Clozapine regulation of p90RSK and c-Fos signaling via the ErbB1-ERK pathway is distinct from olanzapine and haloperidol in mouse cortex and striatum

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Abstract

Treatment of the positive psychotic symptoms of schizophrenia with standard antipsychotic drugs (APDs) is ineffective in a proportion of cases. For these treatment resistant patients the alternative is the APD clozapine which is superior to other agents but carries serious side effects. Why clozapine is uniquely effective is unknown, but we have previously postulated may involve G-protein coupled receptor (GPCR) and epidermal growth factor (EGF) receptor (ErbB1) transactivation signaling to the mitogen-activated protein kinase-extracellular signal regulated kinase (MAPK-ERK) cascade. This was based upon clozapine induced initial down-regulation and delayed ErbB1 mediated activation of the cortical and striatal ERK response *in vivo* distinct from other APDs. This study investigated if modulation of the ErbB1-ERK1/2 pathway by clozapine, olanzapine and haloperidol affected expression of the ERK substrates p90RSK and c-Fos, factors that regulate transcription of proteins associated with neuroplasticity and synapse formation in C57Bl/6 mice. In cortex and striatum, acute clozapine treatment induced biphasic p90RSK phosphorylation via MEK that paralleled ERK phosphorylation independent of EGF receptor blockade. By contrast, olanzapine and haloperidol caused p90RSK phosphorylation that was not concomitant with ERK signaling over a 24-hour period. For c-Fos, clozapine elevated expression 24 hours after administration, a timeframe consistent with ERK activation at 8 hours. Alternatively, haloperidol stimulation of c-Fos levels limited to the striatum was in accord with direct transcriptional regulation through ERK. The unique spatio-temporal expression of downstream nuclear markers of the ErbB1-ERK pathway invoked by clozapine may contribute to its effectiveness in treatment resistant schizophrenia.

Keywords Antipsychotic drugs; signaling; ERK; p90RSK; c-Fos; schizophrenia

Abbreviations ANOVA, analysis of variance; APD, antipsychotic drug; BSA, bovine serum albumin; CREB, cAMP-response element-binding protein; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ErbB1, EGF receptor; GPCR, G-protein coupled receptor; HRP, horseradish peroxidase; hrs, hours; IgGs, immunoglobulins; IP, intraperitoneal; MAPK-ERK, mitogen activated protein kinase-extracellular signal regulated kinase; MEK, MAPK/ERK kinase; mins, minutes; PFC, prefrontal cortical; PKA, protein kinase A; s, seconds; PP2A, protein phosphatase 2A; p90RSK, 90 kDa ribosomal s6 protein kinase; P-p90RSK, phosphorylated p90RSK; SEM, standard error of the mean; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBST, tris buffered saline Tween

1. Introduction

The antagonism of dopamine principally at the D2 receptor is a unifying property of all antipsychotic drugs (APDs) used to treat schizophrenia (Kapur and Seeman, 2001; Masri et al., 2008). While such a mechanism may contribute to the amelioration of psychosis in about one half of patients, side effect tolerability and poor patient compliance further limit the effectiveness of APDs (Miyamoto et al., 2005). For the remaining patients who exhibit persistent psychotic symptoms, the atypical APD clozapine which demonstrates superior efficacy relative to other APDs (Leucht et al., 2009; McEvoy et al., 2006; Tandon et al., 2008) may be the only effective option. This implies that the mechanism underpinning the antipsychotic action of clozapine is unique. We have proposed that the selective efficacy of clozapine may involve a convergence of G-protein coupled receptor (GPCR) and epidermal growth factor (EGF) receptor/(ErbB1) signaling through the extracellular signal-regulated kinase (ERK) cascade (Pereira et al., 2009). The ERK cascade then phosphorylates proteins associated with an array of neuronal functions which govern aspects of memory formation, social behavior and emotion (Valjent et al., 2001; Sweatt, 2004), adaptive processes impaired in schizophrenia. In this regard, we have observed that distinct from olanzapine and haloperidol, clozapine induced initial inhibition and subsequent activation of the cortical and striatal ERK response *in vitro* and *in vivo*, mediated by the EGF receptor (ErbB1) (Pereira et al., 2009; Pereira et al., 2011). Modulation of the EGF receptor by clozapine points to APD action through alternate pathways and supports recent findings of developmental disturbances in EGF system signaling at neuregulin 1-ErbB4 (Hahn et al., 2006; Harrison and Law, 2006; Bertram et al., 2007) and EGF-ErbB1 receptors in the neonate and adult brain (Futamura et al., 2003; Kato et al., 2011; Sotoyama et al., 2007). Likely key distal targets

of APD regulation of the EGF receptor-ERK pathway are proteins that invoke gene transcription and may be involved in accounting for differences in APD related clinical outcomes such as 90 kDa ribosomal s6 protein kinase (p90RSK) and c-Fos.

p90RSK is a downstream substrate of the ERK cascade activated in response to growth factors, peptide hormones and neurotransmitters (Frodin and Gammeltoft, 1999). Three p90RSK isoforms; RSK1, RSK2 and RSK3 induce immediate-early gene expression of transcriptional regulators including c-Fos, cAMP-response element-binding protein (CREB) and CREB-binding protein (Xing et al., 1996). In addition, ERK1 knock-out mice show decreased phosphorylation of RSK1 in prefrontal cortex (PFC) and striatum and display region-specific ERK signaling deficits (Engel et al., 2009). There remains a paucity of literature however, on the effects of *in vivo* APD treatment on modulation of p90RSK levels. A single study in rat frontal cortex documented that haloperidol caused time and dose-dependent changes in p90RSK phosphorylation in concert with ERK1/2 changes via protein phosphatase 2A (PP2A), induced by alterations in dopaminergic transmission (Kim et al., 2008). Likewise, c-Fos expression which functions as a sensor for ERK signal duration is increased following mitogenic stimulation and p90RSK activation and is differentially affected by APDs (Deutch, 1994; Deutch and Duman, 1996; Sgambato et al., 1998). For example, haloperidol-induced increases in c-Fos in the striatum localized to D2 receptor output neurons (Bertran-Gonzalez et al., 2008), has been linked to its propensity to cause extrapyramidal side effects (Robertson and Fibiger, 1996; Marchese et al., 2008). By comparison, clozapine more consistently increased c-Fos levels in the PFC which may underlie its greater efficacy in treating negative symptoms (Robertson and Fibiger, 1992) but had limited effect in the striatum (Robertson et al., 1994; Wirtshafter and Asin, 2003; Verma et al., 2007). Olanzapine a congener of clozapine also increased c-Fos activity in the PFC

(Robertson and Fibiger, 1996), indicating cortical c-Fos induction is not a property unique to clozapine. Notwithstanding these data, an understanding of how clozapine and other APDs affect p90RSK and c-Fos neuronal signaling via ERK and the related EGF receptor system remains unexplored.

In view of this and based upon our assertion that a novel mechanism of action of clozapine involving the EGF receptor coupled with regulation of ERK phosphorylation may define its singular efficacy, these studies extend our previous *in vivo* findings (Pereira et al., 2009; Pereira et al., 2011) to (i) determine whether acute clozapine treatment differentially regulates p90RSK phosphorylation that coincides with ERK phosphorylation in mouse PFC and striatum in a manner distinct from olanzapine and haloperidol, (ii) ascertain whether acute clozapine, olanzapine or haloperidol treatment alters c-Fos expression over a 24-hr period in PFC or striatum and (iii) establish if any changes observed in p90RSK or c-Fos levels following APD treatment in cortex or striatum are EGF receptor mediated.

2. Methods

2.1. Drugs and reagents

All reagents including clozapine, haloperidol, SL327 and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Missouri, USA unless indicated otherwise. Olanzapine was supplied by Eli Lilly, Indiana, USA and AG1478 (EGF receptor inhibitor) obtained from A.G.

Scientific, Inc., California, USA. Antibodies such as phospho-p90RSK (P-p90RSK), RSK1/RSK2/RSK3 and β -Actin were provided by Cell Signaling Technology, Massachusetts, USA; goat anti-mouse and goat anti-rabbit horseradish peroxidase (HRP)-conjugated immunoglobulins (IgGs) by DAKO, NSW, Australia and c-Fos by Assay Designs, Michigan, USA.

2.2. *Animals*

All experimental procedures on mice were performed in accordance with The University of Melbourne Animal Ethics Committee guidelines. Groups of 7 week old male C57B/6 mice were housed under standard laboratory conditions on a 12-hr light-dark cycle (lights on 07:00 hrs) with food and water available ad libitum. Animals were acclimatized for one week to the laboratory environment; were handled daily to reduce acute stress before drug injection and were weighed prior to treatment.

2.3. *APD time course studies*

Acute time course experiments were conducted on groups (n=4-7) of C57Bl/6 mice administered with the APDs clozapine (2.5 mg/kg), olanzapine (1 mg/kg) or haloperidol (0.25 mg/kg), dissolved in 0.9% saline acidified with 0.1N HCl or vehicle (1% v/v) as a single dose via intraperitoneal (IP) injection. Treatments for each drug were for 20, 60, 240, 480 mins and 24 hrs; whilst clozapine was also examined at 120, 180 mins and 16 hrs and olanzapine at 120 min

and 16 hrs following injection. Olanzapine was examined in the PFC and not in the striatum since our previous data indicated no significant effects on striatal ERK1/2 phosphorylation through 24 hrs of drug treatment (Pereira et al., 2011). The doses chosen were mid-range of those used in mouse studies and reflect APD dose in humans. Doses were also the highest tolerated without sedation and generate effects consistent with antipsychotic mouse models of psychosis (Pozzi et al., 2003; Beshpalov et al., 2007; Pereira et al., 2009). After the time interval indicated mice were killed by decapitation, their heads immersed in liquid nitrogen for 6 s and brains rapidly removed and dissected within 20 s at ice-cold temperatures. Prefrontal cortical (PFC) and striatal (ventral and dorsal) tissue were sonicated in 1% SDS (750 μ l) and boiled for 10 mins; lysates were frozen at -80°C until required. Lysates were centrifuged at 14000 X g for 5 mins at 4°C to remove insoluble material prior to protein measurement using Bio-Rad Protein Assay (California, USA), with BSA as standard. Brain lysates were assayed for P-p90RSK and c-Fos expression as outlined.

2.4. Inhibitor treatment studies

To assess the effect of MEK inhibition on clozapine-induced p90RSK phosphorylation and c-Fos expression, SL327 (MEK inhibitor) at 30 mg/kg (Browning et al., 2005) dissolved in 36.5% DMSO was administered to C57Bl/6 mice (n=4) 10 mins before IP injection of clozapine or vehicle. SL327 was also injected at 180 and 360 mins after the experiment commenced to maintain adequate plasma levels with the experiment ending at 480 mins and 24 hrs later. Given that we had observed that clozapine significantly inhibited P-p90RSK at 60 mins and increased P-p90RSK levels at 480 mins in striatum (Fig. 1), we examined the effect of EGF receptor

inhibition on P-p90RSK and c-Fos expression, in mice (n=4-7) treated with AG1478 (EGF receptor inhibitor) at 25 mg/kg dissolved in 50% DMSO 10 mins preceding clozapine or vehicle administration for 60 and 480 mins. For the 480 min experiments, AG1478 was injected four times 2.5 hrs apart to maintain sufficient plasma concentrations (Ellis et al., 2006). Upon termination of treatment, PFC and striatum were extracted as earlier described.

2.5. *p90RSK and c-Fos assay*

30 μ g of protein lysate was separated by SDS-PAGE and immunoblotted using standard procedures. Briefly, proteins were electrotransferred to nitrocellulose membrane (Osmonics, Minnesota, USA) and blocked at room temperature in 5% skim milk powder, TBST (20 mM Tris-Base pH 7.5, 150 mM NaCl, 0.01% Tween-20). Membranes were exposed to phospho-p90RSK (Thr359/Ser363) antibody at 1:1000 in 5% BSA overnight and goat anti-rabbit HRP-conjugated IgGs at 1:2000 in blocking buffer for 90 mins at 4°C. For c-Fos determination, c-Fos (8B5) mouse monoclonal antibody at 1:500 and goat anti-mouse HRP-conjugated IgGs at 1:2000 in blocking buffer were utilized. Following primary and secondary antibody exposure, membranes were washed twice in TBST for 15 mins at room temperature. Immunoreactivity was detected using ECL Western Blotting Detection Reagents, (Amersham Biosciences, Buckinghamshire, UK) and Hyperfilm ECL (Amersham Biosciences). To control for loading, membranes were stripped in buffer containing 62.5 mM Tris-HCl at pH 6.7, 2% SDS and 100 mM β -mercaptoethanol at 50°C for 30 mins and re-probed with RSK1/RSK2/RSK3 (32D7) rabbit monoclonal antibody at 1:1000 in 5% BSA and goat anti-rabbit HRP-conjugated IgGs at 1:2000 in blocking buffer for measurement of total RSK levels as described above.

Alternatively, since c-Fos (8B5) detected endogenous levels of total c-Fos protein, β -Actin (13E5) rabbit monoclonal antibody at 1:2000 in 5% BSA was used as a loading control. Proteins were quantified using Multi Gauge Software (Fujifilm V3.0). The optical densities of phosphorylated p90RSK (P-p90RSK) or c-Fos immunoreactive bands were measured, normalized to the optical densities of total RSK and β -Actin, respectively, and expressed as a percentage of vehicle treated control.

2.6. Data analysis

Data was pooled accordingly with each treatment group repeated in quadruplicate and the mean $\pm$ standard error of the mean (SEM) calculated using GraphPad Prism 5 software (GraphPad Software Inc., California, USA). Two-way analysis of variance (ANOVA) was used to determine whether p90RSK and c-Fos levels were affected by factors of time and brain region after clozapine or haloperidol treatment. Bonferroni corrected multiple comparison tests were performed to discriminate differences between experimental groups. Variables were also assessed using one-way ANOVA followed by post hoc Bonferroni tests to determine the source of variation between experimental measures or Dunnett's multiple comparison tests to establish significant differences between control and treated groups. Unpaired Student's (2-tailed) *t*-tests for comparison between pairs of variables were used when necessary.

3. Results

3.1. Effect of antipsychotic drugs over 24 hrs on p90RSK phosphorylation in mouse prefrontal cortex and striatum

Clozapine-induced p90RSK phosphorylation was significantly affected by time ($F_{(13, 95)} = 4.88$, $p < 0.0001$) but not brain region ($F_{(1, 95)} = 0.24$, $p = 0.6238$) across 24 hours with no significant interaction observed between the factors ($F_{(13, 95)} = 0.90$, $p = 0.5588$). More specifically, clozapine caused an initial decrease in cortical p90RSK phosphorylation at 20 (vehicle $100 \pm 3\%$ vs clozapine $79 \pm 3\%$, $p < 0.01$) and 60 mins (vehicle $100 \pm 5\%$ vs clozapine $53 \pm 5\%$, $p < 0.01$), followed by a non-significant elevation above baseline and decline at 24 hrs which was significant (vehicle $100 \pm 7\%$ vs clozapine $76 \pm 3\%$, $p < 0.01$) (Fig. 1A). A biphasic pattern of p90RSK phosphorylation was also observed in the striatum with clozapine significantly reducing P-p90RSK levels at 60 (vehicle $100 \pm 9\%$ vs clozapine $60 \pm 4\%$, $p < 0.01$) and 120 mins (vehicle $100 \pm 11\%$ vs clozapine $63 \pm 4\%$, $p < 0.05$), then increasing P-p90RSK levels at 480 mins (vehicle $100 \pm 7\%$ vs clozapine $130 \pm 9\%$, $p < 0.05$) with a return to baseline at 24 hrs (Fig. 1B). Olanzapine however, did not alter PFC p90RSK phosphorylation over a 24-hr period relative to vehicle treated animals (Fig. 2). By comparison, haloperidol administration resulted in a significant overall interaction between the factors of time and brain region ($F_{(9, 55)} = 2.08$, $p = 0.0468$) with each factor contributing significantly to the total variance seen (Time: ($F_{(9, 55)} = 3.72$, $p = 0.0011$; Region: ($F_{(1, 55)} = 5.27$, $p = 0.0256$). Subsequent post hoc comparisons indicated that haloperidol produced a modest decrease in cortical p90RSK phosphorylation at 60 mins (vehicle $100 \pm 2\%$ vs haloperidol $83 \pm 5\%$, $p < 0.05$) but no significant changes thereafter (Fig. 3A). Furthermore, haloperidol caused a significant decrease in P-p90RSK levels in the striatum at 20 (vehicle $100 \pm 11\%$ vs haloperidol $79 \pm 10\%$, $p < 0.05$) and 60 mins (vehicle $100 \pm$

31% vs haloperidol $65 \pm 10\%$, $p < 0.05$) and 24 hrs after drug administration (vehicle $100 \pm 28\%$ vs haloperidol $49 \pm 17\%$, $p < 0.05$) (Fig. 3A and B).

3.2. *Effect of clozapine on p90RSK phosphorylation in the absence and presence of SL327 and AG1478 in mouse prefrontal cortex and striatum*

p90RSK phosphorylation induced by clozapine at 480 min in PFC and striatum was significantly attenuated by SL327, a MEK inhibitor (Fig. 4A–D). Whilst the decrease in p90RSK phosphorylation in response to clozapine at 60 mins in PFC (vehicle $100 \pm 3\%$ vs clozapine $67 \pm 7\%$, $p < 0.001$) and striatum (vehicle $100 \pm 4\%$ vs clozapine $69 \pm 5\%$, $p < 0.01$) was verified, this effect was not significantly altered by the presence of the EGF receptor inhibitor AG1478 in either brain region, despite a trend for reversal in PFC (clozapine $67 \pm 7\%$ vs clozapine + AG1478 $85 \pm 3\%$, $p > 0.05$) (Fig. 5A–D). Likewise, the observed increase in striatal P-p90RSK levels 480 mins after clozapine treatment was unaffected by AG1478 (Fig. 6A and B).

3.3. *Effect of antipsychotic drugs over 24 hrs on c-Fos expression in mouse prefrontal cortex and striatum*

Characterization of c-Fos expression in response to clozapine in mouse PFC and striatum over the designated time course indicated no significant overall interaction between the factors of time and region ($F_{(13, 99)} = 1.07$, $p = 0.3941$). When assessed independently, significance was attributed to time ($F_{(13, 99)} = 9.58$, $p < 0.0001$) but not region ($F_{(1, 99)} = 0.00$, $p = 0.9789$). Thus a similar c-Fos pattern was observed in both regions with an initial increase in c-Fos levels within

20 min followed by a decrease to 120 mins and subsequent activation at 24 hrs (Fig. 7A and B, respectively). After olanzapine administration, c-Fos levels showed some non-significant changes similar to clozapine in the PFC but were significantly lowered following 16 hrs exposure (Fig. 8). Haloperidol induced time ($F_{(9, 46)} = 12.99, p < 0.0001$) and region-specific changes ($F_{(1, 46)} = 13.48, p = 0.0006$) in c-Fos levels with significant reductions in the PFC at 20 min and 24 hrs and a 5-fold increase above vehicle in striatal tissue at 240 min (Fig. 9A and B, respectively).

3.4. Effect of clozapine in the absence and presence of SL327 and AG1478 on c-Fos expression in mouse prefrontal cortex and striatum

c-Fos protein levels induced by clozapine at 480 min and 24 hrs later in PFC and striatum were not significantly altered in the presence of SL327 in either brain region (data not shown). Consistent with the results of our clozapine time course experiment, clozapine inhibited c-Fos expression in the PFC after 60 mins of treatment ($p < 0.01$) (Fig. 10A and B). Pre-treatment with AG1478 and addition of clozapine nominally increased c-Fos levels, however, this effect was not significant compared to clozapine treatment alone (clozapine $70 \pm 10\%$ vs clozapine + AG1478 $84 \pm 8\%$, $p > 0.05$). AG1478 itself also produced no significant changes to c-Fos expression relative to vehicle (Fig. 10A and B). At 480 mins, non-significant reduction in c-Fos levels after exposure to clozapine was not altered by AG1478 (Fig. 10C and D). Striatal data followed similar trends to that recorded in the PFC and hence are not shown.

A summary of the P-p90RSK and c-Fos findings in mouse PFC and striatum following clozapine, olanzapine and haloperidol treatment over 24 hrs is provided in Table 1.

4. Discussion

In cortex and striatum, clozapine induced a biphasic profile of p90RSK phosphorylation that paralleled ERK phosphorylation as reported earlier (Pereira et al., 2009; Pereira et al., 2011). Thus initial clozapine suppression of p90RSK activity for up to 120 mins was followed by a tendency for delayed activation by 480 min and a moderate decline 24 hrs later. Whilst p90RSK phosphorylation at 480 min caused by clozapine was blocked with SL327 a MEK inhibitor, it appeared unaltered by the EGF receptor inhibitor, AG1478, although this also non-significantly increased p90RSK phosphorylation by itself. By contrast, olanzapine had no appreciable effect on cortical p90RSK levels whereas haloperidol induced p90RSK phosphorylation that was discordant with ERK phosphorylation. These data suggest that p90RSK is a target of the ERK pathway stimulated by clozapine but that olanzapine and haloperidol differ from clozapine in the manner in which they impact downstream transcriptional events.

We have demonstrated changes to ERK phosphorylation in response to *in vivo* clozapine treatment of about 40 percent (Pereira et al., 2011). This may influence phosphorylation of ERK substrates such as p90RSK and account for the modest but significant decrease at 60 mins and increase at 480 mins in phospho-RSK expression levels seen after drug administration. Furthermore, given that clozapine-induced p90RSK activation in PFC and striatum was not affected by AG1478 our data highlight the potential difficulty in using a pharmacological agent that targets an upstream component of the pathway, namely the EGF receptor, whilst measuring a distal response that is not sequentially linked. Additionally, the phospho-p90RSK antibody used

measured the total pool of phosphorylated p90RSK1/2/3 when phosphorylated at either Thr359 or Ser363. Therefore, isoform specific changes in p90RSK activity may be masked by the level of phosphorylation of the global pool of p90RSK. In line with this, Engel et al., (2009) reported that RSK1 but not RSK3 levels were decreased in PFC and striatum but not hippocampus of ERK1 knock-out mice indicating p90RSK and ERK isoform and regional specificity which should be specifically examined in subsequent studies. The picture is further complicated by a study demonstrating coincident upregulation of hippocampal pERK1/2 and p90RSK during associative learning paradigms in mice (Sananbenesi et al., 2002) suggesting a possible behavioural effect for clozapine given that it produced analogous signal transduction effects in cortex and striatum.

While clozapine induced similar patterns of p90RSK and ERK phosphorylation, haloperidol induced p90RSK activity that was not concomitant with ERK activity. Specifically, haloperidol caused a significant decrease in p90RSK phosphorylation in striatum at 60 min and 24 hrs later, which contrasted with sustained increases in striatal pERK1 levels noted before (Pereira et al., 2011). Differences between changes in ERK1/2 and p90RSK phosphorylation in response to haloperidol may suggest simultaneous activation of other direct substrates of ERK such as Elk-1 which haloperidol is known to target (Pozzi et al., 2003). Despite methodological differences, our haloperidol results in the PFC are comparable to a study in rat, where 0.5 and 1 mg/kg haloperidol increased cortical p90RSK phosphorylation at 15 mins and produced a prolonged decrease from 30 to 120 mins mediated by PP2A and ERK (Kim et al., 2008). However this study did not examine cortical p90RSK activity after 120 mins or RSK activity in the striatum (Kim et al., 2008).

In relation to c-Fos, clozapine increased levels at 20 min, inhibited expression thereafter but caused marked activation 24 hrs later. This time delay is consistent with the fact that ERK activation occurred 480 mins after clozapine administration (Pereira et al., 2011) and that, as a regulator of gene transcription, the synthesis of c-Fos protein may lag behind the activation of more proximal signaling events such as ERK. In addition, the initial inhibition of c-Fos by clozapine at 60 min was not reversed by AG1478 to any significant extent in keeping with our ERK findings. At 480 min however, a generalised non-significant decrease in c-Fos levels was noted across experimental treatments, which was not influenced by the EGF receptor inhibitor AG1478. In light of this, we cannot discount that an EGF receptor mediated effect on c-Fos levels following clozapine administration, could occur subsequent to 480 min consistent with the delayed c-Fos activation observed at 24 hrs. This latter c-Fos stimulation by clozapine is relevant given that the immediate early gene in transducing neural activity is known to influence dendritic growth through a mechanism gated by AP-1 (Hartwig et al., 2008) and regulate synaptic plasticity and memory consolidation (Fleischmann et al., 2003; Igelstrom et al., 2010; Sarantis et al., 2012). For haloperidol, the c-Fos expression profile generated had a closer temporal fit to ERK induction seen earlier (Pereira et al., 2011). Thus an initial reduction in PFC c-Fos levels at 20 min corresponded with decreases in ERK phosphorylation within the first 60 mins of haloperidol treatment, and similarly, increases in striatal c-Fos expression at 240 min matched ERK activation at this time point.

Evidence suggests that there may be variability in c-Fos activity in response to APDs between mice and rats (Wirtshafter and Asin, 2003). Our finding that clozapine inhibited c-Fos at 60 and 120 mins in mouse contrasts with a dose-dependent induction of c-Fos levels in rat PFC at 2 hrs

when dosed at 10, 20 and 30 mg/kg (Robertson and Fibiger, 1992) and may be due to such species differences. The dose range of clozapine used in rats covers the dose of 2.5 mg/kg administered to mice in our study when body weight is adjusted for and hence is unlikely to explain the observed differences in c-Fos activity at the same time point. Regional differences in c-Fos induction by clozapine and haloperidol have been reported whereby clozapine increased c-Fos in the PFC and had minimal effect in the striatum, while haloperidol markedly elevated c-Fos expression in the striatum but had no effect in the PFC (Robertson and Fibiger, 1992; Wirtshafter and Asin, 2003). As well, chronic clozapine treatment induced persistent expression of c-Fos and increased AP-1 activity in rat cortical regions highlighting more lasting effects of treatment (Kontkanen et al., 2002). As hypofrontality is a key determinant of cognitive functioning, the variable modulation of prefrontal c-Fos expression by clozapine and haloperidol may underscore their differential effectiveness in treating the negative and cognitive symptoms of schizophrenia (Weinberger, 1988; Robertson et al., 1994). In this regard, although not incontrovertible the observation that clozapine administration mildly improves the cognitive domains of delayed recall and processing speed in schizophrenia (Woodward et al., 2005) may be of relevance. Similarly, striatal increases in c-Fos expression in the presence of haloperidol but not with clozapine may be associated with the higher frequency of extrapyramidal and motor side effects associated with haloperidol use (Robertson et al., 1994; Robertson and Fibiger, 1996; Wirtshafter and Asin, 2003; Verma et al., 2007). Moreover, co-administration of delta-9-tetrahydrocannabinol with haloperidol but not clozapine increased striatal c-Fos expression possibly explaining increased cataleptic states seen with haloperidol (Marchese et al., 2008), albeit dependent on dose. Notwithstanding these observations, to account for the delayed therapeutic effects of APDs would require repeated dosing given possible differences between

the acute versus chronic responses of these agents in regulation of long-term neuronal effects such as synaptic proliferation and plasticity.

5. Conclusion

In summary, we have established that APDs exert dissimilar effects on expression of p90RSK and c-Fos, downstream markers pivotal to the translation of repeated short term coupling of drug with receptor into long-term neuronal adaptive changes. For clozapine, p90RSK phosphorylation matched ERK phosphorylation in timeframe but the direct involvement of the EGF system was not confirmed. Conversely, olanzapine and haloperidol induced distinctive patterns of p90RSK activity that did not mimic ERK activation. Hence olanzapine exerted limited effects on p90RSK expression whilst haloperidol caused a significant decrease in striatal p90RSK levels, contrary to the sustained increases in striatal pERK1 levels previously observed. On the other hand, delayed elevation in c-Fos expression 24 hrs after clozapine treatment was a lag time consistent with a more distal signaling event. By comparison, haloperidol stimulation of c-Fos expression in striatum in line with ERK activation suggested transcriptional regulation as a direct consequence of drug induced ERK signaling. Thus the temporal and regional properties of clozapine in modulating expression of nuclear targets of the ERK cascade distinct from olanzapine and haloperidol demonstrate a novel pathway of signaling that may be relevant in explaining the utility of clozapine in cases where other APDs have failed.

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Figure legends

Fig. 1. Effect of clozapine on p90RSK phosphorylation in C57Bl/6 mouse prefrontal cortex and striatum. (A) Clozapine treatment (2.5 mg/kg) over a 24 hr period – prefrontal cortex. (B) Clozapine treatment (2.5 mg/kg) over a 24 hr period – striatum. At each time point treated samples were expressed relative to vehicle control standardized to 100 percent. Data represent the mean $\pm$ SEM of 4 to 7 mice per experimental group. * $p < 0.05$; ** $p < 0.01$, statistical differences between tissue in the absence (vehicle) and presence of clozapine.

Fig. 2. Effect of olanzapine (1 mg/kg) on p90RSK phosphorylation in C57Bl/6 mouse prefrontal cortex over a 24 hr period. At each time point treated samples were expressed relative to vehicle control standardized to 100 percent. Data represent the mean $\pm$ SEM of 4 to 7 mice per experimental group. Statistical comparisons were made between tissue in the absence (vehicle) and presence of olanzapine.

Fig. 3. Effect of haloperidol on p90RSK phosphorylation in C57Bl/6 mouse prefrontal cortex and striatum. (A) Haloperidol treatment (0.25 mg/kg) over a 24 hr period – prefrontal cortex. (B) Haloperidol treatment (0.25 mg/kg) over a 24 hr period – striatum. At each time point treated samples were expressed relative to vehicle control standardized to 100 percent. Data represent the mean $\pm$ SEM of at least four mice per experimental group. * $p < 0.05$, statistical differences between tissue in the absence (vehicle) and presence of haloperidol.

Fig. 4. The effect of the MEK inhibitor SL327 on clozapine-induced p90RSK phosphorylation at 480 min in C57Bl/6 mouse prefrontal cortex and striatum. Representative blots indicate immunoreactive bands of phospho-p90RSK (upper panel) and RSK1/RSK2/RSK3 (lower panel) in (A) prefrontal cortex and (C) striatum following *in vivo* treatment with clozapine (2.5 mg/kg) $\pm$ SL327 and correspond with the bar graphs below. (B) $\pm$ SL327 treatment – prefrontal cortex (D) $\pm$ SL327 treatment – striatum. Data are expressed relative to clozapine treatment which has been standardized to 100 percent and represent the mean $\pm$ SEM of at least four mice per experimental group. * p <0.05, statistical differences compared with clozapine alone. Cloz=Clozapine

Fig. 5. The effect of the EGF receptor inhibitor AG1478 on clozapine-induced p90RSK phosphorylation at 60 mins in C57Bl/6 mouse prefrontal cortex and striatum. Representative blots indicate immunoreactive bands of phospho-p90RSK (upper panel) and RSK1/RSK2/RSK3 (lower panel) in (A) prefrontal cortex and (C) striatum following *in vivo* treatment with clozapine (2.5 mg/kg) $\pm$ AG1478 and correspond with the bar graphs below. (B) $\pm$ AG1478 treatment – prefrontal cortex (D) $\pm$ AG1478 treatment – striatum. Data are expressed relative to vehicle control standardized to 100 percent and represent the mean $\pm$ SEM of 7 mice per experimental group. ** p <0.01, *** p <0.001, statistical differences between tissue in the absence (V) and presence of clozapine are indicated. V=Vehicle, Cloz=Clozapine

Fig. 6. The effect of the EGF receptor inhibitor AG1478 on clozapine-induced p90RSK phosphorylation at 480 mins in C57Bl/6 mouse striatum. (A) Representative blots indicate immunoreactive bands of phospho-p90RSK (upper panel) and RSK1/RSK2/RSK3 (lower panel) following *in vivo* treatment with clozapine (2.5 mg/kg) $\pm$ AG1478 and correspond with the bar

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Fig. 7. Effect of clozapine on c-Fos expression in C57Bl/6 mouse prefrontal cortex and striatum. (A) Clozapine treatment (2.5 mg/kg) over a 24 hr period – prefrontal cortex. (B) Clozapine treatment (2.5 mg/kg) over a 24 hr period – striatum. At each time point treated samples were expressed relative to vehicle control standardized to 100 percent. Data represent the mean $\pm$ SEM 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 are indicated.

Fig. 8. Effect of olanzapine (1 mg/kg) on c-Fos expression in C57Bl/6 mouse prefrontal cortex over a 24 hr period. At each time point treated samples were expressed relative to vehicle control standardized to 100 percent. Data represent the mean $\pm$ SEM of 4 to 7 mice per experimental group. $*p<0.05$, statistical differences between tissue in the absence (vehicle) and presence of olanzapine are indicated

Fig. 9. Effect of haloperidol on c-Fos expression in C57Bl/6 mouse prefrontal cortex and striatum. (A) Haloperidol treatment (0.25 mg/kg) over a 24 hr period – prefrontal cortex. (B) Haloperidol treatment (0.25 mg/kg) over a 24 hr period – striatum. At each time point treated samples were expressed relative to vehicle control standardized to 100 percent. Data represent

the mean $\pm$ SEM of at least four mice per experimental group. $*p<0.05$; $***p<0.001$, statistical differences between tissue in the absence (vehicle) and presence of haloperidol are indicated.

Fig. 10. The effect of the EGF receptor inhibitor AG1478 on clozapine-induced c-Fos expression at 60 and 480 mins in C57Bl/6 mouse prefrontal cortex. Representative blots indicate immunoreactive bands of c-Fos (upper panel) and β -Actin (lower panel) at (A) 60 mins and (C) 480 mins following *in vivo* treatment with clozapine (2.5 mg/kg) $\pm$ AG1478 and correspond with the bar graphs below. (B) $\pm$ AG1478 treatment – 60 mins (D) $\pm$ AG1478 treatment – 480 mins. Data are expressed relative to vehicle control standardized to 100 percent and represent the mean $\pm$ SEM of 4 to 7 mice per experimental group. $**p<0.01$, statistical differences between tissue in the absence (V) and presence of clozapine are indicated. V=Vehicle, Cloz=Clozapine

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Table 1 Summary of the P-p90RSK and c-Fos findings in mouse prefrontal cortex and striatum following clozapine, olanzapine and haloperidol treatment over 24 hours.

APD	Brain region	Protein	20 min	60 min	120 min	180/240 min	480 min	16/24	24/24
Clozapine	Prefrontal cortex	P-p90RSK	↓	↓	—	—	—	—	↓
		c-Fos	↑	↓	↓	—	—	—	↑
	Striatum	P-p90RSK	—	↓	↓	—	↑	—	—
		c-Fos	—	↓	↓	—	—	—	↑
Olanzapine*	Prefrontal cortex	P-p90RSK	—	—	—	—	—	—	—
		c-Fos	—	—	—	—	—	↓	—
Haloperidol	Prefrontal cortex	P-p90RSK	—	↓	—	—	—	—	—
		c-Fos	↓	—	—	—	—	—	↓
	Striatum	P-p90RSK	↓	↓	—	—	—	—	↓
		c-Fos	—	—	—	↑	↓	—	—

↓ = significantly decreased protein phosphorylation

↑ = significantly increased protein phosphorylation

— = no significant change

APD=antipsychotic drug, min= minutes

* The effect of olanzapine on p90RSK and c-Fos levels was not studied in the striatum since no significant changes in upstream ERK1/2 phosphorylation within 24 hrs of drug treatment were observed in this brain region (Pereira et al., 2011).

Research Highlights

- Antipsychotic drugs differentially target the ERK substrates p90RSK and c-Fos
- Clozapine induces biphasic p90RSK phosphorylation that mimics ERK phosphorylation
- Olanzapine and haloperidol phosphorylate p90RSK discordant with ERK signaling
- Elevation of c-Fos by clozapine is consistent with timeframe of ERK activation
- Spatio-temporal effect of clozapine on p90RSK/c-Fos may contribute to efficacy in treatment resistant schizophrenia

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