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ARTICLE

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Clozapine induction of ERK1/2 cell signalling via the EGF receptor in mouse prefrontal cortex and striatum is distinct from other antipsychotic drugs

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Abstract

Treatment resistance remains a major obstacle in schizophrenia, with antipsychotic drugs (APDs) being ineffective in about one third of cases. Poor response to standard therapy leaves the APD clozapine as the only effective treatment for many patients. The reason for the superior efficacy of clozapine is unknown, but as we have proposed previously it may involve modulation of neuroplasticity and connectivity through induction of interconnected mitogenic signalling pathways. These include the mitogen-activated protein kinase-extracellular signal regulated kinase (MAPK-ERK) cascade and epidermal growth factor (EGF)/ErbB systems. Clozapine, distinct from other APDs, induced initial inhibition and subsequent activation of the ERK response in prefrontal cortical (PFC) neurons *in vitro* and *in vivo*, an action mediated by the EGF receptor (ErbB1). Here we examine additionally the striatum of C57Bl/6 mice to determine if clozapine, olanzapine, and haloperidol differentially regulate the ERK1/2 pathway in a region or time-specific manner conditional on the EGF receptor. Following acute treatment, only clozapine caused delayed striatal ERK phosphorylation through EGF receptor phosphorylation (tyrosine 1068 site) and MEK that paralleled cortical ERK phosphorylation. Olanzapine induced initial pERK1-specific blockade and an elevation 24-h later in PFC but had no effect in the striatum. By contrast, haloperidol significantly stimulated pERK1 in striatum for up to 8 h, but exerted limited effect in PFC. Clozapine but not olanzapine or haloperidol recruited the EGF receptor to signal to ERK. These *in-vivo* data reinforce our previous findings that clozapine's action may be uniquely linked to the EGF signalling system, potentially contributing to its distinctive clinical profile.

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Key words: Antipsychotic drug, clozapine, EGF receptor, ERK, schizophrenia.

Introduction

Antipsychotic drugs (APDs) exert variable efficacy in treating the positive psychotic symptoms of schizophrenia. This therapeutic effect is attributed in part to affinity for central dopamine D_2 receptors (D_2Rs), a feature of all APDs in clinical use (Kapur & Seeman, 2001; Masri *et al.* 2008; Seeman, 2002). However, APDs are less able to improve the negative symptoms and cognitive deficits of schizophrenia (Miyamoto *et al.* 2005) with this extending to positive symptoms where in about one third of cases they may be of limited benefit (Lieberman *et al.* 2005; Pantelis & Lambert, 2003). This refractoriness to treatment may be addressed in a proportion of cases by the atypical APD clozapine which is demonstrably more effective than other agents (Leucht *et al.* 2009; Lewis *et al.* 2006; McEvoy *et al.* 2006; Stroup *et al.* 2006; Tandon *et al.* 2008). The efficacy of clozapine in treating

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schizophrenia where other APDs have failed suggests that clozapine has a unique signalling profile, plausibly initiated via G-protein coupled receptor (GPCR) binding and activation of intraneuronal pathways distinct from other APDs. Supportive of the involvement of alternative pathways have been recent findings identifying perturbations in the epidermal growth factor (EGF)-neuregulin 1 (NRG1) ErbB system in the pathology of schizophrenia (Buxbaum *et al.* 2008) indicating disease processes modulated through pathways other than the D_2-Gi/o PKA or 5-HT_{2A}-Gq phospholipase-C signalling cascades characteristically linked to APD action.

One candidate pathway that can integrate signalling between APDs and the EGF/ErbB receptor system and its ligands is the mitogen-activated protein kinase-extracellular signal regulated kinase (MAPK-ERK) cascade (Britsch, 2007; Pozzi *et al.* 2003). Activation of the MAPK-ERK pathway phosphorylates proteins involved in transcriptional and translational regulation, dendritic organization, cellular excitability, long-term potentiation and depression, neuronal survival, synaptogenesis and neurotransmitter release (Engel *et al.* 2009). In this way, ERK activation contributes to synaptic plasticity and connectivity, processes impaired in schizophrenia (Harrison & Weinberger, 2005; Konradi & Heckers, 2001). Stimulation of the ERK pathway may be directly by growth factors such as the EGF ligand family (including EGF, the neuregulins) and brain-derived neurotrophic factor (BDNF), principally through activation of receptor tyrosine kinases (RTKs). ERK activation is also regulated by the activity of dopamine, serotonin and glutamate receptors (Valjent *et al.* 2005) of which APDs are known modulators (Miyamoto *et al.* 2005). The mechanism by which APDs regulate ERK phosphorylation can involve direct binding to GPCRs. This occurs through Gi/o/Gs modulation of adenylate cyclase/protein kinase A (PKA) activity, Gq-stimulation of phospholipase-C or transactivation as is the case for clozapine (Pereira *et al.* 2009).

Evidence suggests that APDs differentially mediate the ERK cascade *in vitro* and *in vivo*, dependent on cell and tissue type (Ahmed *et al.* 2008; Fumagalli *et al.* 2006). For instance, haloperidol increased ERK1/2 levels in cultured hippocampal neurons (Yang *et al.* 2004) while clozapine exerted similar effects in 5-HT_{1A} receptor transfected CHO cells (Cussac *et al.* 2002) and along with olanzapine induced ERK activation and neurite outgrowth in PC12 cells (Lu & Dwyer, 2005; Lu *et al.* 2004). Furthermore, long-term *in-vivo* exposure to olanzapine up-regulated ERK1/2 phosphorylation in subcellular compartments of rat prefrontal cortex (PFC) (Fumagalli *et al.* 2006). By comparison, *in vivo* clozapine reduced and haloperidol increased ERK activation in mouse dorsal striatum (Pozzi *et al.* 2003) with opposing effects observed in rat PFC (Ahmed *et al.* 2008). In relation to animal behaviour, ERK modulation by clozapine affected conditioned avoidance response, an index of antipsychotic efficacy (Browning *et al.* 2005) and repeated clozapine treatment corrected a methamphetamine-induced cognitive deficit in mice (Kamei *et al.* 2006) in a manner distinct from other APDs. Such studies considered to model schizophrenia symptomatology provide plausible justification that differential regulation of ERK signalling by clozapine may be related to the unique clinical profile of the drug. Therefore while not unequivocal, mild remediation of cognitive deficits in schizophrenia in the domains of learning and processing speed observed with clozapine (Woodward *et al.* 2005), may be conceivably linked to release of dopamine and acetylcholine in the PFC and hippocampus (Ichikawa *et al.* 2002; Kuroki *et al.* 1999) and signalling pathways such as ERK.

In accord with this, we have previously reported that clozapine and other APDs acutely inhibited ERK1 and ERK2 activation in PFC neurons *in vitro* but only clozapine stimulated ERK with sustained treatment *in vitro* and *in vivo* (Pereira *et al.* 2009). This stimulation was selectively mediated by the EGF receptor rather than by Gi/o/q coupled receptors, PKA or phospholipase-C-linked signalling systems (Pereira *et al.* 2009). Moreover, the Gi/o inhibitor pertussis toxin did not affect clozapine-induced ERK activation in cortical neurons (Pereira *et al.* 2009), distinguishing clozapine from its congener olanzapine, which mediates ERK phosphorylation through a pertussis toxin-sensitive pathway (Lu & Dwyer, 2005). Here we expand our *in-vivo* studies to (i) establish whether clozapine signalling via the EGF receptor is cortical-specific or extends to mouse striatum, (ii) determine whether olanzapine and haloperidol differentially regulate the ERK1/2 pathway in PFC and striatum following acute drug treatment and (iii) ascertain whether any APD-induced changes in pERK1/2 levels in cortex or striatum are EGF-receptor dependent.

Material and methods

Drugs and reagents

All agents including clozapine, haloperidol, SL327 and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA) unless stated otherwise. Olanzapine was a generous gift provided by Eli Lilly

(USA) and AG1478 (EGF receptor inhibitor) was obtained from Calbiochem (USA). Unless indicated otherwise, antibodies were supplied by Cell Signalling Technology (USA).

Animals

Animal care and experimental procedures were performed in accordance with The University of Melbourne Animal Ethics Committee guidelines. Groups ($n=5$) of 7-wk-old male C57BL/6 mice were housed under standard laboratory conditions on a 12-h light/dark cycle (lights on 07:00 hours) with food and water available *ad libitum*. Animals were acclimatized for 1 wk prior to drug injection and weighed before treatment.

APD time-course studies

For time-course experiments, mice were treated via intraperitoneal (i.p.) injection with clozapine (2.5 mg/kg), olanzapine (1 mg/kg) and haloperidol (0.25 mg/kg) dissolved in 0.9% saline acidified with 0.1 N HCl or vehicle (1% v/v) as a single dose. Animals were assayed at 20, 60, 240, 480 min or 24 h after haloperidol treatment; while for clozapine and olanzapine additional time-points of 120, 150, 180, 300 min and overnight (15 h) were examined. The doses chosen were mid-range of those used in mouse studies and parallel APD dose in humans. Doses were also selected because they were known to produce effects consistent with antipsychotic mouse models of psychosis without sedation (Bespalov *et al.* 2007; Pereira *et al.* 2009; Pozzi *et al.* 2003). Immediately after the time interval specified, mice were decapitated, the head immersed in liquid nitrogen for 6 s, the brain rapidly removed and PFC and striatum (ventral and dorsal) dissected out within 20 s on ice. Brain tissue was sonicated in 1% SDS (750 μl), boiled for 10 min and lysates frozen at -80 °C. Before protein determination, lysates were centrifuged at 14 000 ***g*** for 5 min at 4 °C to remove insoluble material. Protein content of lysates was measured by Bio-Rad Protein Assay (USA) using BSA as standard. Brain lysates were assayed for phosphorylated and total ERK1 and ERK2 as described.

Inhibitor treatment studies

To examine the effect of MEK inhibition on clozapine-induced ERK phosphorylation, SL327 (MEK inhibitor) at 30 mg/kg (Browning *et al.* 2005) dissolved in 36.5% DMSO was administered 10 min before i.p. injection of clozapine or vehicle. Since the experiment spanned

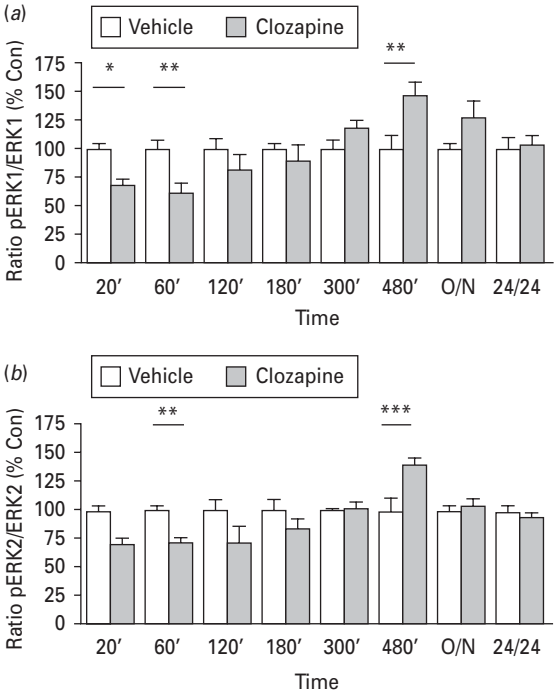

Fig. 1. Effect of clozapine on ERK phosphorylation in C57BL/6 mouse striatum. (*a*) Clozapine treatment (2.5 mg/kg) over a 24-h period – pERK1. (*b*) Clozapine treatment (2.5 mg/kg) over a 24-h period – pERK2. At each time-point treated samples were expressed relative to vehicle control (Con) standardized to 100%. Data represent the mean ± S.E.M. of at least four mice per experimental group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, statistical differences between tissue in the absence (vehicle) and presence of clozapine.

480 min three injections of SL327 were given 3 h apart to maintain sufficient plasma concentrations. Similarly, to study the effect of EGF receptor inhibition on ERK phosphorylation, mice were treated with AG1478 (EGF receptor inhibitor) at 25 mg/kg dissolved in 50% DMSO 10 min prior to APD or vehicle administration. For AG1478 experiments with clozapine, 60- and 480-min time-points were chosen since we had previously demonstrated that clozapine significantly inhibited ERK phosphorylation at 60 min and increased ERK levels at 480 min in striatum. In the case of haloperidol, co-treatment with AG1478 was undertaken at 60 and 240 min, time-points at which the drug had significantly activated ERK above vehicle. For experiments over a 480-min period, four injections of AG1478 were performed 2.5 h apart to sustain adequate plasma levels (Ellis *et al.* 2006). Experiments were terminated and brain tissue extracted as noted.

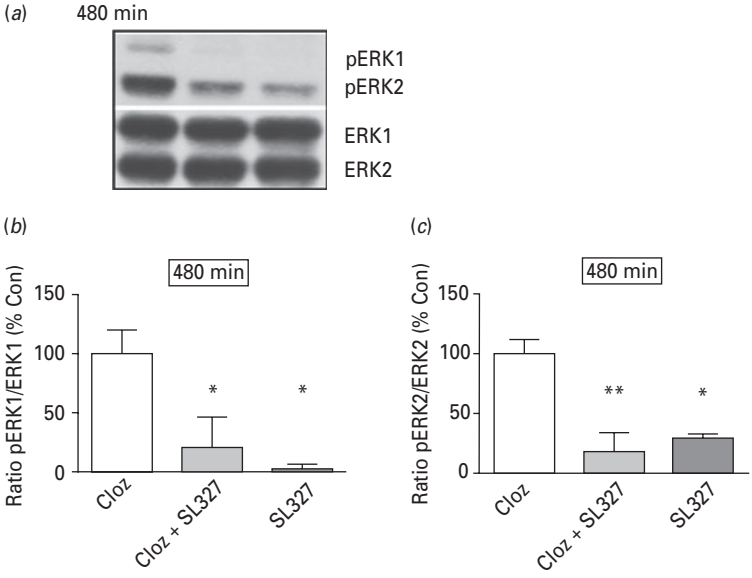

Fig. 2. The effect of the MEK inhibitor SL327 on clozapine-induced ERK phosphorylation at 480 min in C57BL/6 mouse striatum. (*a*) SL327 pre-treatment (*b*) SL327 pre-treatment – pERK1 (*c*) SL327 pre-treatment – pERK2. Representative blots (*a*) indicate immunoreactive bands of phosphorylated ERK1 and ERK2 (upper panel) and total ERK1 and ERK2 (lower panel). Data are expressed relative to clozapine treatment which has been standardized to 100% and represent the mean ± S.E.M. of at least four mice per experimental group. * $p < 0.05$, ** $p < 0.01$, statistical differences compared with clozapine alone. Cloz, Clozapine.

ERK1/2 assay

pERK1/2 levels were measured in PFC and striatal tissue using standard SDS–PAGE and Western immunoblotting methods. Ten μg of protein lysate was denatured at 90 °C in sample loading buffer, separated by PAGE electrophoresis (5% stacking gel, 10% resolving gel) and transblotted to nitrocellulose (Osmonics, USA). Membranes were blocked in 5% skim milk, TBST [20 mM Tris-base (pH 7.5), 150 mM NaCl, 0.01% Tween-20] and incubated overnight with anti-phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}) (E10) antibody (1:2000) in blocking buffer at 4 °C. Membranes were washed in TBST (2 × 15 min) and incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated immunoglobulins (IgGs) (Dako, Australia) (1:2000) in blocking buffer at 4 °C. Membranes were washed with TBST (2 × 15 min) and detection undertaken by chemiluminescence imaging using Enhanced Chemiluminescence (ECL) detection reagents and hyperfilm ECL (Amersham Biosciences, UK). Next blots were stripped [62.5 mM Tris–HCl (pH 6.7), 2% SDS and 100 mM β-mercaptoethanol] and re-probed with anti-p44/42 MAP kinase antibody (1:1000) and goat anti-rabbit-HRP conjugated IgGs (Dako) (1:2000) for assessment of total ERK1 and total ERK2 levels. Proteins were quantified by densitometry using Multi Gauge Software (Fujifilm v. 3.0). The optical densities of phosphorylated ERK1 (pERK1) and phosphorylated ERK2 (pERK2) immunoreactive bands were measured, normalized to the optical densities of total ERK1 (ERK1) and total ERK2 (ERK2), respectively, and then expressed as a percentage of vehicle-treated control.

EGF receptor (Tyr^{1068}) assay

Twenty μg of protein lysate was separated by SDS–PAGE and immunoblotted using modification of the procedures outlined. Briefly, samples were separated on a 5% stacking gel, 8% resolving gel at 160 V for 70 min. Overnight transfer to nitrocellulose membrane was undertaken at 30 V, 4 °C followed by an increase to 70 V for 60 min. Electrotransferred membranes were exposed to EGF receptor (Tyr^{1068}) (1H12) mouse antibody (1:1000) and goat anti-mouse HRP-conjugated IgGs (Dako, Australia) (1:2000) for detection of phosphorylated EGF receptor (Tyr^{1068}) and EGF receptor antibody in 5% BSA (1:1000) and goat anti-rabbit-HRP conjugated IgGs (Dako) (1:2000) for detection of total EGF receptor levels. EGF receptor proteins were detected and measured as described previously.

Data analysis

Animal data was pooled with each treatment group repeated in quadruplicate and the mean ± standard

error of the mean (S.E.M.) calculated using GraphPad Prism 5 software (GraphPad Software Inc., USA). One-way analysis of variance (ANOVA) was used to discriminate differences between variables and *post-hoc* Bonferroni-corrected multiple comparison tests or Dunnett's multiple comparison tests applied to establish significant differences between treatment groups or control and treated groups, respectively. Two-way ANOVA was also used to determine whether ERK1/2 levels were affected by factors of time or brain region. Bonferroni *post-hoc* tests were then performed to ascertain the source of variation between experimental measures. Unpaired Student's (two-tailed) *t* tests were applied as appropriate.

Results

Time-course of clozapine and olanzapine effects on ERK phosphorylation in mouse PFC and striatum

Clozapine treatment caused initial reduction at 20 and 60 min, subsequent activation at 480 min and normalization of the striatal pERK1 response over a 24-h period analogous to effects seen in the cortex (pERK1: $F_{15,59}=5.12$, $p<0.0001$) (Fig. 1*a*). For pERK2, levels were decreased at 60 min, increased at 480 min and returned to baseline thereafter (pERK2: $F_{15,58}=10.87$, $p<0.0001$) (Fig. 1*b*). Moreover, significant attenuation of clozapine phosphorylation of both ERK isoforms at 480 min with SL327 may indicate that phosphorylation had occurred via the precursor MEK (pERK1: $F_{2,8}=6.477$, $p=0.0212$; pERK2: $F_{2,7}=15.32$, $p=0.0028$) (Fig. 2*a–c*) but does not exclude ERK phosphatase activity in modulating the effects observed. By contrast, time-course findings for olanzapine in the PFC were pERK1-specific with blockade at 60 and 240 min and an elevation at 24 h (pERK1: $F_{13,42}=1.995$, $p=0.0459$) with no significant differences observed in pERK2 levels relative to vehicle (Fig. 3*a, b*). Furthermore, olanzapine induced no significant effects on ERK1/2 phosphorylation in the striatum within 24 h of drug treatment (data not shown).

Effect of clozapine in the absence and presence of AG1478 on ERK and EGF receptor (Tyr^{1068}) phosphorylation in mouse striatum

At 60 min, significant reductions in ERK1 and ERK2 phosphorylation caused by clozapine were not altered by AG1478 (Fig. 4*a–c*). However at 480 min, clozapine-induced pERK1 and pERK2 activation was significantly attenuated by AG1478 (Fig. 4*d–f*). Furthermore, clozapine treatment at 480 min triggered a concomitant increase in EGF receptor (Tyr^{1068}) phosphorylation above vehicle control which was lowered by AG1478 (Fig. 5*a, b*). Similarly, in the presence of AG1478 alone the amount of EGF receptor (Tyr^{1068}) activation was minimal and equated with vehicle-treated control (Fig. 5*a, b*).

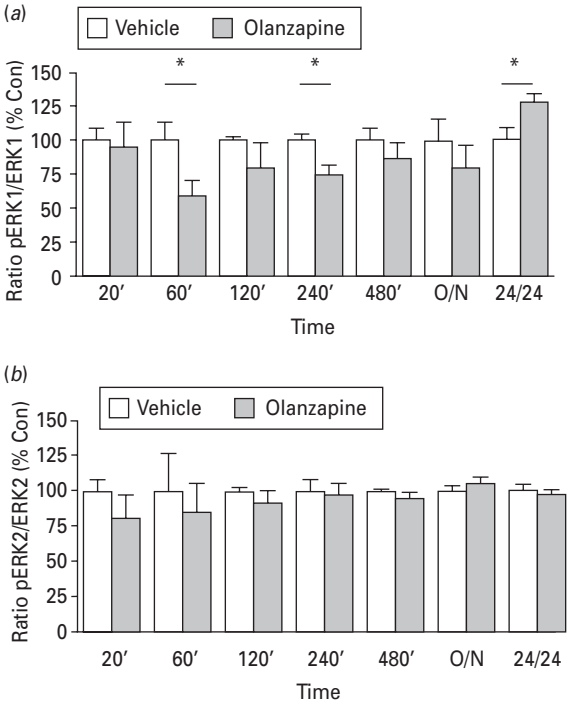

Fig. 3. Effect of olanzapine on ERK phosphorylation in C57BL/6 mouse prefrontal cortex. (*a*) Olanzapine treatment (1 mg/kg) over a 24-h period — pERK1. (*b*) Olanzapine treatment (1 mg/kg) over a 24-h period — pERK2. At each time-point treated samples were expressed relative to vehicle control (Con) standardized to 100%. Data represent the mean ± S.E.M. of at least four mice per experimental group. * $p<0.05$, statistical differences between tissue in the absence (vehicle) and presence of olanzapine.

Effect of haloperidol over 24 h on ERK phosphorylation in mouse PFC and striatum

Following haloperidol administration, PFC activation of pERK1 was not significantly different between vehicle- and drug-treated mice at all time-points examined (data not shown). However, pERK2 levels were significantly decreased at the early 20- and 60-min stages ($F_{9,33}=3.999$, $p=0.0016$) but subsequently returned to baseline (Fig. 6*a*). By comparison in striatum, haloperidol stimulated pERK1 ($F_{9,32}=9.375$, $p<0.0001$) over a 60–480 min period (Fig. 6*b*) but did not alter pERK2 to any significant extent (data not shown).

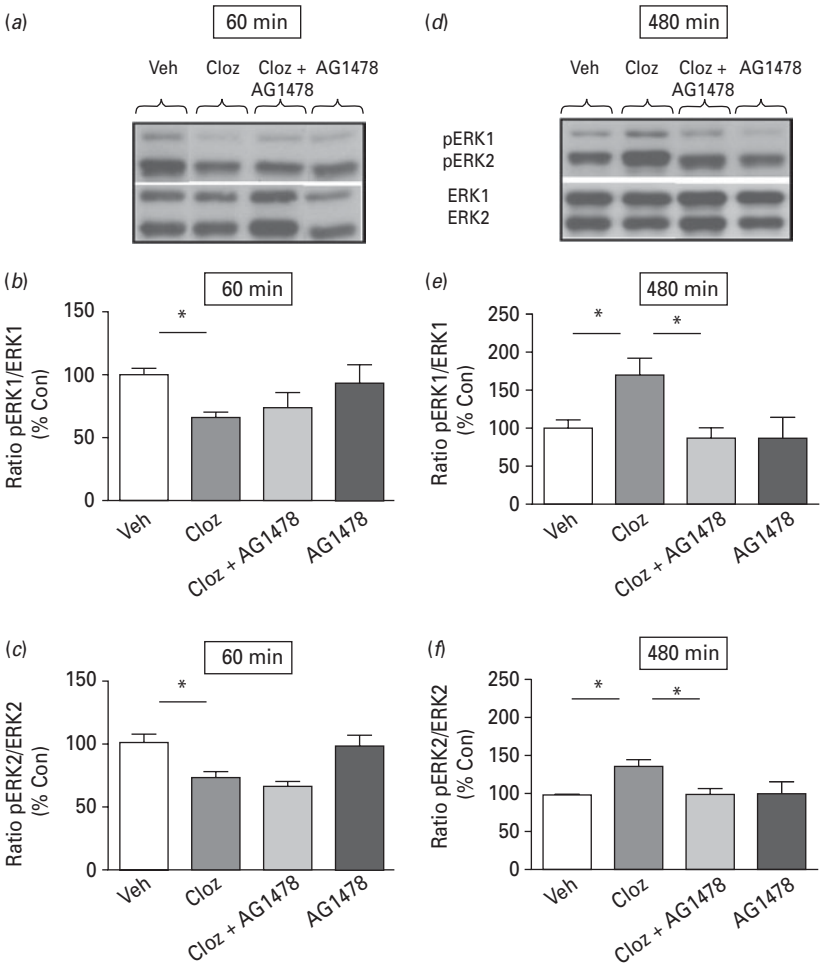

Fig. 4. Effect of clozapine on ERK phosphorylation in C57BL/6 mouse striatum in the absence or presence of AG1478 (EGF receptor inhibitor). (*a*) Effect of AG1478 on clozapine induced ERK phosphorylation at 60 min. (*b*) Effect of AG1478 on clozapine induced pERK1 phosphorylation at 60 min. (*c*) Effect of AG1478 on clozapine induced pERK2 phosphorylation at 60 min. (*d*) Effect of AG1478 on clozapine induced ERK phosphorylation at 480 min. (*e*) Effect of AG1478 on clozapine induced pERK1 phosphorylation at 480 min. (*f*) Effect of AG1478 on clozapine induced pERK2 phosphorylation at 480 min. Representative blots (*a, d*) indicate immunoreactive bands of phosphorylated ERK1 and ERK2 (upper panel) and total ERK1 and ERK2 (lower panel) in mouse striatum following *in-vivo* treatment and correspond with the bar graphs below (*b, c, e, f*). At each time-point treated samples were expressed relative to vehicle control (Con) standardized to 100%. Data represent the mean ± S.E.M. of at least three mice per experimental group. * $p < 0.05$, statistical differences between tissue in the absence (Veh) and presence of clozapine, and clozapine in the absence and presence of AG1478 are indicated. Veh, Vehicle; Cloz, clozapine.

The effect of brain region on haloperidol mediated ERK phosphorylation over time showed significant interaction between the factors of region and time for each ERK isoform (pERK1: $F_{9,66} = 2.84$, $p = 0.0070$; pERK2: $F_{9,66} = 2.47$, $p = 0.0172$). For pERK1, however, the factors of time and region independently just failed to reach statistical significance. For pERK2, significance was attributed to time but not region. Subsequent *post-hoc* pERK2 comparisons between vehicle- and haloperidol-treated mice at each time-point demonstrated that this was due to significant differences at 60 min in PFC ($p < 0.01$) (Fig. 6) and striatum ($p < 0.05$) (data not shown).

Effect of haloperidol in the absence and presence of AG1478 on ERK phosphorylation in mouse striatum

Haloperidol produced a significant increase in ERK1 phosphorylation in mouse striatum at 60 and 240 min (60 min: $F_{3,25} = 8.371$, $p < 0.0005$; 240 min: $F_{3,10} = 6.358$,

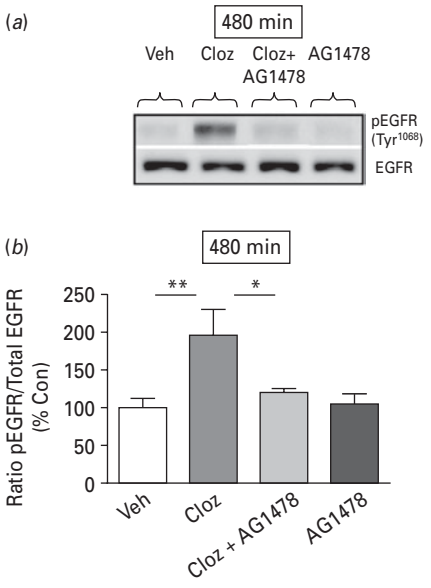

Fig. 5. Effect of clozapine (2.5 mg/kg) on striatal levels of EGF receptor (Tyr[1068]) phosphorylation in C57BL/6 mice in the absence or presence of AG1478 (EGF receptor inhibitor) at 480 min. Representative blots (*a*) indicate immunoreactive bands of phosphorylated EGF receptor (Tyr[1068]) (upper panel) and total EGF receptor levels (lower panel) following clozapine ± AG1478 treatment and correspond with the bar graph below. (*b*) Effect of AG1478 on clozapine induced EGF receptor phosphorylation at 480 min. Data are expressed relative to vehicle control (Con) standardized to 100% and represent the mean ± S.E.M. of at least four mice per experimental group. * $p<0.05$, ** $p<0.01$, statistical differences between tissue in the absence (Veh) and presence of clozapine and clozapine in the absence and presence of AG1478 are indicated. Veh, Vehicle; Cloz, clozapine.

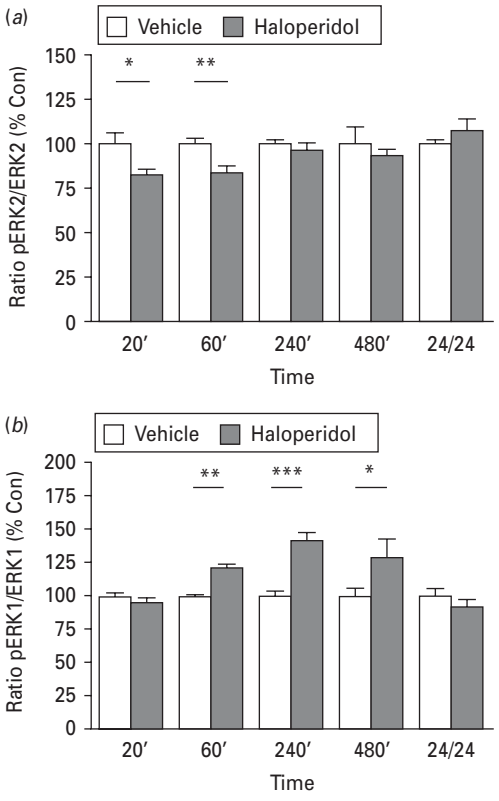

Fig. 6. Effect of haloperidol on ERK phosphorylation in C57BL/6 mouse prefrontal cortex (PFC) and striatum. (*a*) PFC: haloperidol treatment (0.25 mg/kg) over a 24-h period – pERK2. (*b*) Striatum: haloperidol treatment (0.25 mg/kg) over a 24-h period – pERK1. At each time-point treated samples were expressed relative to vehicle control (Con) standardized to 100%. Data represent the mean ± S.E.M. of at least four mice per experimental group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, statistical differences between tissue in the absence (vehicle) and presence of haloperidol are indicated.

$p<0.0110$) confirming our previous time-course findings. This haloperidol-induced ERK1 phosphorylation, however, was not significantly altered by prior treatment with AG1478. Similarly AG1478 itself did not significantly change pERK1 levels relative to vehicle control (Fig. 7*a–d*).

A summary of the significant pERK1/2 findings in mouse PFC and striatum following clozapine, olanzapine and haloperidol treatment over 24 h is provided in Table 1.

Discussion

Clozapine treatment *in vivo* exerted biphasic time-dependent effects on ERK1/2 phosphorylation in mouse striatum similar to its ERK transduction profile in PFC (Pereira *et al.* 2009) but distinct from that of olanzapine, its structural derivative. Thus clozapine caused initial blockade of the striatal pERK response at 20 and 60 min, subsequent activation at 480 min and return to baseline by 24 h. By comparison, olanzapine did not affect ERK1/2 phosphorylation in the striatum but altered cortical pERK1 signalling in an isoform-specific manner. Moreover, decrease in clozapine-induced ERK phosphorylation with SL327, a MEK inhibitor, suggested that activation had occurred via the obligate precursor MEK. Our data also indicated that the initial ERK phosphorylation decrease by clozapine was EGF receptor independent while the later ERK phosphorylation increase was EGF receptor dependent. Furthermore we verified that sustained clozapine treatment increased EGF receptor phosphorylation at the Tyr[1068] site in accord with its involvement in ERK signalling and denoting a

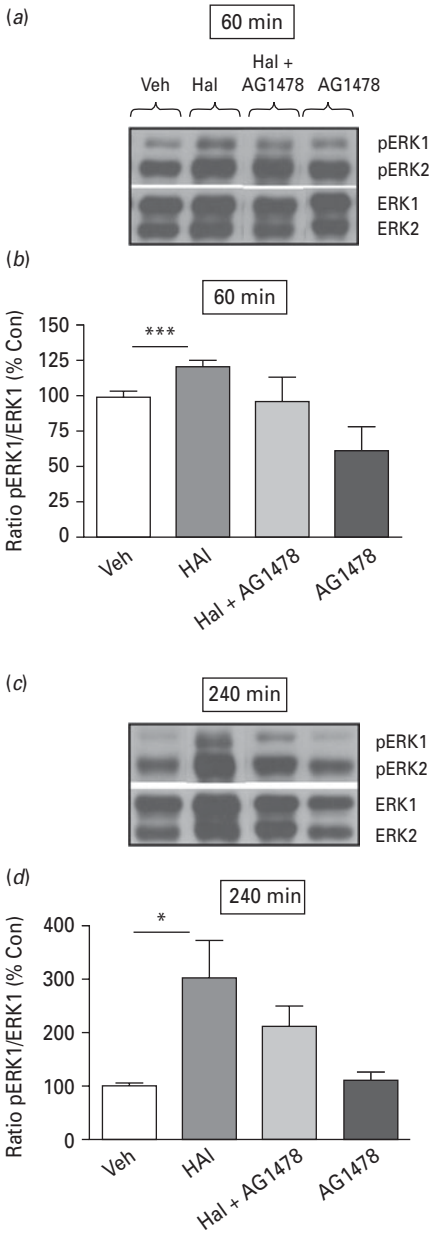

Fig. 7. Effect of haloperidol on ERK phosphorylation in C57BL/6 mouse striatum in the absence or presence of AG1478 (EGF receptor inhibitor). Representative blots (*a*) indicate immunoreactive bands of phosphorylated ERK1 (upper panel) and total ERK1 (lower panel) levels at 60 min following haloperidol (0.25 mg/kg) ± AG1478 treatment and correspond with the bar graphs below. (*b*) Effect of AG1478 on haloperidol induced ERK1 phosphorylation at 60 min. Representative blots (*c*) indicate immunoreactive bands of phosphorylated ERK1 (upper panel) and total ERK1 (lower panel) levels at 240 min following haloperidol (0.25 mg/kg) ± AG1478 treatment and correspond with the bar graphs below. (*d*) Effect of AG1478 on haloperidol induced ERK1 phosphorylation at 240 min. Data are expressed relative to vehicle control (Con) standardized to 100% and represent the mean ± S.E.M. of at least four mice per experimental group. * $p < 0.05$, *** $p < 0.001$, statistical differences between tissue in the absence (Veh) and presence of haloperidol are indicated. Veh, Vehicle; Hal, haloperidol.

mechanism of EGF receptor transactivation (Prenzel *et al.* 1999). These findings demonstrate that clozapine stimulation of ERK in striatal neurons engages the EGF system that we recently tied to APD action (Pereira *et al.* 2009).

The biphasic pattern of ERK phosphorylation observed in striatum following clozapine treatment *in vivo* parallels and extends our previous *in vitro* and *in vivo* findings in PFC neurons (Pereira *et al.* 2009). Although our striatal tissue comprised of both ventral and dorsal regions, our data were in agreement with a previous study conducted in mouse dorsal striatum, where acute 60-min clozapine administration reduced pERK1/2 levels consistent with its relative inability to induce extrapyramidal side-effects (Pozzi *et al.* 2003). The effect at subsequent times, however, was not investigated. Only a few other studies have examined clozapine regulation of striatal ERK signalling but direct comparisons require qualification given differences in study design, such as species tested, drug dose used, duration of study (acute or chronic) and outcome measures. In this regard, clozapine did not affect ERK activation in CD-1 mouse dorsal striatum 15 min after injection (Valjent *et al.* 2004) but induced ERK2 phosphorylation in rat ventrolateral caudate putamen following chronic (21-d) treatment (Ahmed *et al.* 2008). In the present study, changes in ERK phosphorylation were first recorded 20 min after drug injection at which point pERK1 was reduced, while elevation in pERK1/2 levels at 480 min is in accord with previous data (Ahmed *et al.* 2008) notwithstanding the different experimental paradigms used. Similarly, our olanzapine data which indicated reduced pERK1 levels between 1 h and 4 h after injection and an elevation 24 h later, limited to the PFC, concurs with findings in the rat where a decrease in cortical ERK1/2 phosphorylation in nuclear, cytosol and membrane fractions was noted at 2 h, whereas an increase in ERK phosphorylation restricted to the membrane fraction was observed 24 h after long-term exposure (Fumagalli *et al.* 2006). These data also coincide with behavioural outcomes whereby acute olanzapine treatment reduced baseline and amphetamine-induced hyperactivity in ERK1-ablated

Table 1. Summary of the significant pERK1/2 findings in mouse prefrontal cortex and striatum following clozapine, olanzapine and haloperidol treatment over 24 h

Antipsychotic drug	Prefrontal cortex	Striatum
Clozapine		
ERK1	↓ 20 min ↓ 60 min ↑ 480 min	↓ 20 min ↓ 60 min ↑480 min
ERK2	↓ 60 min	↓ 60 min ↑ 480 min
Olanzapine		
ERK1	↓ 60 min ↓ 240 min ↑ 24 h	
ERK2		
Haloperidol		
ERK1		↑ 60 min ↑ 240 min ↑ 480 min
ERK2	↓ 20 min ↓ 60 min	

↓, Significantly decreased ERK phosphorylation; ↑, significantly increased ERK phosphorylation.

mice that showed deficits in RSK1 signalling, an ERK substrate (Engel *et al.* 2009).

Haloperidol exerted differential regulation of ERK signalling in PFC and striatum, indicating dissimilarity with the atypical drugs, clozapine and olanzapine in these regions. For haloperidol, region and pERK isoform-specific changes were observed, including cortical decreases in pERK2 at 60 min and sustained striatal pERK1 increases at 480 min. Significant variability in pERK isoform levels within regions at the times tested may suggest that pERK1 and pERK2 pools are functionally different. Our cortical haloperidol data are consistent with effects seen in rat (Kim *et al.* 2008) and parallel findings that the drug did not improve an ERK-mediated cognitive impairment induced by methamphetamine (Kamei *et al.* 2006) or affect ERK activation following 21-d treatment (Ahmed *et al.* 2008). These findings may account for the ineffectiveness of haloperidol in treating cognitive deficits in schizophrenia. In striatum, our haloperidol data concur with other *in-vivo* mouse studies where a significant increase in pERK1 but no changes in pERK2 levels over baseline were seen at 60 min (Pozzi *et al.* 2003). Of particular interest is our observation of prolonged striatal pERK1 activation, given the association of haloperidol with extrapyramidal side-effects relative to the lower incidence of these symptoms seen with atypical APDs. Haloperidol antagonism of D_2R in the striatum, leading to phosphorylation of the transcription factor Elk-1 by an ERK dependent mechanism, may underlie these motor side-effects (Pozzi *et al.* 2003). Furthermore, elevated striatal ERK phosphorylation recorded with haloperidol is in line with volumetric and ultrastructural changes in synapse morphology (Konradi & Heckers, 2001) and maximal gene induction in neurotransmitter, GPCR and transcription factor signalling pathways elicited by the drug in striatum (Girgenti *et al.* 2010).

In mouse striatum, clozapine-induced ERK1/2 inhibition at 60 min was unaffected by EGF receptor blockade whereas clozapine-induced ERK1/2 activation at 480 min was significantly reduced via the EGF receptor, similar to findings reported in mouse PFC (Pereira *et al.* 2009). Thus we postulated that clozapine may be unique in recruiting the EGF receptor growth factor system to activate ERK and that this may have significance for clozapine's unmatched ability to treat refractory schizophrenia. Consistent with our hypothesis, ERK induction by haloperidol and olanzapine was found to be independent of EGF receptor activity. The modulation of the EGF receptor by clozapine is a novel mechanism of APD action and may have implications for the treatment of schizophrenia. For instance, EGF ligand and receptor levels in brain, serum and cerebrospinal fluid of patients with schizophrenia have been measured and notwithstanding some inconsistencies, studies have generally found decreased EGF ligand levels and compensatory up-regulated receptor levels in patients (Futamura *et al.* 2002; Ikeda *et al.* 2008). While genetic association studies have identified the EGF and NRG1 genes as risk candidates for schizophrenia (Stefansson *et al.* 2002), the EGF receptor A61G single nucleotide polymorphism has been linked with early-onset schizophrenia in male patients (Hanninen *et al.* 2007). Although animal studies have suggested that schizophrenia may be a delayed sequelae to a disruption in early neonatal EGF signalling (Futamura *et al.* 2003; Kato *et al.* 2011; Sotoyama *et al.* 2007), the EGF system can also be invoked later in development in the regulation of synaptic plasticity in the adult brain (Wong & Guillaud, 2004). In line with this, clozapine

corrected persistent cognitive and behavioural dysfunction induced by early abnormal EGF receptor stimulation in adult rats (Futamura *et al.* 2003) and has been associated with mild improvements in cognitive domains such as verbal fluency and delayed recall in schizophrenia (Woodward *et al.* 2005) with regulation of the EGF system being a potential determinant of such effects. Thus the present study suggests that treatment with APDs such as clozapine could potentially restore impaired EGF receptor signalling that may occur in some patients with schizophrenia.

Just which GPCR is utilized by clozapine to undertake EGF receptor phosphorylation is not known. We have, however, demonstrated previously that clozapine-induced transactivation of the EGF receptor occurred independently of the D_2 or 5-HT_{2A} receptor in mouse PFC neurons (Pereira *et al.* 2009). Furthermore, the transactivation pathway that signals to the EGF receptor through Src-family kinases, matrix metalloproteinases or β-arrestin-mediated endocytosis (Wetzker & Bohmer, 2003) remains to be defined for clozapine bearing in mind that there may be regional differences. Therefore, future studies will seek to identify the GPCR used by clozapine to recruit the EGF receptor and the transactivation mechanism downstream of receptor binding.

In summary, we have established that APD activation of ERK was differentially regulated by the EGF receptor in a temporal and region-specific manner. For clozapine, delayed striatal ERK phosphorylation mediated by the EGF receptor paralleled our previous cortical data. As distinct from clozapine, striatal ERK stimulation by haloperidol and cortical ERK phosphorylation by olanzapine were EGF receptor independent. This unique spatio-temporal pattern of ERK activation by clozapine involving EGF receptor transactivation may provide a mechanism of potential relevance to explain the superior effectiveness of clozapine in treatment-resistant schizophrenia.

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Statement of Interest

None.

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