologically, the switch in NMDAR expression in developing cortical neurons (McKay et al., 2012). The mechanism of TCN 213 appears to be paradoxical in nature; whilst the compound selects for GluN2A-containing NMDARs, the potency of TCN 213 block is dependent on the concentration of glycine and not that of glutamate. Using Schild analysis, TCN 213 was found to possess an equilibrium constant (Kg) of 2 μM. Although estimates of the concentration of glycine (or α-serine) in vivo have not been determined, unequivocally, they are likely to be in the micromolar range, while typically concentrations of at least 30 μM are contained in artificial cerebrospinal fluid solutions. Performing assays of NMDAR function to ensure saturation of the GluN1 binding site. Use of glycine (or α-serine) at this concentration, which is equal to 20 × its EC50 value at GluN1/GluN2A NMDARs (Chen et al., 2008), limits the effectiveness of TCN 213 due to this antagonist’s comparatively low affinity. Therefore relatively high concentrations of TCN 213 need to be used to achieve substantial block of GluN2A NMDAR-mediated responses (McKay et al., 2012).

The present study reports the pharmacological characterization of TCN 201—an antagonist suggested to be more potent than TCN 213 (Bettini et al., 2010) while still discriminating between GluN1/GluN2A and GluN1/GluN2B NMDARs. Our data show that TCN 201 is indeed more potent than TCN 213, but like TCN 213, its antagonism is also GluN1 co-agonist dependent. Furthermore the nature of its antagonism is not competitive and our results are consistent with it having an allosteric modulatory effect on glycine (or α-serine) binding. Complementary to our recent genetic approach to elucidate the GluN2 subunit dependency of NMDAR excitotoxicity (Martel et al., 2012), TCN 201 can also be used to assess the contribution of GluN2A subunits and adds to the list of new GluN2A-selective ligands in the pharmacological toolbox that can be used to elucidate NMDAR subunit composition and function.

2. Materials and methods

2.1. Plasmid constructs, cRNA synthesis and receptor expression in oocytes

Nomenclature of NMDA receptor subunits follows Collingridge et al. (2009) and Alexander et al. (2011). pSP64T-based plasmid constructs containing cDNA coding for rat GluN1-1a (i.e. the splice variant that lacks exon 5, but contains exons 21 and 22), hereafter termed GluN1 and wild-type rat GluN2A receptor subunits were prepared as described by (Chen et al., 2005). The rat GluN2B-containing cRNA expression vector was a gift by Stephen Traynelis (Emory University, Atlanta, GA). cRNA was synthesized as runoff transcripts as previously detailed (Chen et al., 2005; 2008; Erreger et al., 2007). Fluorescence intensity in ethidium bromide-stained agarose gels was employed to confirm the fidelity and yield of synthesized cRNAs. For recombinant receptor expression, GluN1 and GluN2B cRNAs were mixed at a nominal ratio of 1:1 and diluted with nuclease-free water to 5 ng μl−1. Oocytes (Stage V–VI) were removed from Xenopus laevis that had been killed in accordance with current UK Home Office protocols and defolliculated by initial collagenase treatment, then manually using forceps. 23 nl of cRNA mix was applied to isolated GluN2A-containing NMDAR-mediated responses and action potential driven excitatory/inhibitory postsynaptic events, respectively. Access resistances were monitored and recorded where this changed by >20% were discarded. Currents were filtered at 2 kHz and digitized online at 5 kHz via a BNC-2090A/PCI-6251 DAQ board interface (National Instruments, Austin, TX, USA) and analyzed using WinEDR 3.19 software (Dr John Dempster, University of Strathclyde, Glasgow, UK).

2.5. Assessment of antagonist potencies

The concentration of TCN 201 required to inhibit 50% (IC50) of agonist-evoked responses was determined by fitting data to the equation:

\[
I - I_\text{max} \left(1 - \left(\frac{I_\text{max} - I_0}{1 + (B/IC_{50})^n}\right)\right) = I_0
\]

where I is current response, I0 is predicted maximum current in the absence of antagonist, I0 is the predicted minimum current in the presence of an infinite concentration of antagonist [8] is the concentration of the antagonist and n represents the Hill coefficient. To obtain an overall mean IC50 value, data points were normalized to the predicted maximum, pooled and refitted with the equation, with the maximum of each curve being constrained to asymptote to 1 but with the minimum fitted as a free parameter (Otton et al., 2011; Wrighton et al., 2008). In addition we fitted data points where both the maximum and minimum were constrained (to 1 and 0, respectively). The F ratio was calculated from the following equation:

\[
F = \frac{SSR - SSR_{\text{adj}}}{DF_{\text{adj}} - DF} / \frac{SSR_{\text{adj}}}{DF}\]
unconstrained fits respectively and $DF_1$ and $DF_2$ are the degrees of freedom associated with each of the two fits.

2.6. Schild analysis

TCN 201 antagonism at the GluN1/GluN2A NMDAR was examined by the Schild method (Arunlakshana and Schild, 1959; Wyllie and Chen, 2007). Dose-ratios ($r$) were defined as the ratio of the concentration of agonist, in the presence of a fixed concentration of antagonist, required to evoke the same response that was obtained in the absence of the antagonist. Dose-ratios from individual oocytes were determined at low agonist concentrations by constructing a partial concentration-response curve generated in the absence of antagonist and in the presence of a series of increasing antagonist concentrations. Due to the lack of sufficient perfusion lines, the dose-ratio estimate for the higher concentrations of TCN 201 (3 and 10 μM) was determined in a separate series of experiments from those carried out for the lower two concentrations (0.3 and 1 μM).

Each series of two-point concentration-response curves were plotted on a log–log scale and the slope of the line used to fit the initial (agonist-free) two-point concentration-response curve was used to fit the remaining curves. These parallel fits were used to calculate an overall mean $r$ value for each antagonist concentration [8], which were then used to construct a Schild plot. On a log–log scale the gradient of the line used to fit such data is predicted by the Schild equation to be unity for a competitive antagonist. Thus, if the slope of a ‘free’ fit was not significantly different from 1, then the results were taken to be consistent with the Schild equation, and the data were refitted with the slope fixed at 1, i.e. they were refitted with the Schild equation,

$$r - 1 = \frac{[B]}{K_P}$$

in which the only free parameter is the intercept on the x-axis and which gives the equilibrium constant for antagonist binding, $K_P$. For ‘allosteric’ antagonism at equilibrium, the Schild plot deviates from unity at higher concentrations of antagonist (Christopoulos and Benikin, 2002). Such data can be fitted with the following modified equation:

$$r - 1 = \frac{[B](1 - \alpha)}{[B] + K_P}$$

where $\alpha$ is the allosteric constant and $K_P$ is the estimated allosteric antagonist dissociation constant. Note that if $\alpha = 0$, this equation simply reduces to the Schild equation. In addition, and unlike the Schild equation, $K_P$ does not give an $r$ value equal to 2 when $K_P$ equals [8] but rather will give an $r$ value less than 2 for $0 < \alpha < 1$ and thus $K_P$ should not be interpreted in the same way as the $K_B$ of a competitive antagonist.

2.7. Chemicals

Glutamate, glycine and $D$-serine were purchased from Sigma–Aldrich (Poole, UK). TCN 201, TCN 213, NMDA, ifenprodil, picrotxin and tetrodotoxin were purchased from Tocris Bioscience (Bristol, UK). Stock solutions (3 mM) of TCN 201 were made by dissolving the antagonist in DMSO. Due to the limited solubility of TCN 201 the maximum concentration used in our experiments was 10 μM – at higher concentrations a noticeable precipitate was observed in the external recording solutions used in either the TEVC experiments or whole-cell patch-clamp experiments.

2.8. Statistical analysis

Results are presented as mean ± standard error of the mean and statistical comparison between datasets was assessed using either Student’s t-test (paired where appropriate) or analysis of variance (ANOVA) tests to determine whether differences between mean values were significant ($p < 0.05$). Analysis of the fitting inhibition curves to unconstrained or constrained minima was assessed using an F-test. Microcal Origin v8.0 software was used for graphical presentation.

3. Results

3.1. TCN 201 demonstrates potent but GluN1 co-agonist concentration-dependent inhibition of GluN1/GluN2A NMDAR-mediated responses

Fig. 1ai shows two representative TEVC current traces recorded from a $X$. laevis oocyte and mediated by recombinantly expressed GluN1/GluN2A NMDARs. The extent of the antagonism exhibited by TCN 201 is dependent on the glycine concentration present in the external recording solution and noticeably less inhibition is present in the righthand trace ([glycine] = 30 μM; 20 × its EC$_{50}$ value). Fig. 1aii illustrates a comparable experiment where TCN 213, rather than TCN 201, was used – again note the decrease in the level of inhibition when glycine is present at the higher concentration. Fig. 1aiii shows the mean inhibition observed in a series of experiments for both TCN 201 and TCN 213 antagonism of GluN1/GluN2A NMDAR-mediated currents recorded in the presence of the lower (10 μM) or higher (30 μM) external glycine concentration. On average, and with glycine at 10 μM, TCN 201 produced 82.4 ± 1.1% ($n = 12$) inhibition whereas TCN 213 gave significantly less block (44.0 ± 2.2%; $n = 11$) of GluN1/GluN2A NMDAR-mediated currents ($p < 0.001$). Although both TCN 201 and TCN 213 gave less inhibition of currents evoked in the presence of the higher glycine concentration (50.7 ± 1.1%; $n = 8$ and 17.3 ± 1.4%; $n = 9$, respectively) the difference in potency between the two antagonists is still clearly evident ($p < 0.001$).

In contrast to the observed glycine dependency, the potency of block of TCN 201 (10 μM) was not dependent on the glutamate concentration used to evoke GluN1/GluN2A NMDAR-mediated currents (Fig. 1b). In these experiments glutamate was applied at either 3, 10 or 30 μM, while the glycine concentration was fixed at 30 μM. For each of the glutamate concentrations tested, the extent of block by TCN 201 was not significantly different ($F_{(2,14)} = 1.839$, $p = 0.19$, one-way ANOVA), with the mean values for the percentage inhibition being 54.5 ± 3.4% ($n = 5$; 3 μM), 52.2 ± 3.7% (n = 6; 10 μM) and 45.8 ± 2.8% (n = 6; 30 μM).

To assess TCN 201’s selectivity for GluN1/GluN2A NMDAs a similar series of experiments to those illustrated in Fig. 1ai were performed but in oocytes that expressed GluN1/GluN2B NMDARs. As shown in Fig. 1c, TCN 201 (10 μM) produced only slight inhibition of GluN1/GluN2B NMDAR-mediated currents. For glycine concentrations of 3, 10 and 30 μM the extent of block by TCN 201 was not significantly different ($F_{(2,15)} = 0.8116$, $p = 0.46$, one-way ANOVA), with the mean values for the percentage inhibition being 1.8 ± 0.6% (n = 6; 3 μM), 3.1 ± 1.0% (n = 6; 10 μM) and 3.1 ± 0.8% (n = 6; 30 μM). Thus our data demonstrate that TCN 201 selectively block GluN1/GluN2A NMDARs but in a manner that is dependent on the concentration of the GluN1-site agonist.

3.2. Inhibition curves for TCN 201 acting at GluN1/GluN2A NMDARs activated by glutamate and glycine or $D$-serine

We quantified the potency of TCN 201 by determining its IC$_{50}$ value at GluN1/GluN2A NMDARs under conditions where either glycine or $D$-serine was used as the GluN1-site agonist. Additionally, since IC$_{50}$ values are dependent on the concentration of the agonist used to evoke responses (Wyllie and Chen, 2007) three concentrations of each agonist were examined. As glycine and $D$-serine display the same potency at GluN1/GluN2A NMDARs (Chen et al., 2008) we were able to use the same representative data for each agonist in this series of experiments. Fig. 2ai shows a representative TEVC current trace where the NMDAR-mediated current was evoked by a saturating concentration of glutamate (30 μM; (Erreger et al., 2007)) and glycine (3 μM; 2 × its EC$_{50}$ value). Increasing concentrations (0.03–10 μM) of TCN 201 were applied cumulatively to inhibit the current. Fig. 2aii illustrates a similar experiment but under conditions where the glycine concentration was increased (30 μM; 20 × its EC$_{50}$ value). It is evident that TCN 201 causes substantially less inhibition of the TEVC current trace illustrated in Fig. 2aii. The mean data obtained from a series of such experiments are shown in Fig. 2b. As described in Materials and Methods these data were fitted in two ways – either the predicted minimum value of the fit was left unconstrained (solid lines) or constrained to asymptote to zero (dashed lines). For each of the glycine concentrations examined fitting the data points with curves with an unconstrained minima led to significantly improved the fits ($F_{(4,3)} = 10.34, p = 0.0422$; 3 μM; $F_{(4,3)} = 17.43, p = 0.0204$; 10 μM; $F_{(4,3)} = 36.28, p = 0.0071$; 30 μM) which leads to the prediction that TCN 201 will not completely inhibit responses mediated by GluN1/GluN2A NMDARs. The
calculated IC\textsubscript{50} values (which in this context is the concentration of antagonist that gives 50\% of the maximal amount of inhibition that can be achieved) and Hill slopes were 0.446 ± 0.026 μM, 1.42 ± 0.061 (n = 15, [glycine] = 3 μM), 0.746 ± 0.133 μM, 1.49 ± 0.21 (n = 5, [glycine] = 10 μM) and 3.89 ± 1.06 μM, 1.17 ± 0.11 (n = 10, [glycine] = 30 μM). We carried out a similar series of experiments and analyses using α-serine as the GluN1-site agonist (Fig. 2c, d) and again found that fitting the datasets with curves where the minima were left unconstrained produced significantly better fits than those where the minima were constrained to asymptote to zero (F(4,3) = 106.95, p = 0.0014; 3 μM; F(4,3) = 18.74, p = 0.0184; 10 μM; F(4,3) = 38.63, p = 0.00065; 30 μM). The respective calculated IC\textsubscript{50} values and Hill slopes obtained from these fits were 0.326 ± 0.018 μM, 1.57 ± 0.078 (n = 6, [α-serine] = 3 μM), 0.816 ± 0.231 μM, 1.33 ± 0.21 (n = 6, [α-serine] = 10 μM) and 1.92 ± 0.25 μM, 1.17 ± 0.07 (n = 6, [α-serine] = 30 μM).

Thus each of the datasets obtained at either the lower, intermediate or higher GluN1-site agonist concentration were better fitted with curves in which their minima were unconstrained. This indicates that TCN 201 will not produce complete block of NMDAR-mediated responses and is consistent with the notion that the antagonism is non-competitive in nature. Furthermore, the mean values for TCN 201 inhibition of GluN1/GluN2A NMDAR-mediated currents were significantly different (p = 0.0119, two-tailed t-test) when α-serine, rather than glycine was used as the GluN1-site agonist at the lowest concentration examined (3 μM). This ‘agonist-dependency’ of IC\textsubscript{50} values is not unexpected given that IC\textsubscript{50} values are dependent on agonist affinity and efficacy. Thus, we therefore decided to investigate further the nature of TCN 201’s antagonism by carrying out Schild analysis which does not suffer from such limitations (Wyllie and Chen, 2007).
Fig. 2. Inhibition curves for TCN 201 antagonism of GluN1/GluN2A NMDAR-mediated responses activated by co-agonists glycine or D-serine. (ai), TEVC trace recorded from an oocyte expressing GluN1/GluN2A NMDARs and voltage-clamped at −30 mV. The upper bar in this trace and in panels (a(ii), (c) and (c(ii)) indicates the duration of the bath application of glutamate/glycine, while the shaded bar in this panel (and in (ai), (ci) and (cii)) indicates the co-application TCN 201. Increasing concentrations of TCN 201 were applied, cumulatively, as indicated by the arrowheads. In (ai), as in (a), but currents were evoked using a higher concentration of glycine (30 μM). Note that TCN 201-mediated inhibition is less at this higher glycine concentration. (b), mean normalised inhibition curves for TCN 201 block of GluN1/GluN2A NMDAR-mediated currents evoked by glutamate (30 μM) and either 3 mM (n = 15; ■), 10 mM (n = 5; ▲) or 30 mM (n = 10; ●) glycine. The solid curves show the fit with the minimum fitted as a free parameter, whereas the dashed curves show the fit of the data points when the minimum value was constrained to 0 (see Materials and methods). (c), as in (ai) but where currents were evoked by glutamate (30 μM) and D-serine (3 mM), again increasing concentrations of TCN 201 (0.03–10 μM) were applied, cumulatively, as indicated by the arrowheads. In (ci), as in (c), but where currents were the D-serine was 30 μM. (d), mean normalised inhibition curves for TCN 201 block of GluN1/GluN2A NMDAR-mediated currents activated by glutamate (30 μM) and either 1 μM (n = 6; ■), 10 μM (n = 6; ▲) or 30 μM (n = 6; ●) D-serine.

3.3. Schild analysis of TCN 201 antagonism of GluN1/GluN2A NMDAR-mediated currents

Schild analysis (Arulnachaln and Schild, 1959; Wyllie and Chen, 2007) allows the unambiguous determination of the equilibrium constant (K0) for a competitive antagonist and is independent of the nature and concentration of agonist. Fig. 3a, b shows TEVC current traces that exemplify experiments carried out to determine the dose ratio (r) for shifts in glycine potency in the presence of increasing (0.3–10 μM) concentrations of TCN 201. Specifically, Fig. 3a depicts a series of recordings from a single oocyte where two concentrations of glycine (in the presence of glutamate (30 μM)) are applied in the absence (upper trace) or presence (middle and lower traces) of TCN 201 (0.3 and 1 μM). Fig. 3b shows a similar experiment but when the TCN 201 concentration was increased (3 and 10 μM). From these we constructed two-point concentration-response curves (Fig. 3c, d) and estimated r for each concentration of antagonist (n = 7). The mean r values obtained were 5.19 ± 0.4, 16.7 ± 2.1, 41.1 ± 1.5 and 49.0 ± 2.0 for 0.3, 1, 3 and 10 μM TCN 201, respectively. Note that the shift in the concentration-response curve in the presence of the highest concentration of TCN 201 (10 μM) is considerably less than would be predicted for competitive antagonism. Indeed this smaller than predicted shift is clearly observed in the Schild plot (Fig. 3e). However the first three data points (0.3, 1 and 3 μM TCN 201) appear to give a straight line. A linear regression fit of these data gives a line with a slope of 0.98 (95% confidence interval: 0.85–1.14). The fit of the data (solid line) illustrated in Fig. 3e shows a line whose slope has been constrained to unity and allows the derivation of the K0 for TCN 201 (70 nM). For comparison, data obtained from our study of TCN 213 antagonism (McKay et al., 2012) is illustrated — note the lower potency of this antagonist (K0 = 2 μM). Clearly however, the entire dataset for TCN 201 antagonism is not satisfactorily described by the Schild equation and this can be taken to indicate that TCN 201 is not acting in a competitive manner at the GluN1 agonist binding site (as indeed is also suggested by the data presented in Fig. 2). Thus, the dotted line (Fig. 3e) shows the complete dataset fitted with a modified equation (Christopoulos and Kenakin, 2002) which has previously been used to describe agonist-surnountable ‘allosteric’ non-competitive antagonism at G-protein coupled receptors. This fit better describes the data and gives an allosteric K0 of 56 nM and an
allosteric constant (\(a\)) of 0.0123. This indicates that the maximum shift in the dose ratio that can be obtained with TCN 201 is around 80-fold (= 1/\(a\)). However such a shift would only be achieved with a TCN 201 concentration of approximately 275 \(\mu\)M which is well above its limit of solubility. In addition we carried out Schild analysis using \(\beta\)-serine, rather than glycine, as the GluN1-site agonist (Fig. 3f). A linear regression fit of the data points obtained with 0.3, 1 and 3 \(\mu\)M TCN 201 gives a line with a slope of 0.87 (95% confidence interval: 0.71–1.03). The fit of the data (solid line) illustrated in Fig. 3e shows a line whose slope has been constrained to unity and gives a \(K_B\) for TCN 201 of 81 nM – comparable to the value obtained when glycine was used as the GluN1-site agonist. Similar to our findings when glycine was used as the GluN1-site agonist, the data point at the highest concentration of TCN 201 (10 \(\mu\)M) gives an \(r\) value that deviates considerably from the linearity. Again using the modified equation we estimated an allosteric \(K_B^a\) of 66 nM and an allosteric constant of 0.0106. Given the good agreement between these values with those obtained when glycine was used as the GluN1-site agonist and data reported in a recent study which has also examined the mechanism of action of TCN 201 (Hansen et al., 2012) it would seem unlikely that the deviation from linearity of the Schild plot arises from the limitation of TCN 201's solubility.

3.4. Antagonism by TCN 201 of NMDAR-mediated responses in rat cortical neurones

In a final series of experiments we assessed the ability of TCN 201 to antagonize NMDA-mediated currents in rat cortical neurones to assess the utility of this compound in identifying NMDAR subunit populations. It should be noted that as we used NMDA (rather than glutamate) in these experiments we determined whether TCN 201 (10 \(\mu\)M) blocked, to the same extent, GluN1/GluN2A NMDAR-mediated currents elicited by NMDA/glycine compared with currents elicited by glutamate/glycine. We observed significantly less inhibition of NMDA/glycine-evoked currents (69.9 ± 4.1%; \(n = 12, p < 0.001\), data not shown) compared to glutamate/glycine-evoked currents (91.2 ± 1.1%; Fig. 2b). This emphasizes the importance of considering the nature of the agonist used to evoke responses but establishes the maximum inhibition expected for TCN 201 block of NMDA/glycine-evoked currents for a population of NMDARs comprised solely of GluN1 and GluN2A NMDAR subunits.

Fig. 4a shows a series of NMDAR-mediated currents recorded from rat cortical neurones under three separate conditions. Fig. 4ai shows a representative recording from a neurone (DIV 9–10) where the predominantly expressed GluN2 subunit is anticipated to be
GluN2B. As is illustrated in the righthand trace and quantified in Fig. 4b, ifenprodil (3 μM) produces substantial block (80% ± 3%, n = 7) of this current as would be anticipated for a NMDAR population that contains a high proportion of GluN1/GluN2B NMDARs. Importantly, TCN 201 gives only a modest block of this current (5% ± 2%) which is comparable to the extent of TCN 201 block seen at recombinant GluN1/GluN2B NMDARs (Fig. 1c). To increase the number of GluN2A-containing NMDARs we transfected neurones with pCis-GluN2A plasmids (Rutter and Stephenson, 2000). NMDAR-mediated currents recorded from these neurones (Fig. 4aii) now displayed a reduced sensitivity to ifenprodil (24% ± 3% block, n = 6; Fig. 4b) but increased sensitivity to TCN 201 (47% ± 4% block, n = 6; Fig. 4b). Finally, the developmental increase in GluN2A expression was confirmed by recording NMDAR-mediated currents from neurones that had been cultured for longer time periods (Fig. 4aiii). In these neurones we observed that currents were less sensitive to ifenprodil (57% ± 5% block, n = 9; Fig. 4b) compared to those from DIV 9–10 cultures. This reduction in ifenprodil sensitivity was accompanied by an increase in the extent of the block produced by TCN 201 compared to that observed for currents in DIV 9–10 (non-transfected) cultures (16% ± 3% block, n = 9; Fig. 4b, p < 0.01). Furthermore, while the range of ifenprodil and TCN 201 block of currents showed considerable variation within each of these groups there was a strong inverse correlation (R² = 0.91) in the sensitivities of currents to these two NMDAR subtype-selective antagonists (Fig. 4c). A similar observation has been documented for ifenprodil and TCN 213 block of NMDAR-mediated currents in cortical neurones (McKay et al., 2012).

4. Discussion

Three main points in regard to antagonism of NMDARs by TCN 201 are made in this study. Firstly, TCN 201 is a selective GluN1/GluN2A NMDAR antagonist showing little antagonism at GluN1/GluN2B NMDARs. Secondly, TCN 201 acts in a non-competitive manner with the extent of its block being dependent on the concentration of the GluN1-site agonist (glycine or D-serine) present in the external salt solution. Thirdly, TCN 201 antagonism of NMDAR-mediated currents shows a negative correlation with their ifenprodil sensitivity and therefore, in combination with ifenprodil, can be used to monitor, pharmacologically, the expression levels of GluN2A and GluN2B NMDAR subunits in neuronal populations.

4.1. GluN1-site agonist-dependency of TCN 201 antagonism

TCN 201 antagonism of GluN1/GluN2A NMDAR-mediated currents is glycine (D-serine) dependent. In this respect TCN 201 antagonism resembles that produced by the chemically-related compound TCN 213 (Bettini et al., 2010; McKay et al., 2012). When compared with our recent study of TCN 213 antagonism of NMDARs, the data presented here show that TCN 201 is a more...
potent antagonist (as was predicted in binding assays performed by Bettini et al., 2010). Furthermore, our data also suggest that TCN 201 is unable to block completely NMDAR-mediated responses under conditions where the GluN1-site agonist is present at concentrations that may reflect those found in cerebro-spinal fluid in vivo (Fig. 2b, d). The data obtained were fitted with curves whose minima were unconstrained predicted a maximal inhibition, in the presence of saturating glycine (or α-serine), of around 40%. Due to the limited solubility of TCN 201 in our solutions we were unable to verify the precise level of maximum inhibition experimentally but the predicted incomplete block of NMDARs when glycine (or α-serine) is present at ≥30 μM needs to be borne in mind when using this novel GluN2A-selective antagonist.

4.2. TCN 201 antagonism is not competitive

Quantitative analysis of antagonist action is best carried out using Schild analysis (Arumalakshana and Schild, 1959) to determine the nature of the antagonism and (if appropriate) the Kᵦ of the antagonist. Our analysis reveals that TCN 201 does not act in a competitive manner at GluN1/GluN2A NMDARs since the Schild plots for TCN 201 when either glycine or α-serine was used as the GluN1-site agonist deviated from linearity at the highest antagonist concentration used in this study (10 μM). While we were able to fit a line with unity slope to the first three data points of the TCN 201 Schild plots (Fig. 3e, f) the intercept on the abscissa perhaps more properly describes the concentration of TCN 201 that gives a dose ratio of 2 (the negative logarithm of which will equal the pA₂ for TCN 201) rather than strictly its Kᵦ value which should be reserved for purely competitive antagonists (Wyllie and Chen, 2007). Comparison of pA₂ values for TCN 201 and TCN 213 (7.15 versus 5.69) shows that TCN 201 is approximately 30-times more potent at GluN1/GluN2A NMDARs.

The hyperbolic nature of the TCN 201 Schild plot is indicative of an allosteric non-competitive antagonism and the data points were better fitted by an alternative equation that has previously been associated with the description of action of agonist-surnountable non-competitive inhibitors acting at G-protein coupled receptors (Christopoulos and Kenakin, 2002). In the case of these surmountable non-competitive inhibitors, deviations away from unity at higher concentrations of antagonist are due to the saturable nature of the antagonism. Since saturable behaviour can be achieved, allosteric antagonists will shift the agonist concentration-response curve to the right, in a manner similar to competitive antagonism, but according to a defined limit (Christopoulos and Kenakin, 2002). Our estimates of an allosteric Kᵦ of 56 nM (66 nM) and an allosteric constant of 0.0123 (0.1016), when glycine (α-serine) is used as the GluN1-site agonist, are in excellent agreement with a recent study that has employed a similar analysis of TCN 201 antagonism at GluN1/GluN2A NMDARs since the amount of block obtained was similar to that seen when TCN 201 antagonized NMDA-evoked responses in oocytes expressing GluN1/GluN2A NMDARs. Finally in older cultures the extent of TCN 201 block was variable but always (negatively) correlated with that of ifenprodil and indicated an increased expression of GluN2A-containing NMDARs, as is to be expected with this stage of development. What remains to be determined is the relative contribution of heterodimeric (GluN1/GluN2A) and heterotrimeric (GluN1/GluN2A/GluN2B) NMDAR combinations in this population. Related to this is the fact that we do not know the potency of TCN 201 when it acts at GluN2A/B-containing heterotrimeric NMDARs—a combination thought to represent a substantial proportion of NMDARs in the adult forebrain (Chazot and Stephenson, 1997; Rauner and Kohr, 2011).

5. Conclusion

TCN 201 is a potent selective inhibitor of GluN1/GluN2A NMDARs that displays only minimal activity at GluN1/GluN2B NMDARs. The potency of its antagonism is dependent on the concentration of the GluN1-site agonist and not that of the GluN2-site agonist. Schild analysis demonstrates that the nature of its antagonism is not competitive but rather is consistent with the notion that TCN 201 acts by an allosteric non-competitive mechanism. TCN 201, like TCN 213 (Mckay et al., 2012), can be used to monitor, pharmacologically, the change in GluN2 NMDAR subunit expression levels in developing central neurones. Nevertheless, while TCN 201 offers the opportunity to block selectively GluN2A-containing NMDARs care must be taken in experimental designs to take account of (1) TCN 201 block is strongly-dependent on the GluN1-site agonist concentration and (2) TCN 201 possesses a comparatively low solubility in salt solutions that are commonly used when assessing NMDAR function.
Conflict of interest
The authors state no conflict of interest.

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