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Pharmacology of $5HT_{2C}$ receptor-mediated ERK1/2 phosphorylation: Agonist-specific activation pathways and the impact of RNA editing

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ABSTRACT

We have previously characterized a mechanism of 5HT-stimulated extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation via the non-RNA-edited isoform of the serotonin $5HT_{2C}$ receptor ($5HT_{2C}$R-INI) in a CHO cell line. We have now used CV1 cells, which endogenously express epidermal growth factor receptors (EGFRs), to investigate whether the mechanisms underlying ERK1/2 activation by the $5HT_{2C}$R change in a time-, agonist-, and cell background-dependent manner. Interrogation of the CV1 $5HT_{2C}$R-INI ERK1/2 signaling pathway, using a variety of pathway-selective inhibitors, revealed a clear time-dependence in the involvement of specific pathway components such as phosphatidylinositol 3-kinase, EGFR, matrix metalloproteases and protein kinase C. The contribution of these components to the overall response also varied with the agonist used to stimulate the receptor, providing further evidence for the ability of $5HT_{2C}$R-INI to signal in an agonist-specific manner. We also investigated the impact of $5HT_{2C}$R RNA editing on this phenomenon. Although we found no alteration in antagonist pharmacology, the partially edited VSV and fully edited VGV isoforms of the $5HT_{2C}$R exhibited altered temporal and pharmacological characteristics, including the degree of dependence on specific effectors, in signaling to ERK1/2 in comparison to the $5HT_{2C}$R-INI. In conclusion, we provide evidence for remarkable flexibility in $5HT_{2C}$R-mediated ERK1/2 signaling that can be pharmacologically and mechanistically distinct depending on the agonist or edited isoform involved and on the duration of receptor activation.

1. Introduction

The serotonin $5HT_{2C}$ receptor ($5HT_{2C}$R) is a member of the seven-transmembrane domain G protein-coupled receptor (7TMR) superfamily. This receptor is expressed at high levels in various brain regions including the choroid plexus and the hippocampus and is involved in the regulation of mood and feeding behavior [1,2]. Dysregulation of the receptor has been implicated in various psychological disorders, such as anxiety, depression, schizophrenia, and also in the development of obesity and epilepsy [3]. However, it is not clear which facets of $5HT_{2C}$R signaling are important to its physiological functions.

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Much of the uncertainty in identifying the mode of action of the $5HT_{2C}R$ relates to the promiscuity with which the receptor couples to G proteins [1]. This pleiotropic coupling to multiple intracellular pathways has been used to demonstrate ligand-directed trafficking of receptor stimulus (LDTRS), a phenomenon characterized by the generation of agonist-specific receptor conformational states [4,5]. Pharmacological orders of potency and/or efficacy thus become effector pathway-dependent, rather than receptor subtype-dependent [6–8]. Recently, data from other 7TMR systems have shown that signals to extracellular signal-regulated kinases 1 and 2 (ERK1/2) can be directed to either G protein-dependent or -independent arms of the ERK1/2 signaling network in an agonist-specific manner [9,10], suggesting exquisite sensitivity of the effector system to discrete receptor active conformations. Extending this paradigm, there is scope for an agonist to direct signals along a very specific route even through the G protein-dependent arm of the ERK1/2 network according to the combination of G proteins that are activated by that agonist, especially within the exceedingly complex network of effectors connecting 7TMRs to ERK1/2 stimulation [11].

In addition to its ability to mediate LDTRS, the $5HT_{2C}R$ is also pharmacologically interesting in that it is the first and currently only 7TMR identified to undergo RNA editing [2,12]. This process involves the deamination of five specific adenosine residues in the $5HT_{2C}R$ pre-mRNA [13], which affects the identity of amino acids that are incorporated into the mature protein at positions 156, 158 and 160 (corresponding to the amino acids isoleucine (I), asparagine (N) and isoleucine (I), respectively, in the non-edited isoform, $5HT_{2C}R$-INI), and can result in, potentially, 24 different receptor isoforms. Importantly, the affected amino acid positions constitute part of the second intracellular loop of the $5HT_{2C}R$, a region critical to G protein coupling. Consequently, RNA editing alters various facets of $5HT_{2C}R$ signaling, including phosphoinositide, calcium and arachadonic acid responses [12,14], constitutive receptor activity [15,16], Rho activation [17] and receptor desensitization [18]. RNA editing has been suggested to be one of the possible modes of perturbation leading to certain $5HT_{2C}R$-dependent pathophysiologies [19–21].

Recently, we presented the first characterization of the coupling of the $5HT_{2C}R$ to the phosphorylation of ERK1/2 [7]. That study provided further evidence for the ability of the $5HT_{2C}R$ to mediate LDTRS, and characterized a mechanism by which the endogenous agonist, 5HT, stimulates ERK. However, we only utilized the non-edited $5HT_{2C}R$-INI isoform, and mechanistic information was not obtained for alternative agonists. The aim of the present study, therefore, was to dissect the mechanisms underlying ERK1/2 activation by different $5HT_{2C}R$ agonists at the $5HT_{2C}R$-INI to ascertain whether the route taken through the G protein-signaling network to ERK1/2 activation varied in an agonist-dependent manner. We also aimed to investigate the effects of RNA editing on the temporal, pharmacological and mechanistic aspects of $5HT_{2C}R$-mediated ERK1/2 signaling; for this, we chose the fully edited VGV isoform and the partially edited, but most abundant, VSV isoform. We find that the identity of the stimulating agonist, and also the duration of agonist stimulation, impart on the ERK1/2 response distinct profiles of activation. We conclude that this may affect the spatial localization and physiological outcome of the resultant ERK1/2 signal in a time- and agonist-dependent manner. We also find that RNA editing significantly changes the potency and efficacy with which agonists activate ERK1/2 via the $5HT_{2C}R$ and the pathway components involved in this activation. These data support the concept that differential expression of different RNA-edited $5HT_{2C}R$ isoforms, or stimulation of the receptor with synthetic ligands, may have profound influence on the magnitude, duration, location and target of the signaling arising from receptor activation.

2. Materials and methods

2.1. Materials

Fetal bovine serum was from JRH (Lenexa, KS, USA). Dulbecco's minimal essential medium (DMEM), penicillin–streptomycin additive and hygromycin were obtained from Invitrogen (Victoria, Australia). The pENTR/D-TOPO and pEF5/FRT/V5-DEST vectors, LR Clonase™ enzyme and Lipofectamine™ 2000 were also from Invitrogen. QuikChangeII® mutagenesis kits were supplied by Stratagene (through Integrated Sciences, Melbourne, Victoria, Australia). [^{3}H]mesulergine (73 Ci/mmol) was from NEN Life Sciences Products, Inc. (Boston, MA, USA). 4-2-Hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), U0126, serotonin, DOI, quipazine, mianserin (MSN) and clozapine (Cloz) were supplied by Sigma–Aldrich (NSW, Australia). GM6001, Rö318220, AG1478, and LY294002 were from Calbiochem (NSW, Australia). The *SureFire*® AlphaScreen® kit used for all estimations of ERK1/2 phosphorylation was from TGR BioSciences (Thebarton, SA, Australia).

2.2. Cell line generation and culture

The non-RNA-edited $5HT_{2C}R$ cDNA sequence was inserted into a Gateway entry clone pENTR/D-TOPO vector, and multiple-point mutagenesis was carried out using the QuikChangeII® Site-Directed Mutagenesis kit to create the two $5HT_{2C}R$ edit forms. The forward primer (5′ → 3′) used for conversion of INI to VSV was: CG CTG GAT CGG TAT GTA GCA **G**TA CGT A**G**C CCT **G**TT GAG CAT AGC CGT TTC AAT TCG. Shown in bold are the editing sites targeted (A, C and D). The forward (5′ → 3′) primer used for conversion of INI to VGV was: CG CTG GAT CGG TAT GTA GCA **G**TA CGT **GG**T CCT **G**TT GAG CAT AGC CGT TTC AAT TCG. Shown in bold are the editing sites targeted (A, C′, C and D). Editing of site B (underlined in both) was not necessary in either construct as this is a redundant site in these instances that does not change the desired incorporation of valine into position 156. Constructs were cloned into the pEF5/FRT/V5-DEST vector using LR Clonase™ according to the manufacturer's instructions. Constructs were transfected into Flp-In-CV1 cells (an African green monkey kidney epithelial cell line). Stable transfections were performed using Lipofectamine™ 2000 and 1 μg of each DNA together with 9 μg of recombinase-encoding plasmid, pO44G. Cells expressing either INI, VSV or VGV forms of the $5HT_{2C}R$ were selected using 400 μg/ml hygromycin and grown in DMEM containing 10%

heat-inactivated fetal bovine serum, 2 mM HEPES, penicillin (50 U/ml) and streptomycin (50 mg/ml) in a humidified CO_2-enriched (5%) atmosphere. Once all non-expressing cells were eliminated, the concentration of hygromycin was reduced to 200 μg/ml to maintain the purity of the stable cell lines during passaging.

2.3. Saturation binding assay

CV1 cells were harvested and washed with high ionic strength HEPES buffer (in mM; NaCl, 110.0; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 1.0; glucose, 25.0; HEPES, 20.0; sucrose, 58.4) before centrifuging (400 × *g*, 5 min). The resulting pellet was resuspended in ice-cold low ionic strength HEPES buffer (in mM; HEPES, 50; $MgCl_2$, 2.5; EGTA, 2.0, pH 7.4), homogenized using 3 × 10 s bursts separated by 30 s on ice with a hand held tissue homogenizer (PT-DA 1205/2EC Polytron Aggregate; Kinematica), and centrifuged (400 × *g*, 10 min). The supernatant was then centrifuged (31,000 × *g*, 30 min, 4 °C) using a Model J2-M1 centrifuge (Beckman, Fullerton, CA, USA) and the resulting pellet was resuspended in 3 ml ice-cold low ionic strength HEPES buffer, assayed for protein content using the method of Bradford [22] with bovine serum albumin as the standard, and frozen at −80 °C until required for experiments.

[^{3}H] mesulergine saturation assays were performed as described previously [23]. Briefly, assays were performed in triplicate in a volume of 500 μl low ionic strength HEPES buffer containing 1 mg/ml ascorbic acid, 100 μg of membrane protein from CV1 cells, and various concentrations of [^{3}H]mesulergine. Incubation (1 h, 37 °C), in a shaking water bath, was terminated by the addition of 2 ml ice-cold 0.9% (w/v) NaCl solution followed by rapid filtration using a Brandel cell harvester (Gaithersburg, MD, USA) and washing with 2× 2 ml ice-cold 0.9% (w/v) NaCl solution. Filters (Whatman GF/C) were pre-soaked in 0.9% (w/v) NaCl solution containing 1% polyethyleneimine (PEI) at 4 °C for 1 h prior to filtering. Filters were placed in 7 ml scintillation vials (Wallac, Gaithersburg, MD, USA), followed by addition of 5 ml of scintillation cocktail (Ultima Gold LSC-cocktail, Packard). Vials were then left to stand until the filters became uniformly translucent (at least 2 h) before the radioactivity was determined using a Model 1409 DSA Liquid Scintillation counter (Wallac).

2.4. ERK1/2 phosphorylation

The combined levels of both isoforms of phosphorylated ERK1/2 were measured using the *SureFire*® AlphaScreen® (Amplified Luminescent Proximity Homogeneous Assay-Screen)-based assay. Data showing a comparison of *SureFire*® results against western blot-determined values in the $5HT_{2C}$R-expressing Chinese hamster ovary (CHO) cell line described previously [7] are shown in Fig. 1A. We have also validated the kit extensively against the more traditional western blot technique at other 7TMRs [24].

Cells were plated into 96-well plates at 40,000 cells per well and grown to approximately 80–90% confluence before serum starvation overnight. At the relevant time prior to assay, pharmacological inhibitors (where utilized) were added and incubated until assay at 37 °C (see Section 3 inhibitors and concentrations). Agonist concentration–response curves were constructed using the time to peak ERK1/2 phosphorylation, as determined in separate time course experiments (see Section 3). For Schild-type analyses using the antagonists MSN or Cloz, each antagonist was pre-equilibrated with cells for 30 min prior to stimulation with 5HT for 5 min. For the subsequent pharmacological pathway inhibitor studies, three different time points of agonist stimulation were investigated: 5, 10 or 30 min. In each instance, pharmacological inhibitors of

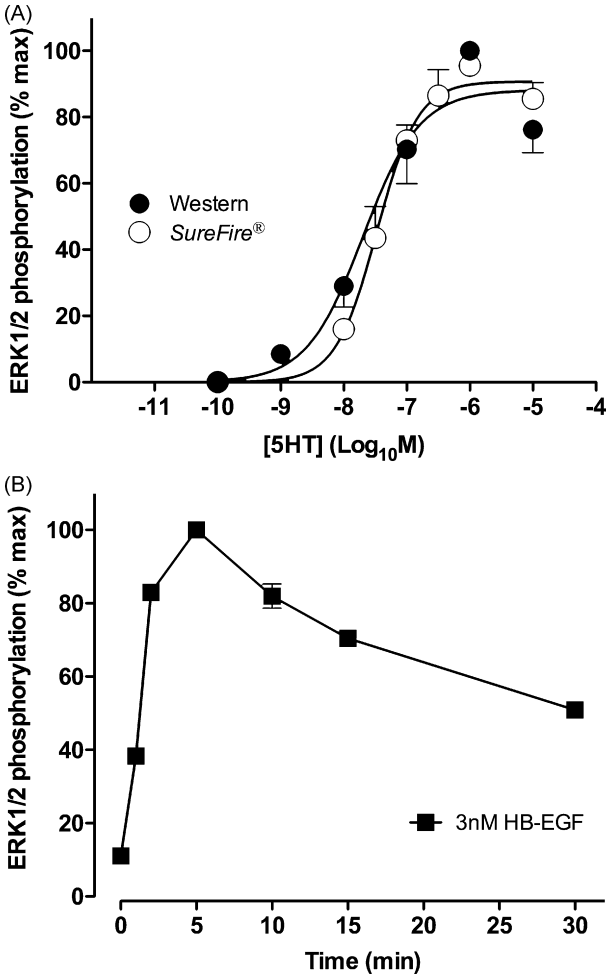

Fig. 1 – (A) Comparison of *SureFire*® phospho-ERK1/2 detection kit with more traditional western blotting procedures. CHO-$5HT_{2C}$R-INI cells were stimulated with increasing concentrations of 5HT for 5 min and processed either by western blot procedures (for methods see Ref. [7]) or by the phospho-ERK1/2-specific *SureFire*® kit used in the current study. Data represent combined quantification of both isoforms (1 and 2) of phospho-ERK and are expressed as a percentage of the maximal response to 5HT (i.e., 1 μM). Further validation of the kit has been performed previously [24]. (B) Confirmation of epidermal growth factor receptor expression in CV1 cells. CV1-$5HT_{2C}$R-INI cells were stimulated for various periods of time with the EGFR ligand, heparin-binding EGF-like growth factor (HB-EGF; 3 nM) and ERK1/2 phosphorylation assessed using the *SureFire*® ERK1/2 kit. Data are expressed as a percentage of the maximal response to HB-EGF.

selected signaling pathways were first pre-equilibrated with cells for 30 min prior to agonist stimulation, such that the total exposure time to inhibitor was 35, 40 or 45 min, respectively; there were no significant effects of any of the inhibitors on basal ERK phosphorylation (not shown). The reaction was then terminated by removal of the media followed by lysis using the proprietary lysis buffer supplied with the *SureFire*® kit. After processing these samples following the manufacturer's instructions, we used a Fusion-α^{TM} plate reader (PerkinElmer) to excite the donor beads and to read the light emitted from the acceptor beads following energy transfer. All assays included a positive and negative control for ERK1/2 activation in cells (5 min incubation with 1 μM 5HT or buffer, respectively), and a cell-free control, containing only kit components and buffer, to estimate energy transfer arising from random collisions between beads (approx. 500 relative fluorescence units (RFU)).

2.5. Data analysis

Saturation binding data were analyzed according to a one site hyperbolic binding model using Prism 5.01 (GraphPad Software, San Diego, CA) to derive estimates of [^{3}H]mesulergine B_{max} (total receptor density) and K_D (radioligand-receptor equilibrium dissociation constant). For the functional ERK1/2 experiments, data were normalized either against the basal signal (''fold of basal'') or, for the 5HT–antagonist interaction experiments, as a percentage of the maximal agonist response (after baseline subtraction) determined in the absence of antagonist (''% control 5HT''), as indicated in each figure. For the initial agonist characterization experiments, concentration–response curves were fitted by non-linear regression with Prism to the following four-parameter logistic equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{\log(EC_{50} - [A])n_H}} \quad (1)$$

where Y is the response; [A] is agonist concentration; Top and Bottom are the upper and lower asymptotes, respectively; EC_{50} is the midpoint location (potency) parameter; and n_H is the Hill slope [25]. However, subsequent 5HT–antagonist interaction experiments were characterized by a saturable depression of agonist maximal effect with increasing antagonist concentrations (see Section 3). Thus, Eq. (1) was not utilized to determine agonist potency values for further analysis of the antagonistic effect because the EC_{50} values obtained under such conditions do not represent equi-effective concentrations (see Ref. [26]). Rather, agonist concentration–response curves, determined in the absence and presence of antagonist, were normalized as described above and fitted to the following sigmoidal concentration–response equation:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log EC_F - \log[F/100 - F]) - \log[A]}} \quad (2)$$

where F represents an arbitrary percent response level between 0 and 100 [25]. For our analysis, this value was fixed to 20, thus allowing for the estimation of the logarithm of the agonist concentration that produces the equivalent of 20% of the maximal control agonist response at all antagonist concentrations. The estimated ''log $EC_{20\%}$'' values were then converted to negative logarithms ''$pEC_{20\%}$'' and fitted to the following equation of agonist–antagonist interactions by non-linear regression:

$$Y = -\log([B]^S + 10^{-pA_2 \times S}) - P \quad (3)$$

where Y is the $pEC_{20\%}$; [B] is the antagonist concentration; S is a logistic slope factor analogous to the Schild slope; pA_2 is the negative logarithm of the concentration of antagonist needed to shift the concentration–response curve twofold to the right; and P is a fitting constant [25,27]. This analysis permitted the estimation of both the slope and pA_2 values for each inhibitor. An F-test was employed to determine whether the value of S differed significantly from unity.

2.6. Statistical analysis

All estimates of potency or affinity were determined as logarithms. For inhibitor studies, variations in agonist-mediated ERK1/2 phosphorylation in the presence of different inhibitors over time were tested for statistical significance using repeated measures- or two-way analysis of variance (ANOVA), with a Bonferroni's post-test performed between various time points (see individual figures for specific comparisons). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Characterization of ERK1/2 signaling by $5HT_{2C}$R-INI in CV1 cells

Saturation binding studies using [^{3}H]mesulergine revealed that all three isoforms of the $5HT_{2C}$R were expressed at low levels in the CV1 cells: $5HT_{2C}$R-INI, $B_{max} = 141 \pm 44$ fmol/mg protein, $pK_D = 8.7 \pm 0.6$, $n = 3$; $5HT_{2C}$R-VSV, $B_{max} = 101 \pm 9$ fmol/mg protein, $pK_D = 8.9 \pm 0.5$, $n = 3$; $5HT_{2C}$R-VGV, $B_{max} = 103 \pm 10$ fmol/mg protein, $pK_D = 9.2 \pm 0.6$, $n = 3$. We thus anticipated that under these conditions, we would be unlikely to see any receptor reserve in agonist response.

We have shown previously that the $5HT_{2C}$R-INI is capable of coupling to the ERK1/2 pathway when expressed in Chinese hamster ovary cells [7]. The temporal profile of this coupling was transient, with a peak at 5–10 min and a rapid dephosphorylation of ERK1/2 such that signals at 30 min were no different to basal levels. CHO cells, however, lack appreciable expression of epidermal growth factor receptor (EGFR) [7], transactivation of which can be a major contributor to 7TMR-mediated ERK1/2 activation [28]. In contrast, CV1 cells express endogenous EGFRs (e.g., Ref. [29] and Fig. 1B), and were therefore chosen to further our investigations of $5HT_{2C}$R-mediated ERK1/2 signaling.

We first aimed to characterize the temporal and pharmacological profiles of ligand-stimulated ERK1/2 phosphorylation via the $5HT_{2C}$R-INI in the CV1 background. The temporal profile of 5HT (1 μM), DOI (1 μM) and quipazine (10 μM) was different to that seen previously in CHO cells [7]. Although a rapid increase in ERK1/2 phosphorylation was noted in both cell types, an appreciable level of ERK1/2 activation was still apparent at 30 min in CV1 cells (Fig. 2A), in contrast to the rapid decay in signal intensity seen previously in CHO cells.

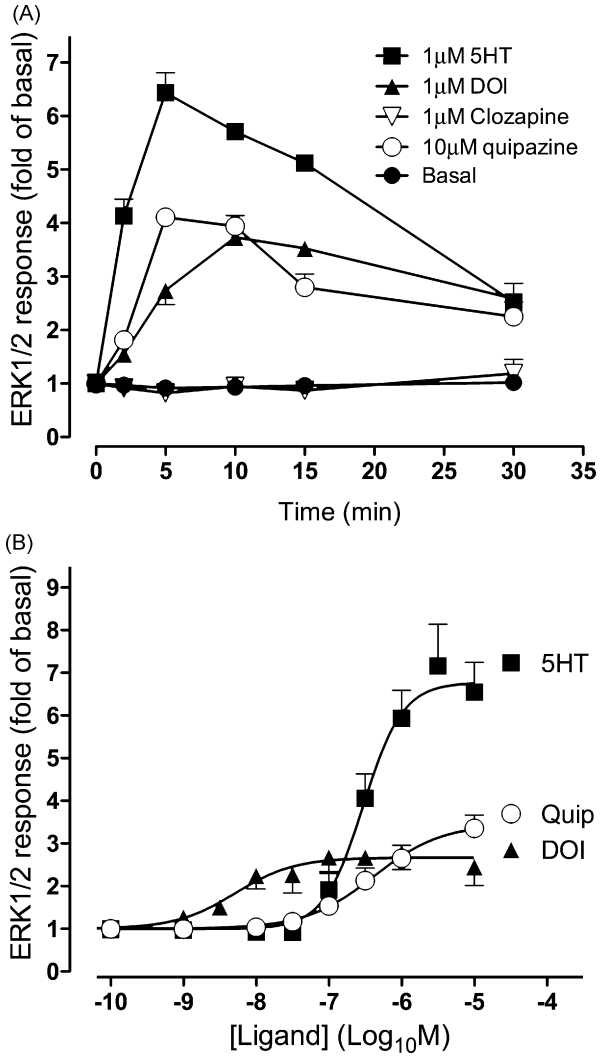

Fig. 2 – (A) Time course of ERK1/2 phosphorylation by various ligands in CV1 cells expressing the INI isoform of the human $5HT_{2C}$R. Basal signaling in this cell line in this series of experiments was 1326 ± 214 RFU. Results are presented as fold of basal response, mean ± S.E.M., *n* = 3–9 separate experiments, with each experimental point determined in duplicate. (B) Concentration–response curves for ligands displaying agonist efficacy at the $5HT_{2C}$-INI receptor. CV1 cells expressing the INI isoform of the $5HT_{2C}$R were stimulated with increasing concentrations of 5HT (100 pM–100 μM), DOI (10 pM–100 μM) or quipazine (100 pM–100 μM) for either 5 min (5HT, quipazine) or 10 min (DOI). Cells were then lysed, and phosphorylated ERK1/2 was quantified using the *SureFire*® kit. Data are presented as fold of basal response, mean ± S.E.M., *n* = 3–7 separate experiments, with each experimental assay point determined in duplicate.

DOI took longer to produce a maximal response (10 min) than did 5HT or quipazine (5 min), consistent with previous results [7]. The inverse agonist activity of clozapine was not apparent in the CV1 cells, probably due to the low level of receptor expression and the consequent absence of any significant degree of agonist-independent receptor activity.

Subsequently, concentration–response curves were constructed using the peak response times determined for each agonist (5HT/quipazine, 5 min; DOI, 10 min; Fig. 2B). As with previous studies, DOI and quipazine were both partial agonists, with relative maximal agonist effects less than 0.5 compared to 5HT. The potencies of DOI (pEC_{50} = 8.28 ± 0.25, *n* = 5) and quipazine (pEC_{50} = 6.42 ± 0.12, *n* = 3) were comparable to those determined in CHO cells, but 5HT was substantially less potent in the current study (pEC_{50} = 6.45 ± 0.12, *n* = 7 in CV1 vs. 7.45 ± 0.13, *n* = 4 in CHO) [7].

3.2. *Effects of RNA editing on $5HT_{2C}$R-mediated ERK1/2 signaling in CV1 cells*

There is evidence from prior studies that RNA editing modulates $5HT_{2C}$R signaling via classical G protein-coupled pathways (see Section 1). To determine whether ERK1/2 activation by $5HT_{2C}$R is also affected by editing, we extended our study to include temporal and pharmacological characterization of the VSV and VGV isoforms of the $5HT_{2C}$R. We found that both edited isoforms of the $5HT_{2C}$R exhibited a dramatically different time course of ERK1/2 activation following agonist stimulation compared to the unedited isoform. Neither edit responded to DOI (1 μM) or quipazine (10 μM) at any time point, while the response to 5HT (1 μM) was greatly reduced in magnitude and duration, being limited to a very transient peak at 5 min followed by a rapid decay to basal levels by 15 min (Fig. 3). The potency of 5HT in mediating ERK1/2 activation was not significantly reduced in the edited isoforms compared to the unedited variant (pEC_{50} = 6.52 ± 0.10, *n* = 3–6 (VSV); 6.62 ± 0.09, *n* = 4 (VGV) vs. 6.45 ± 0.12, *n* = 7 (INI)) (Fig. 4). However, we did note a progressive steepening of the concentration–response curves to the agonist, as manifested in the estimates of the Hill slope parameter, which was 1.5 ± 0.6 for the INI isoform, 2.0 ± 0.6 for the VSV isoform and 2.5 ± 0.5 for the VGV isoform. It is possible that this latter finding reflects differences in the intracellular mechanisms governing transduction of receptor activation to final observed ERK1/2 response for the different isoforms.

3.3. *Lack of effect of RNA editing on antagonist/inverse agonist potency*

Additional experiments were performed to investigate whether RNA editing modulates the potency of antagonists/inverse agonists in inhibiting $5HT_{2C}$R-mediated ERK1/2 signaling, given that a number of such compounds are used clinically. Specifically, we constructed 5HT concentration–response curves in the absence or presence of increasing concentrations of MSN or Cloz at each of the three $5HT_{2C}$R isoforms. As shown in Fig. 5, the antagonism of 5HT-mediated responses in the ERK1/2 assays was characterized by two distinct features. First, the concentration–response curve to 5HT was progressively shifted to the right in a parallel fashion by increasing concentrations of either antagonist, as expected for competitive antagonism. However, the maximal response to 5HT was also depressed by increasing antagonist concentrations (Fig. 5); this latter feature is at odds with the expectations of simple competitive antagonism. Although this may be taken as presumptive evidence of a non-competitive or

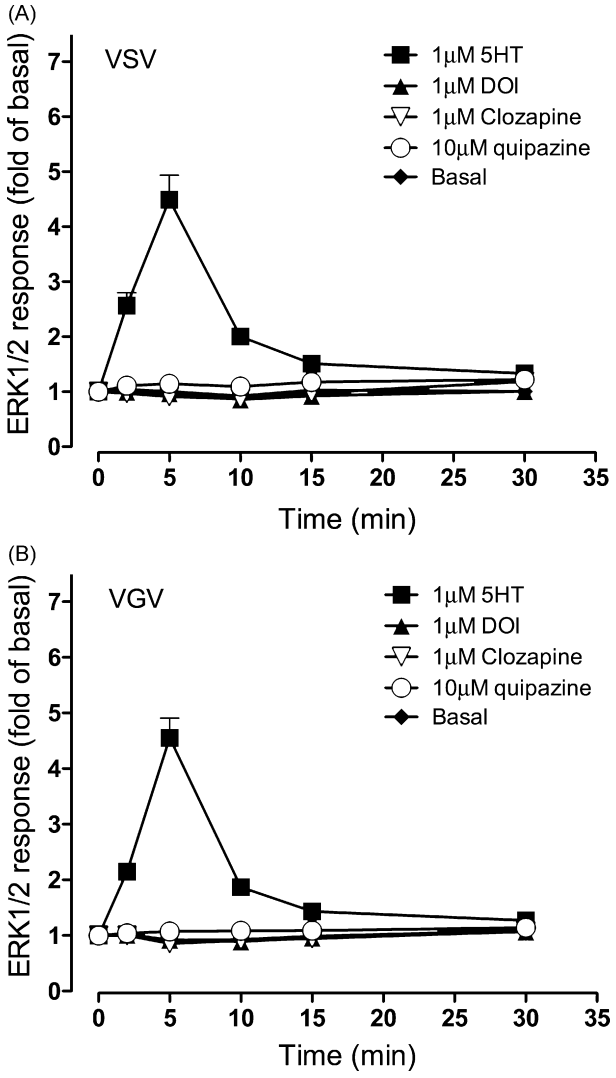

Fig. 3 – Time course of ERK1/2 phosphorylation stimulated by various ligands in CV1 cells expressing either the partially edited VSV (A) or the fully edited VGV (B) isoforms of the human $5HT_{2C}R$. Basal signaling for the VSV isoform in this series of experiments was 1232 ± 246 RFU, whereas basal signaling for the VGV isoform was 1351 ± 348 RFU. Data are expressed as fold of basal, mean ± S.E.M., n = 3–10 separate experiments, with each experimental assay point determined in duplicate.

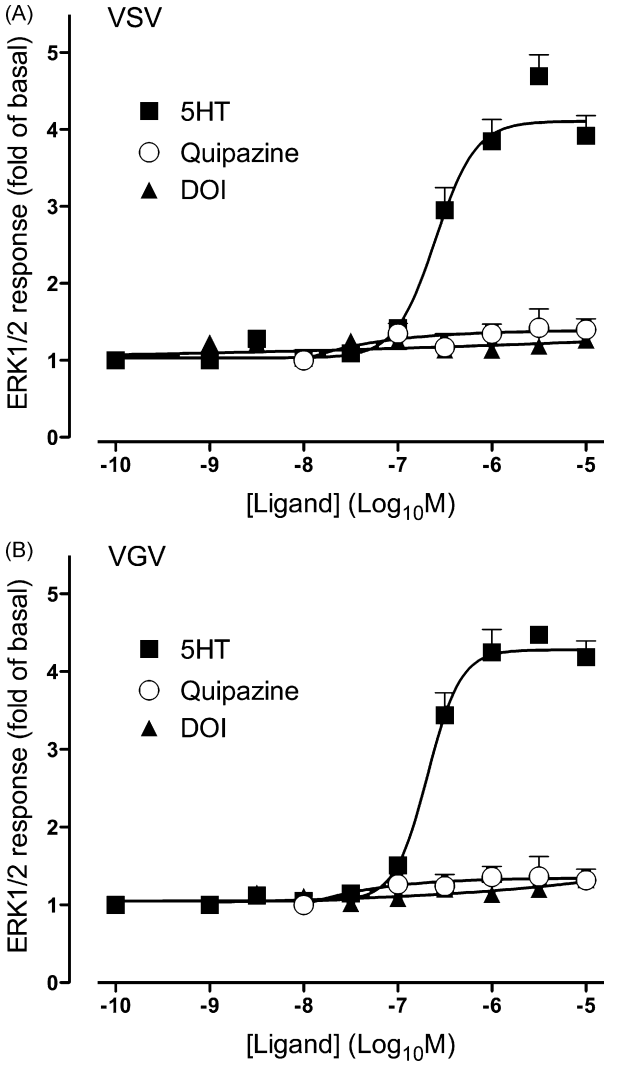

Fig. 4 – Concentration–response curves for ERK1/2 phosphorylation by 5HT, DOI and quipazine at the VSV (A) and VGV (B) isoforms of the $5HT_{2C}R$ expressed in CV1 cells. Data are expressed as fold of basal, mean ± S.E.M., n = 3–6 separate experiments, with each experimental assay point determined in duplicate.

allosteric mode of antagonism of the ERK1/2 pathway, it should be noted that this pattern of antagonism has previously been noted in a number of systems characterized by transient response kinetics, such as calcium mobilization [26,30] and likely represents a hemi-equilibrium between agonist binding, antagonist binding and response time.

We have previously developed a method for determining antagonist potency in such a situation [26], which differs from classic Schild analysis in that it does not utilize changes in EC_{50} values to assess antagonist potency, since these no longer represent equi-effective response levels if the agonist maximum is reduced relative to the control curve. Thus, application of Eq. (3) of Section 2 to data derived from the shifts produced by antagonist at the 20% response level of the control curve (pEC_{20} values), yielded the pA_2 and Schild slope estimates shown in Table 1, where it can be seen that there was no significant deviation of any of the Schild slope estimates from unity, nor was there any significant difference in pA_2 values for either antagonist between the three $5HT_{2C}R$ isoforms.

3.4. *Agonist-specific mechanisms of ERK1/2 activation via $5HT_{2C}R$-INI*

We have previously characterized a pathway that mediates 5HT-stimulated ERK1/2 activation in $5HT_{2C}R$-INI-expressing CHO cells, involving phospholipase D, protein kinase C and the RAF/MEK/ERK module [7]. However, that study was limited to the effects of a single agonist, in a cellular background devoid

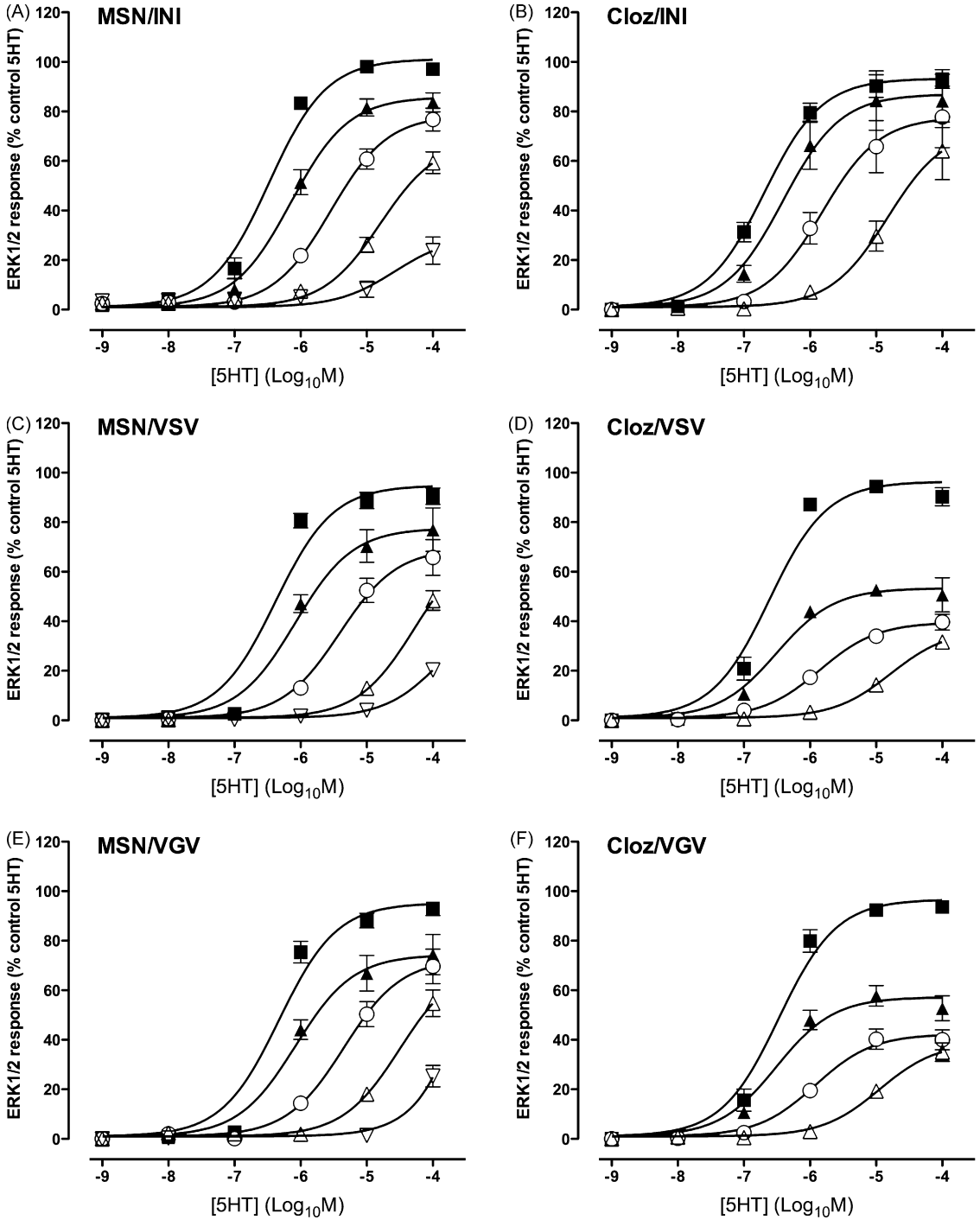

Fig. 5 – Effects of two $5HT_{2C}$R antagonists on 5HT-stimulated ERK1/2 phosphorylation via the three $5HT_{2C}$R receptor variants. CV1 cells expressing either the unedited (INI) or edited (VSV or VGV) isoforms of the $5HT_{2C}$R were pre-incubated for 30 min with either buffer (■) or antagonist (mianserin or clozapine) at a concentration of 10 nM (●), 100 nM (▲), 1 μM (△) or 10 μM (□). Following pre-incubation, cells were stimulated with 1 μM 5HT for 5 min before lysing and estimating ERK1/2 phosphorylation using the *SureFire*® kit. Data were normalized against the response to 5HT in the absence of antagonist and are expressed as mean ± S.E.M., *n* = 3–4 separate experiments, with each experimental assay point determined in duplicate.

of EGFRs, and did not consider the possible temporal impact on receptor coupling to ERK. In the current study, we have used various ERK1/2 pathway inhibitors to delineate some of the components of the 5HT-, DOI- and quipazine-mediated ERK1/2 activation pathways. These inhibitors were chosen on the basis of prior studies and the subsequent determination of complete inhibition curves to ERK1/2 stimulated by 1 μM 5HT (not shown), to ensure their potencies reflect expected activity

Table 1 – Potency of $5HT_2$ antagonists, mianserin and clozapine, to inhibit 5HT-induced ERK1/2 activation in CV1 cells stably expressing the unedited (INI) or edited (VSV or VGV) isoforms of the $5HT_{2C}R$

	pA_2[a]	Slope[b]	df[c]
Mianserin			
INI	8.09 ± 0.16	0.96 ± 0.07	67
VSV	8.12 ± 0.10	1.02 ± 0.04	29
VGV	8.05 ± 0.15	0.99 ± 0.07	26
Clozapine			
INI	8.09 ± 0.21	1.04 ± 0.10	31
VSV	8.23 ± 0.14	1.10 ± 0.08	16
VGV	8.19 ± 0.15	1.04 ± 0.08	15

[a] Antagonist concentration required to shift the agonist concentration–response curve twofold to the right. Eq. (2) was fitted with the Schild slope constrained to unity.
[b] Logistic slope factor, analogous to Schild slope.
[c] Degrees of freedom.

at their primary target proteins in intact cells [7,31–35]. Each inhibitor was then tested for their ability to inhibit agonist-stimulated ERK1/2 activation at three different time points to assess changes in the relative involvement of particular pathway components over time.

The $5HT_{2C}R$ antagonist, mianserin (1 μM), was effective against all agonists at all time points (Fig. 6), as was the MAPK/ERK kinase (MEK) inhibitor, U0126 (10 μM; data not shown), confirming that the $5HT_{2C}R$ and MEK were both integral components in all scenarios, regardless of time or agonist. However, using inhibitors of protein kinase C (PKC; Rö318220, 10 μM), phosphatidylinositol 3-kinase (PI 3-K; LY294002, 10 μM), epidermal growth factor receptor (AG1478, 1 nM) and a broad spectrum matrix metalloprotease (MMP) inhibitor (GM6001, 10 μM), we found that certain other pathway components were regulated in a distinct time- and agonist-dependent manner. 5HT and DOI exhibited almost identical profiles of inhibitor sensitivity. Initially, this was characterized by a total dependence on the EGFR, with a lesser contribution by MMPs, and complete independence from PKC or PI 3-K inputs (Fig. 6). However, by 30 min, this situation had changed: PI 3-K and PKC had both come into play, with ~40–50% of ERK1/2 activation requiring these effectors. In contrast, dependence on the EGFR and MMP pathways had decreased to only ~50% and ~25%, respectively. Statistical analysis (two-way ANOVA with Bonferroni's post-test) showed that the levels of inhibition exerted by AG1478, GM6001, Rö318220 and LY294002 were all significantly ($p < 0.05$) different at 30 min compared to those at 5 min, suggesting that a change in signaling pathway components had occurred during this time.

ERK1/2 activation by quipazine also changed significantly over the 30-min time course of the experiment. However, the profile of pathway components recruited by quipazine was notably different to 5HT and DOI. At early time points, ERK1/2 activation was completely ablated by GM6001, suggesting total dependence on transactivation mechanisms when quipazine is the stimulating agonist. However, the fact that AG1478 was less than 100% effective implies that receptor tyrosine kinases other than EGFR are the target of these transactivation mechanisms (Fig. 6). Statistical tests (two-way ANOVA with Bonferroni's post-test) showed that the ratio of inhibition by AG1478:GM6001 was significantly ($p < 0.05$) different at all time points for quipazine compared to 5HT and DOI, supporting the idea that the nature of the communication with the receptor tyrosine kinase system changes depending on the $5HT_{2C}R$ agonist used.

Furthermore, and again unlike 5HT or DOI, quipazine was able to engage PI 3-K at a low level at 5 min (~25% inhibition by LY294002), but this recruitment was not altered as the experiment progressed (Fig. 6). While LY294002 was ~45% more effective at 30 min than at 5 min against 5HT- or DOI-stimulated ERK1/2 activity, there was no change in its activity against quipazine-stimulated ERK1/2 between 5 and 30 min. Analysis of the magnitude of the change in LY294002 effectiveness between 5 and 30 min for each of the agonists confirmed that the recruitment of PI 3-K by quipazine was significantly different than that by 5HT or DOI ($p < 0.05$; repeated measures ANOVA with Bonferroni's post-test), in agreement with the theory that quipazine communicates to the effector–second messenger system differently to 5HT or DOI.

3.5. *Effects of RNA editing on ERK1/2 activation pathway selection*

The observation that $5HT_{2C}R$-VSV and $5HT_{2C}R$-VGV retained the ability to couple to ERK, albeit with a loss of efficacy and the loss of any sustained phase of activation, suggested that the edited receptors were able to couple to some, but not all, of the components of the previously characterized pathways. We therefore repeated our experiments with AG1478, Rö318220 and LY294002 to investigate the pathways utilized by these edited receptors. Fig. 7 illustrates these data, with data for INI shown already in Fig. 6 reconfigured in bar graph format for ease of comparison. We found that, in common with the unedited INI isoform (Fig. 7A), both VSV (Fig. 7B) and VGV (Fig. 7C) edited isoforms displayed a strong dependence on EGFR transactivation, given the robust inhibition of ERK1/2 response by AG1478 following stimulation of either receptor with 1 μM 5HT. In terms of PKC and PI 3-K involvement, however, the isoforms are divergent: while INI did not utilize either enzyme at early time points, VSV engages both PKC and PI 3-K, while VGV recruits PKC to a significant degree.

4. Discussion

This study has found that the $5HT_{2C}R$ can activate the ERK1/2 signaling cascade via a variety of mechanisms, including transactivation of the EGFR, and that RNA editing of the $5HT_{2C}R$ modifies both the temporal and mechanistic characteristics of the ERK1/2 response. Furthermore, we provide evidence to support the existence of a set of agonist-specific (receptor-conformation-specific) ERK1/2 activation mechanisms whose components' relative involvement in ERK1/2 signaling varies with time. It is likely that this extreme flexibility of the $5HT_{2C}R$-ERK1/2 axis enables it to signal differently depending on which RNA-edited isoform of the receptor predominates. It also suggests the possibility that aberrant 7TMR-mediated ERK1/2 signaling can be modulated using pathway-selective agonists.

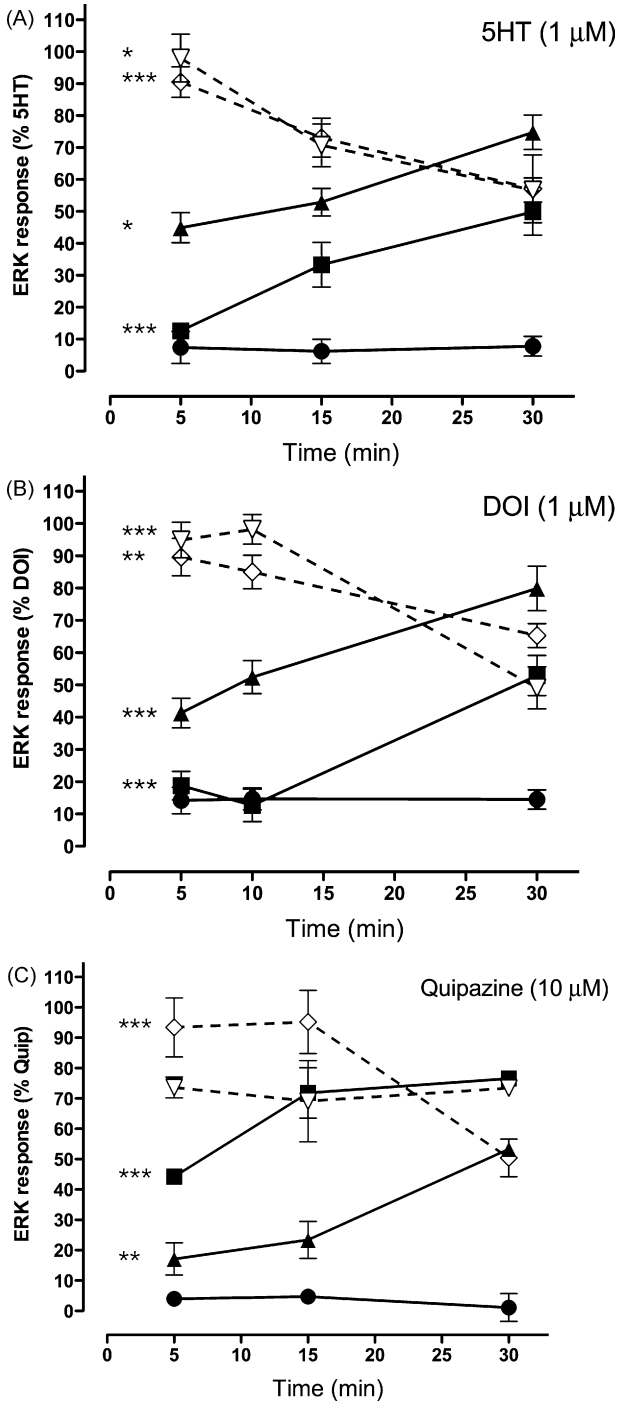

Fig. 6 – Agonist-specific ERK1/2 activation pathways revealed using a variety of ERK1/2 pathway inhibitors. CV1 cells expressing the INI isoform of the $5HT_{2C}$R were pre-incubated with either buffer or one of a panel of inhibitors: MSN (1 μM, 30 min, ●); Rö318220 (10 μM, 30 min, ◇); GM6001 (10 μM, 30 min, ▲); AG1478 (1 nM, 15 min, ■); or LY294002 (10 μM, 30 min, ▽). Stimulation times were determined from the time course data in Fig. 2A, and represent a maximal response, an intermediate-sized response and a late response. Data are normalized against the response to agonist in the absence of inhibitor, mean ± S.E.M., n = 3–13 separate experiments, with each experimental assay point determined in duplicate. Statistical significance between 5 and 30 min time points for each inhibitor was calculated using two-way ANOVA followed by Bonferroni's post-test. Statistical significance is denoted with $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

The network of effectors that convey G protein signals to ERK1/2 activation is labyrinthine [11,28]. The divergent–convergent nature of this network gives agonists the option to traffic signals to ERK1/2 via a variety of different routes. Indeed, some receptors differentially traffic agonist signals between G protein- and β-arrestin-dependent arms of the ERK1/2 pathway [9,10]. Furthermore, the nature of the ERK-activating stimulus can be important in determining the cellular destination of the activated ERK1/2 [36,37] and therefore the physiological outcome of the activation. For instance, ERK-induced proliferation requires nuclear translocation of active ERK, so any factors that cause cytosolic retention of ERK1/2 will limit the cell to non-proliferative responses. It is likely that subtle changes in the way in which ERK1/2 is activated, such as differential recruitment of scaffolding partners, would be sufficient to direct the active ERK1/2 to a different sub-cellular compartment or target organelle, which would have a dramatic effect on the cellular responses to ERK1/2 stimulation by different ligands.

Importantly, the $5HT_{2C}$R can generate differential intracellular signals in an agonist-dependent manner, as evidenced in both 'classical' G protein-mediated pathways and more complex pathways such as ERK1/2 activation [6–8]. This suggests that the receptor is a good candidate to be capable of trafficking signals even more specifically through the G protein-dependent subset of ERK1/2 activation mechanisms. In the present study, we found that the relative involvement of factors such as EGFR, MMPs, PKC and PI 3-K, and the temporal profile of their involvement, can differ in an agonist-specific way.

The initial role of EGFR and, to a lesser extent, MMPs following stimulation with 5HT and DOI suggests a strong dependence on the EGFR pathway. This could be by ectodomain shedding (the release of membrane-tethered receptor tyrosine kinase ligands by 7TMR-activated MMPs), or by alternative means of transactivation such as direct interactions between receptor tyrosine kinases and either 7TMRs or G proteins, as suggested for other systems [28]. Conversely, following 5 min quipazine stimulation, the effects of GM6001 were greater than those of the EGFR inhibitor, AG1478, suggesting that ectodomain shedding leads to transactivation of not only EGFR but perhaps also other receptor tyrosine kinases [38]. Moreover, the sensitivity to PI 3-K inhibition when the receptor is activated by quipazine (but not the 5HT or DOI) for 5 min may implicate a distinct set of PI 3-K-activated MMPs in this mechanism that cleave non-EGFR-acting precursors in the membrane and produce non-EGFR-dependent transactivation. Thus, quipazine appears to recruit a somewhat different ERK1/2 activation pathway than does 5HT or DOI, supporting the idea of agonist-specific routes through the ERK1/2 activation network.

Extending the concept of agonist-specific pathway selection into the therapeutic context suggests that it ought to be possible to therapeutically correct an unbalanced 7TMR-

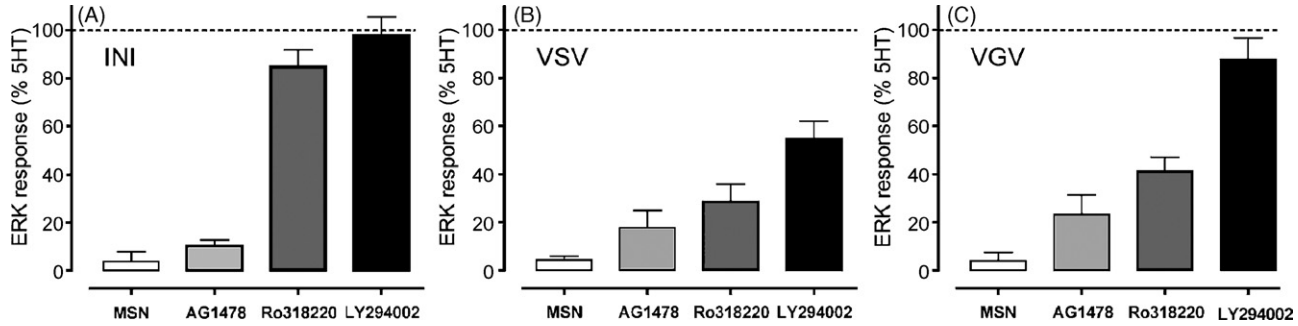

Fig. 7 – RNA editing modulates ERK1/2 pathway selectivity. CV1 cells expressing the INI (A), VSV (B) or VGV (C) isoforms of the $5HT_{2C}$R were pre-incubated with either buffer or one of a panel of inhibitors: MSN (1 μM, 30 min, white bar); Rö318220 (10 μM, 30 min, light grey bar); AG1478 (1 nM, 15 min, dark grey bar); or LY294002 (10 μM, 30 min, black bar). Following pre-incubation, cells were stimulated with 5HT (1 μM, 5 min) before lysis and quantification of ERK1/2 phosphorylation using the *SureFire*® kit. Data are normalized against the response to 5HT in the absence of inhibitor, mean ± S.E.M., $n = 3–6$ separate experiments, with each experimental assay point determined in duplicate.

mediated ERK1/2 response by administering a drug that only activates certain aspects of the ERK1/2 pathway. This would lead to a gain in target specificity, and a reduction in side effects since the non-aberrant facets of the ERK1/2 signal would be maintained. For instance, an over-active proliferative response could be counteracted using a drug that still activates ERK1/2 but specifically in cytosolic locations, thus limiting its impact upon the other signaling aspects of the receptor. While it has yet to be shown that any disease state has an anomalous $5HT_{2C}$R-stimulated ERK1/2 activation as its basis, the possibility that a 7TMR ERK1/2 response could be pharmacologically tailored using the vast flexibility of the ERK1/2 signaling network validates the approach of targeting the ERK1/2 pathway therapeutically [39].

We also found that RNA editing had significant effects on the ability of $5HT_{2C}$R to signal to ERK, changing both its temporal and pharmacological profiles (Figs. 3 and 4). Reduced coupling of the receptor to G protein would be expected to decrease the strength of signals emanating from the receptor, causing a loss of agonist potency and a commensurate dextral displacement of the concentration–response curve, unless receptor reserve is low, in which case the effect would predominantly be manifested as a reduction in maximal response. This is most likely the case in our present study, as the expression level of each of the $5HT_{2C}$R isoforms in the CV1 cells were ~100–150 fmol/mg protein (a more physiologically relevant range). This lack of receptor reserve can also explain the reduced potency of 5HT (~1 order of magnitude) seen in the CV1-$5HT_{2C}$R-INI cell line compared to the CHO-$5HT_{2C}$R-INI cell line used in our previous study [7], which expresses the receptor at higher levels [40]. However, it cannot be ruled out that the loss of efficacy is simply due to the ERK1/2 stimulation being mediated by a different activation pathway (e.g., different G proteins) that has a different ceiling of activation, i.e., that the system determines the maximal response rather than the receptor activity. The implication that editing can influence the selection of signaling pathway, rather than simply muting an existing response without changing receptor coupling specificity, is of potentially profound significance given that small changes in ERK1/2 activation pathway can lead to major changes in the duration, location and ultimate endpoint of ERK1/2 activation. This idea is certainly consistent with the recent finding by Berg et al. [41] that differences (or lack thereof) in the efficiency of coupling of RNA-edited isoforms of the $5HT_{2C}$R depend on the conformation of the receptor being investigated.

In contrast to the number of studies that have focused on the impact of RNA editing on agonist pharmacology, far less is known about the sensitivity of various RNA-edited isoforms to antagonist ligands. RNA editing is known to reduce constitutive receptor activity [15,16], thus affecting the ability of certain $5HT_{2C}$R ligands to act as inverse agonists, but the interaction between such ligands and 5HT agonists in systems lacking appreciable constitutive receptor activity has not been subjected to rigorous quantification. Interestingly, in the current study the patterns of antagonism of the 5HT ERK1/2 concentration–response curve by mianserin and clozapine at each edited isoform of the $5HT_{2C}$R were characterized by different degrees of, apparently saturable, insurmountable antagonism accompanying dextral curve displacement (Fig. 5). Although, on the surface, this may be taken as presumptive evidence of an atypical mode of antagonist action, it is more likely to reflect the fact that the interactions under study are in a hemi-equilibrium state, which is common when agonist–antagonist interactions are studied using systems where responses are transient rather than at steady state [26,42]. Analysis of the data according to a modification of the classic Gaddum/Schild equation of competitive interactions yielded pA_2 estimates that were not significantly different from one another between isoforms, and in good agreement with prior literature values of these compounds using other signaling assays (http://iuphar-db.org/GPCR/ReceptorFamiliesForward). Thus, in contrast to the dramatic effects on agonists, RNA editing does not appear to influence antagonist potency in the absence of appreciable constitutive receptor activity.

In conclusion, we have presented data highlighting the complexity of ERK1/2 signaling, both by the $5HT_{2C}$R and in a wider 7TMR context, and the flexibility afforded by LDTRS and RNA editing. The study of $5HT_{2C}$R signaling mechanisms represents a promising field for further investigation into the

molecular basis of these phenomena, and may lead to novel strategies for therapeutically targeting 7TMRs.

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