mRNAs for clozapine-sensitive receptors co-localize in rat prefrontal cortex neurons

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Received 14 August 1998; received in revised form 29 October 1998; accepted 29 October 1998

Abstract

The clinical efficacy of clozapine in treating schizophrenia may stem from its lack of receptor selectivity. If true, several clozapine-sensitive receptors may be co-expressed by neurons dysfunctional in schizophrenia. To test this hypothesis, neurons from the rat medial prefrontal cortex were acutely isolated and subjected to single cell RT-PCR analysis. The co-ordinated expression of five clozapine-sensitive receptors (D₄, m₁, 5-HT₂a, 5-HT₂c, 5-HT₇) was examined in interneurons and pyramidal neurons. Profiling of GABAergic interneurons commonly revealed the co-expression of two or more clozapine-sensitive receptor mRNAs. Although co-expression of these receptors was less extensive in pyramidal neurons, it was also commonly found. These results suggest that clozapine’s therapeutic effects may be mediated by antagonism of dopaminergic, cholinergic and serotonergic signaling pathways at the single cell level. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved

Keywords: Prefrontal cortex; Single cell RT-PCR; Interneurons; GABA; Pyramidal neurons; Dopamine; Acetylcholine; Serotonin; Schizophrenia

Disordered dopaminergic signaling within the prefrontal cortex has long been thought to be a critical factor in the etiology of schizophrenia [18]. This view is based upon several key observations. Foremost is the ability of typical neuroleptics that act as D₂ dopamine receptor antagonists to alleviate the positive symptoms of schizophrenia. However, in recent years, the exclusive involvement of dopamine in schizophrenia has been questioned [11,16]. In part, the clinical effectiveness of atypical neuroleptics, like clozapine, have motivated this new line of thought. Clozapine differs from typical neuroleptics in its efficacy in alleviating both negative and positive symptoms, its lack of significant motor side effects, and its ability to alleviate symptoms in patients refractory to typical neuroleptics [12,17]. The reasons for clozapine’s clinical efficacy are unclear at present.

One hypothesis is that clozapine’s ability to antagonize both dopamine D₄ receptors and serotonin (5-HT₂a, 5-HT₂c, 5-HT₇) receptors is crucial [12]. In support of this view, a D₄ receptor selective antagonist appears to be clinically ineffective [13], whereas mixed antagonists like risperidone have yielded promising results [17].

If clozapine acts by antagonizing both dopamine D₄ and serotonin receptors, where do these receptors reside? Although there is evidence for alterations in neural function at several levels of the neuroaxis in schizophrenia [2], most experimental evidence points to prefrontal cortex [8]. Both anatomical and functional studies have revealed alteration in the prefrontal cortex of schizophrenics. Furthermore, clozapine appears to have a profound impact on this region. For example, clozapine induces expression Fos in the rat prefrontal cortex while classical neuroleptics have little or no effect in this area [7]. Also, chronic treatment with clozapine leads to down-regulation of D₄ dopamine receptors in primate prefrontal cortex, whereas typical neuroleptics do not [15]. Although clozapine’s effects may be distributed across several cell types leading to complex alterations in

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circuit function, it is also possible that clozapine preferentially targets a particular class of cells. This class of neuron would be distinguished by its co-expression of several clozapine-sensitive receptors.

To test this possibility, we performed single cell RT-PCR (scRT-PCR) profiling of rat prefrontal cortical neurons. This technique is particularly well-suited to determine the co-ordinated expression of four or more receptor mRNAs in the same neuron, in contrast to conventional in situ hybridization [6,21]. We initially focused on GABAergic interneurons because they have been implicated in schizophrenia, [3] and they are known to express D2 receptors [19]. The methodology used to perform the scRT-PCR experiments have been described previously [20,21]. GABAergic interneurons were identified by their expression of glutamic acid decarboxylase (GAD) and calcium binding proteins (parvalbumin, calbindin D28k and calretinin) [6].Pyramidal neurons were identified by the absence of GAD mRNA and the presence of mRNA for Ca2+/calmodulin-dependent protein kinase II [4]. After identification, neurons were tested for the presence of mRNA for D3 dopamine receptors, 5-HT2a, 5-HT2c, 5-HT7 serotonin receptors and m1 muscarinic receptors. m1 muscarinic receptors were added to our screening because of their high affinity for clozapine and their potential importance in cognitive function of prefrontal cortex [12]. The D2 receptor primers have been published previously [21]. The primers used in the other reactions were developed with commercially available software (Oligo, National Biosciences, Plymouth, MN) and synthesized by Life Technologies (Bedford, MA). Fifty-five cycles of PCR were performed with an annealing temperature of 56°C; all other conditions were as described previously [21]. The primer sequences were: GAD (GenBank accession #M21410) 5′-GTATGACGCTCTCTACGATA-CAGG-3′ and 5′-AGGAAATCGATGTCAGACTGGGTG-3′; CaMKII (GenBank accession #J02942) 5′-ACAAGAA-GATGCGTAGTGATCATAGGTGATGTG-3′; calbindin (D28k) (GenBank accession #M12725) 5′-GGGATTGAA-GCTTGAACCTTGTGAGT-3′ and 5′-GATGCGTAGTGATCATAGGTGATGTG-3′; parvalbumin (GenBank accession #M21410) 5′-ACGTTCATTGGTATG-CCGATAA-GTGTTGCTCCGAGCAAGAAGA-CAGT-3′ and 5′-TTGTTGCTCCGAGCAAGAAGA-CAGT-3′; 5-HT2a (GenBank accession #M30705) 5′-TGGACGGAAAATGCTCCTCTTCA-3′ and 5′-TTCGATCCGGACCTTGTGACTA-3′; 5-HT2c (GenBank accession #M16406) 5′-TCTGAGACACCAGCGAAAGGTGTG-3′ and 5′-TTTCTCTTGCGGACCTGTTCCCT-3′. Controls for genomic amplification and cDNA contamination [21] were run for every batch of neurons processed and were consistently negative.

Neurons were acutely isolated from medial prefrontal cortex obtained from the coronal brain sections of 3–5 weeks old Sprague–Dawley rats. Pyramidal and non-pyramidal neurons were readily distinguished on the basis of shape and somal diameter (Figs. 1B and 2B). Forty-one GAD-positive, presumptive interneurons were identified with scRT-PCR. All had detectable levels of calcium binding protein (CBP) mRNA. Surprisingly, over half our sample (21/41) had detectable levels of more than one CBP mRNA. An extreme example is shown in Fig. 1A (top). This neuron expressed GAD mRNA in addition to mRNA for calbindin, parvalbumin and calretinin. Other neurons expressed detectable levels of only one CBP mRNA (Fig. 1A, bottom). Immunocytochemical studies have suggested that although there is some co-localization (ca. 10–20%), these CBPs are largely segregated in interneuronal populations [9,14,22]. Developmental studies have shown that in the 2nd and 3rd postnatal weeks, calbindin and parvalbumin...
The detection of clozapine-sensitive receptor mRNAs was not strongly correlated with that of CBPs. A scatter plot summarizing the profiling experiments in GABAergic interneurons is shown in Fig. 1C. m1 muscarinic receptor mRNA was the most commonly detected of the mRNAs examined (27/41) (Fig. 1A, top; Fig. 1C). D4 receptor mRNA was found in about a quarter of the sample (11/41). Of the 5-HT receptor mRNAs, 5-HT2a was the most commonly detected (16/41) with 5-HT2c (11/41) and 5-HT7 (8/41) mRNAs being less frequent (Fig. 1A, bottom; Fig. 1C). Insofar as co-expression was concerned, there were several interesting patterns. The majority of neurons (28/41) had detectable levels of two or more receptor mRNAs. Thirty-nine percent (16/41) had detectable levels of three or more mRNAs. However, less than half of the neurons having detectable levels of D4 (4/11) or 5-HT7 (7/16) had detectable levels of m1 muscarinic receptor mRNA. This was in sharp contrast to 5-HT2a and 5-HT2c positive neurons, where m1 muscarinic receptor mRNA was frequently detected (9/11 and 6/8, respectively). These results argue that potentially important subsets of interneuron can be identified based upon the pattern of clozapine-sensitive receptor co-expression.

As a first step toward determining whether co-expression of clozapine-sensitive receptors was limited to GABAergic interneurons, a subpopulation of pyramidal neurons was profiled. These neurons were initially screened using morphological criteria (Fig. 2B) and then unequivocally identified by the absence of GAD mRNA and the presence of Ca2+/calmodulin-dependent kinase II mRNA [4]. Because of the recent identification of prominent 5-HT2a receptor immunoreactivity in primate pyramidal neurons and the potential importance of this receptor in mediating clozapine’s clinical efficacy [10,11,16], cells were initially screened for 5-HT2a mRNA. A group of twenty-nine 5-HT2a-positive neurons were further analyzed. Of these, the vast majority (24/29) had detectable levels of m1 receptor mRNA (Fig. 2A, upper). Although less frequent, many pyramidal neurons had detectable levels of other clozapine-sensitive receptors. An example is shown in Fig. 2A (lower). A scatter plot summarizing these experiments is shown in Fig. 2C. Detectable levels of D4 mRNA were found in 21% (6/29) of this group, 5-HT2c mRNA was detected in 28% (8/29) and 5-HT7 mRNA was found in 24% (7/29). All of the neurons had detectable levels of at least two clozapine-sensitive receptors. Interestingly, all the pyramidal neurons having detectable levels of D4 mRNA co-expressed m1 receptor mRNA. Over one-third (11/29) had detectable levels of three or more receptor mRNAs. These results clearly demonstrate that a prominent subset of pyramidal neurons co-express clozapine-sensitive receptors.

Does the co-expression of detectable levels of mRNAs for clozapine-sensitive receptors imply that functionally significant complements of receptor protein are present? Several lines of evidence support this conclusion. Previous scRT-PCR studies have found strong correlations between mRNA detection and functional protein [20]. Furthermore, immunocytochemical studies of D4 and 5-HT2a receptor expression are largely in agreement with our findings [10,19]. Nevertheless, functional assays of receptor protein will be required to provide definitive evidence.

In spite of this limitation, our results argue that clozapine-sensitive receptors are co-expressed by at least two groups of prefrontal neurons. Although D4 receptor mRNA was not detected frequently in cortical pyramidal neurons (as in previous studies [19]), other clozapine-sensitive receptor mRNAs were prominent. It is possible that in these neurons, D4 dopamine receptors are a more significant target of clo-

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Fig. 2. scRT-PCR profiling of pyramidal neurons revealed the co-expression of clozapine-sensitive receptor mRNAs. The analysis was restricted to a subpopulation of pyramidal cells expressing 5HT2a mRNA. (A) Photographs of two ethidium bromide stained agarose gels in which the RT-PCR amplicons from individual pyramidal neurons have been separated by electrophoresis. In one neuron (top), PCR amplicons for CAM-kinase-type-II alpha subunit, 5HT2a and m1 receptor mRNAs were detected. In the other neuron (bottom), the PCR amplicons for CAM-kinase-type-II alpha subunit, 5HT2a, 5HT2c, 5HT7, m1 and D4 mRNAs were detected. The predicted sizes for CAM-kinase-II alpha subunit, GAD, 5HT2a, 5HT2c, 5HT7, m1 and D4 mRNAs were: 354, 426, 465, 545, 383, 189, and 378 base pairs, respectively. (B) Photomicrographs of neurons corresponding to the gels in (A) are shown. Scale bar, 20 μm. (C) The distribution of the clozapine-sensitive receptors in the subpopulation of 29 5-HT2a positive pyramidal neurons is illustrated. The symbols in the plot denote the mRNA detected.
zapine [5,12]. If true, it would mean that three or more receptors in a single pyramidal neuron would be affected by clinically relevant clozapine concentrations. GABAergic interneurons are clearly another important site of clozapine action. It is of potential interest that D4 receptor mRNA was infrequently detected in interneurons expressing m1, 5-HT2a or 5-HT2c receptor mRNAs, whereas these three receptors were commonly found together. These latter receptors share a linkage to G proteins that stimulate phospholipase C. As a consequence, clozapine should significantly disrupt the regulation of intracellular Ca2+ metabolism by serotonin and acetylcholine in this group of interneurons. Taken together, these findings support the hypothesis that clozapine’s ability to simultaneously disrupt several signaling pathways in cortical pyramidal neurons and GABAergic interneurons is a key determinant of its clinical efficacy.

This work was supported by an Established Investigator award from NARSAD to D.J.S. Much of this work was performed at the University of Tennessee, Memphis, TN. We wish to thank Tatiana Tkatch and Gytis Baranauskas for careful reading of the manuscript and helpful discussions.


