Prototypical antipsychotic drugs protect hippocampal neuronal cultures against cell death induced by growth medium deprivation

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Abstract

Background: Several clinical studies suggested that antipsychotic-based medications could ameliorate cognitive functions impaired in certain schizophrenic patients. Accordingly, we investigated the effects of various dopaminergic receptor antagonists – including atypical antipsychotics that are prescribed for the treatment of schizophrenia – in a model of toxicity using cultured hippocampal neurons, the hippocampus being a region of particular relevance to cognition.

Results: Hippocampal cell death induced by deprivation of growth medium constituents was strongly blocked by drugs including antipsychotics (10^-10-10^-6 M) that display nM affinities for D2 and/or D4 receptors (clozapine, haloperidol, (+)-sulpiride, domperidone, clozapine, risperidone, chlorpromazine, (+)-butaclamol and L-741,742). These effects were shared by some caspases inhibitors and were not accompanied by inhibition of reactive oxygen species. In contrast, (-)-raclopride (10^-6 M) was able to block the neuroprotective effect of the atypical antipsychotic clozapine (10^-6 M).

Conclusion: Taken together, these data suggest that D2-like receptors, particularly the D4 subtype, mediate the neuroprotective effects of antipsychotic drugs possibly through a ROS-independent, caspase-dependent mechanism.

Background

There is clinical evidence of cognitive dysfunction in certain schizophrenic patients that is likely to be independent from psychotic symptoms [1]. This dysfunction does not seem to involve a single brain region but rather a network that includes cortical and sub-cortical regions such as the hippocampus. The therapeutic benefits of various antipsychotic drugs are thought to be predominantly associated with their antagonistic actions on D2-like (D2, D3 and D4) dopamine receptors in the brain [2,3]. Although early studies with typical antipsychotic drugs (e.g. haloperidol, chlorpromazine) mostly failed to report significant improvements of cognitive behaviors in schizophrenic patients [4-6], more recent data especially obtained using atypical antipsychotics (e.g. clozapine, risperidone, olanzapine) demonstrated positive effects [7-12]. For exam-
ple, risperidone has been associated with improved verbal working memory and executive functions whereas clozapine and quetiapine seem to improve verbal fluency [9, 13, 14].

The beneficial effects of antipsychotics on cognitive functions and neuroprotection are supported by *in vitro* and animal studies reporting on the protective effects of these drugs in various models of toxicity including focal ischemia [15-19], serum deprivation [20], oxidative stress [21] and apoptosis [22]. More recently, it has been reported that the atypical antipsychotic olanzapine was neuroprotective against various forms of toxicity through the phosphorylation of kinases such as Akt [23].

In the present study, the possible neuroprotective properties of low concentrations of various antipsychotic drugs and other dopamine receptor antagonists were studied in a model of toxicity using primary cultured neurons of the hippocampus, an area particularly relevant to cognitive processes.

**Results**

**Dopamine receptor transcripts are expressed in mature cultured hippocampal neurons**

We estimated first the number of mature neurons in our 3-day old hippocampal cultures using immunocytochemistry for the neuron-specific marker NeuN [24]. Approximately 75% of the cells were labeled thereby indicating that a high proportion of neurons were mature at this stage.

We determined next if the genes coding for the dopamine receptor subtypes were expressed in these cultures. The primer pairs for the amplification of dopamine receptor subtypes 1 to 5 cDNAs were first tested on RNA extracted from rat striatum using a reverse transcription-multiplex PCR (RT-mPCR). As shown in Fig 1B, all primer pairs were able to generate products of the expected length. RT-mPCR was next performed on samples from untreated 3 day-old primary hippocampal cultures. Transcripts for all five dopamine receptor subtypes were also found to be expressed in these cultures (Fig. 1A). It is of note that band intensities do not necessarily reflect relative expression levels of transcripts for the various dopamine receptor subtypes in the starting extract since no internal standards were used. No products were seen when reverse transcriptase was omitted in the RT step indicating that amplified fragments are from transcribed mRNA. Splice isoforms for the D2 and D3 receptor subtypes were observed as well, in both striatum and hippocampal cultures. Sequencing of hippocampal main PCR products confirmed that amplifications were specific for dopamine receptors and that the D2 primer pair amplified the two alternatively spliced transcripts coding for functionally distinct isoforms D2L and D2S [25, 26].

**Effects of typical and atypical antipsychotics against toxicity induced by N2 constituents-deprivation**

As previously described in rat neuroblastoma cells [27], deprivation of transferrin, one of the major iron transport protein in the blood [28], selenium, an essential nutrient with antioxidant properties [29], as well as putrescine, a drug with growth-stimulatory properties [27], resulted in about 70% of hippocampal neuronal cell death as monitored 3 days later using MTT and NR colorimetric assays. Cell death was strongly reduced, in a concentration-dependent manner, in presence of atypical antipsychotics such as clozapine which preferentially binds to D4 receptors over D2 or D3 receptors (Fig 2A) and risperidone, a D2/D4 receptor antagonist that protected hippocampal neurons at the highest concentration tested here [100 ± 6 (CT) vs 162 ± 12 (CT + risperidone 10^-6 M); p < 0.01]. The effects of these atypical antipsychotics were shared by the classical antipsychotic haloperidol which offered a maximal protection at 10^-6 M (Fig. 2B) while, as expected, a higher concentration (10^-4 M) was toxic on its own to hip-
pocampal neurons (10 ± 2 vs 100 ± 4; p < 0.01). Similar effects were obtained with (±)-sulpiride, a selective D2 dopamine receptor antagonist belonging to the benzamide class (Fig 2C); domperidone, a D2/D3 receptor antagonist (Fig 2D); chlorpromazine, a typical antipsychotic which binds with nM affinities to D2, D3, and D4 receptors (Fig 2E); and (+)-butaclamol, a D2/D4 dopamine receptor antagonist (Fig 2F). Interestingly, a D4 receptor antagonist, L-741,742 (10-6M) [30] somewhat protected neurons [100 ± 5 (CT) vs 186 ± 10 (CT + L-741,742) and vs 156 ± 15 (CT + haloperidol 10-6 M); p < 0.01]. Cells treated with the N2 supplement showed the same magnitude of protection (with MTT values ranging from 205% to 389% vs control groups) as that of cells treated with most of drugs at 10-6 M, suggesting that depletion in growth medium rather than cell washes are responsible for decreases in MTT and NR values.

In contrast, the piperidine metabolite of haloperidol, which is devoid of affinity for D2-like receptor [31] was ineffective (Fig 3A). Similarly, (−)-raclopride, a D2/D3 receptor antagonist, and the D1 receptor antagonist (+)-SCH-23390 failed to protect hippocampal neurons (Fig 3B,C). The D3 dopamine receptor antagonist U 99194 maleate (10-6M) [32] was also ineffective [100 ± 5 (CT) vs 100 ± 6 (CT + U 99194)] (Table 1). Finally, remoxipride, another D2 receptor antagonist with weak D4 receptor affinity, failed to protect neuronal cells [100 ± 3 (CT) vs 108 ± 3 (CT + remoxipride) vs 141 ± 4 (CT + N2)].

Table 1 summarizes the apparent affinities of various dopamine receptor antagonists for the D2 and D4 subtypes with their protective effects on hippocampal neurons.

**D2 but neither sigma nor NMDA receptor antagonists blocked the protective effect of antipsychotics**

Besides its well-known antidopaminergic activity, it has been hypothesized that haloperidol protects neuronal cells[16] through its purported activity at σ₁ [33] or NMDA receptors [34]. However, neither NE-100 (10⁻⁷-10⁻⁵ M), a potent and selective σ₁ receptor subtype antagonist, nor (+)-MK-801 (10⁻⁶-10⁻⁵ M), a non-competitive NMDA antagonist, affected neuronal survival (Table 2). Moreover, these compounds failed to modulate the pro-
The protective effects of antipsychotic drugs may involve caspases but not the inhibition of the production of free radicals

In light of the purported anti-apoptotic effects of atypical antipsychotics drugs [22], we investigated next the effects of various inhibitors of caspases, these enzymes likely playing a pivotal role in apoptosis-related cell death. In our model, the co-administration of the caspase-3 inhibitor DEVDO-CHO (5 μM), the caspase-8 inhibitor IETD-CHO (5 μM) or to a lesser extent the caspase-9 inhibitor LEHD-CHO (5 μM) significantly reduced cell death, DEVDO-CHO being the most potent (Table 3). The protective effects of inhibitors of caspases 3, 8 and 9 were not accompanied by changes in ROS accumulation, as evaluated by the DCF assay (Table 3). It has recently been shown that the atypical antipsychotic olanzapine increased cell viability after an exposure to H₂O₂ [21] suggesting that blockade of peroxide accumulation may be involved in the protective effects of antipsychotics reported here. However, results obtained using the DCF assay indicated that haloperidol (10⁻⁶ M) did not affect intracellular ROS (in particular peroxide) accumulation whereas the well-known Ginkgo biloba extract EGb 761 that displayed potent antioxidant properties [35] strongly reduced ROS production [100 ± 3 (CT) vs 93 ± 3 (CT + haloperidol 10⁻⁶M) and vs 62 ± 3 (CT + EGb 761 50 μg/ml), p < 0.01]. Moreover, haloperidol and other antipsychotic drugs including (±)-sulpiride and chlorpromazine did not protect hippocampal neurons from toxicity induced by H₂O₂ (100 μM) in our model (data not shown).

Discussion

Our data indicate that low concentrations of various antipsychotic drugs protect hippocampal neurons against toxicity induced by growth medium deprivation. To our knowledge, this is the first study that reports (with the exception of haloperidol) on the neuroprotective effects of various neuroleptics having high affinity for the dopamine D₂ and D₄ receptor subtypes in hippocampal neuronal cultures. Neuronal survival is estimated using the MTT and neutral red (NR) colorimetric assays. Values represent mean ± SEM of at least three separate experiments, each performed in quadruplicate.

The protective effects of antipsychotics are not likely to be related to their inhibitory action on σ₁- or NMDA receptor-mediated responses [33,36] since neither NE-100 nor (+)-MK-801 offered protection by themselves nor blocked the neuroprotective effects of haloperidol. On the other hand, our data suggest that D₂ and/or D₄ receptors mediate the effects of antipsychotic drugs in our model. First, RT-PCR data showed that D₂ and D₄ receptors are expressed in hippocampal neurons. These data are in agreement with previous studies reporting on the presence of these receptors subtypes in the hippocampal for-
mation [37,38]. Second, all antipsychotics tested here (with the exception of (-)-raclopride and remoxipride) that display nM affinities for D2 and D4 receptors [40-46] were neuroprotective to hippocampal neurons. Third, (-)-raclopride, a preferential D2 antagonist, almost completely blocked the neuroprotective effects of clozapine, an atypical antipsychotic with a particularly high affinity for the D4 subtype.

A preferential role for the D4 receptor in the neuroprotective effect of the various antipsychotics tested in our model is of special interest. Haloperidol, risperidone, chlorpromazine, (+)-butaclamol, domperidone and clozapine exhibit high nM affinities for this receptor sub-type [39,42,43,46] and are potent neuroprotective agents in our model. Moreover, L-741,742, a rather selective D4 antagonist [30] was found to be neuroprotective in our model while (-)-raclopride and remoxipride which bind with only modest affinities to the D4 subtype [39,44] were not effective. U 99194, a potent and selective D3 receptor antagonist, and SCH 23390, a D1 antagonist, failed to be neuroprotective, suggesting that these two receptor subtypes do not mediate the protective effects of antipsychotic drugs in our model (see Table 1 for details). Interestingly, in the mature mammalian brain, the level of D4 receptors is greater than that of the D2 subtype in the hippocampal formation [37]. It would now be of interest to explore further the respective role of the D2 and D4 receptors in the neuroprotective effects of antipsychotics in hippocampal neurons using molecular approaches such as knock-out animals and siRNA. We cannot exclude however the possibility that their neuroprotective ability may also be due to their purported α1-adrenoceptor antagonist activity [47] which has been suggested to contribute to their clinical effect [48]. It has recently been shown that the atypical antipsychotic olanzapine attenu-

### Table 1: Comparison of the affinities (Ki values, nM) of various receptor antagonists at dopamine D2 and D4 receptors with their protective effects (at 10-6M) on hippocampal neurons (expressed in % of MTT values vs control group).

|                | D2 subtype Affinity (Ki, nM) | D4 subtype Affinity (Ki, nM) | Neuroprotective activity ( % vs control, MTT values) |
|----------------|------------------------------|------------------------------|-----------------------------------------------------|
| (+)-butaclamol  | 0.8                          | 40                           | 464                                                 |
| Clozapine      | 36–190                       | 1.6–40                       | 340                                                 |
| Chlorpromazine | 0.66–1.5                     | 1.15–37                      | 320                                                 |
| Domperidone    | 0.30                         | ND                           | 299                                                 |
| Haloperidol    | 0.35–1                       | 0.84–5.1                     | 271                                                 |
| (-)-sulpiride  | 9.2–15                       | 52–1000                      | 199                                                 |
| L-741,742      | > 1700                       | 3.5                          | 186                                                 |
| Risperidone    | 0.3–5.9                      | 0.25–16                      | 162                                                 |
| Haloperidol metabolite | > 10000                   | ND                           | 108                                                 |
| Raclopride     | 0.64–1.8                     | 237–620                      | 110                                                 |
| Remoxipride    | 30–2000                      | 2800–3690                    | 108                                                 |
| U 99194        | 2281                         | > 10000                      | 100                                                 |

Data from [30-32, 39-45]. ND, not determined.

* S-(-)-sulpiride.

Table 2: Effects of NE-100, (+)-MK-801 and of a co-treatment of raclopride with clozapine in enriched hippocampal neuronal cultures, as estimated by the MTT and NR assays

| Drugs                        | MTT (% of control) | NR (% of control) |
|------------------------------|--------------------|-------------------|
| Control                      | 100 ± 6            | ND                |
| + N2                         | 177 ± 10           | ND                |
| + Raclopride (10-4 M)        | 103 ± 5            | ND                |
| + Clozapine (10-4 M)         | 199 ± 13           | ND                |
| + Raclopride (10-4 M) + Clozapine (10-4 M) | 117 ± 8 | ND                |
| Control                      | 100 ± 6            | 100 ± 4           |
| + NE-100 (10-7 M)            | 97 ± 6             | 93 ± 3            |
| + NE-100 (10-4 M)            | 112 ± 9            | 95 ± 4            |
| + NE-100 (10-3 M)            | 127 ± 9            | 82 ± 5*           |
| Control                      | 100 ± 5            | 100 ± 8           |
| + (+)-MK-801 (10-6 M)        | 123 ± 9            | 128 ± 6           |
| + (+)-MK-801 (10-5 M)        | 130 ± 12           | 112 ± 14          |

Data represent mean ± SEM of at least three separate experiments. *p < 0.01, control versus drugs-treated cells. †p < 0.01, cells treated with clozapine alone versus cells co-treated with raclopride and clozapine. ND: not determined.
ated cell death produced by H$_2$O$_2$ in PC12 cells through a mechanism that involves the upregulation of the antioxidant enzyme superoxide dismutase [21]. Although the effects of D$_2$-like receptor antagonists were shared by antioxidants such as Trolox [49] and Egb 761 (data not shown), we found that they were ineffective against toxicity induced by H$_2$O$_2$ (haloperidol, (+)-sulpiride and chlorpromazine) and did not attenuate intracellular ROS production (haloperidol), suggesting that the protective effects of antipsychotic drugs are not due to an antioxidant activity in our model. Moreover, studies from animal models reported that olanzapine and risperidone, but not haloperidol, stimulated neurogenesis in rat brain areas (e.g. hippocampus) [50] and preserved cholinergic pathways and cognitive function, possibly by increasing levels of nerve growth factor (NGF) [51]. This suggests that the promoting effects of antipsychotics -particularly atypical ones- on neuronal function may be also due to other mechanisms including stimulatory effect on neurotrophic factors. In support of this hypothesis, it has been demonstrated that olanzapine and other atypical antipsychotics including clozapine, quetiapine and risperidone exerted protective effects in PC12 cells, possibly by decreasing the expression of the gene encoding for the neurotrophin receptor p75 [20,52]. This is of particular interest here since the p75 receptor has been reported to mediate hippocampal neuronal loss, possibly via the activation of caspases [53]. In support of an anti-apoptotic effect of antipsychotic drugs in our model, inhibitors of caspases 3, 8 and 9 were found to exert neuroprotective effects without affecting ROS production. Interestingly, it has recently been shown that clozapine and risperidone prevented apoptosis and DNA damage induced by the apoptotic agent N-methyl-4-phenylpyridinium in PC12 cells, possibly by attenuating the activation of an enzyme known as glycosylase [22].

Although only obtained using an in vitro model, our data are in accordance with the view that treatment with atypical antipsychotics may improve cognitive functions in schizophrenia [7-11,13,54,55]. Interestingly, only low concentrations of the various antipsychotics tested here, (with the exception of chlorpromazine that is only effective at 1 µM), were needed in our model to offer neuroprotection, much lower than those (i.e. 10–50 µM) used by others mostly in PC12 cells [20,23,56]. Considering tissue penetration and the purported levels of antipsychotics found in rodent brains [57], it is likely that upon repeated treatments, these drugs can reach levels that are sufficient to be neuroprotective.

**Conclusion**

In conclusion, our data show that various D$_2$-like receptor antagonists were able to protect primary hippocampal cultured neuronal cells against cell death induced by medium deprivation. Further studies are necessary to confirm the role of D$_2$-like (D$_2$ and/or D$_4$) dopamine receptors and subsequent intracellular signaling pathways such as the inhibition of apoptosis-related effectors. Our findings also support the hypothesis that antipsychotics could modulate, via their neuroprotective properties, cognitive status in schizophrenic patients.

**Methods**

**Materials**

Materials used for cell cultures and Reverse Transcription-PCR were purchased from Invitrogen-Gibco BRL (Burlington, Ontario, Canada) and from Sigma Chemical Co. (Oakville, On, Canada). Haloperidol, (-)-eticlopride, raclopride, chlorpromazine and risperidone were obtained from Sigma Chemical Co. (Oakville, On, Canada). U 99194 maleate and L-741,742 hydrochloride were obtained from Tocris (Ellisville, MO, USA). The ginkgo biloba extract Egb 761 was kindly provided by IPSEN laboratories (Paris, France). Unless stated otherwise, other chemicals were purchased from Sigma-RBI (Natik, MA, USA). All drugs were freshly prepared on the day of the experiment in a final concentration of ethanol or DMSO that does not exceed 0.01%.

**Neuronal hippocampal cell cultures**

Enriched neuronal hippocampal cells were prepared from E19 fetuses obtained from Sprague-Dawley rats (Charles River Canada, St-Constant, Québec, Canada) as described previously [58]. Animal care was according to protocols

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**Table 3: Effects of inhibitors of caspases 3, 8 and 9 against toxicity and ROS accumulation after serum deprivation in enriched hippocampal neuronal cultures, as estimated by MTT and DCF assays, respectively.**

| Drugs | MTT values (% of control) | DCF values (% of control) |
|-------|--------------------------|--------------------------|
| Control | 100 ± 4                   | 100 ± 6                  |
| + Inhibitor of caspase 3 (DEVDO-CHO, 5 µM) | 171 ± 12 *               | 85 ± 4                   |
| + Inhibitor of caspase 8 (IETD-CHO 5 µM) | 141 ± 9 *               | 96 ± 5                   |
| + Inhibitor of caspase 9 (LEHD-CHO, 5 µM) | 135 ± 10 *              | 89 ± 6                   |
| + Clozapine (1 µM) | 140 ± 8 *               | ND                      |

Data represent mean ± SEM of at least three separate experiments. *p < 0.01 compared to vehicle-treated cells. ND: not determined.
and guidelines of the McGill University Animal Care Committee and the Canadian Council for Animal Care.

Hippocampal cells were plated at day 0 at a density of approximately 12 × 10^4 viable cells per well in 96-well plates. They were grown in Dulbecco’s modified Eagles medium (D-MEM) medium supplemented with 20 mM KCl, 15 mM HEPES and 1% (v/v) serum-free growth medium N2 (final composition: 5 µg/ml insulin, 100 µM putrescine, 20 nM progesterone, 100 µg/ml transferrin, 30 nM selenium), and maintained at 37°C in a 95% air/5% CO₂ humidified atmosphere during 3 days.

**Immunocytochemistry**

On day 0, hippocampal neurons were plated on poly d-lysine (25 µg/mL)-coated 12 mm glass coverslips (Fisher, Nepean, On, Canada) placed in multiwell plates and grown in the same medium as described above. On day 3, the medium was removed, the cells rinsed with PBS and fixed with 4% paraformaldehyde at room temperature (RT) for 15 min. Cells were pre-treated with 0.1% Triton X-100 for 20 min followed by a blocking step with 5% normal donkey serum (NDS)/bovine serum albumine (BSA) 5%/0.1% Triton X-100 in PBS for 20 min at RT. The cells were then incubated overnight at 4°C with a mouse anti-NeuN monoclonal antibody (1:250; Chemicon, Temecula, CA, USA) in PBS supplemented with 0.1% Triton X-100, NDS (5%) and BSA (0.5%). After several washes in PBS, the secondary antibody (Alexa Fluor 568 goat anti-mouse IgG1, 1:200; Invitrogen) diluted in the same buffer as the primary antibody was added and incubation proceeded for 2 hrs at RT. The coverslips were washed several times then mounted on slides with DAPI-containing Vectashield (Vector Laboratories, Burlington, On, Canada). Hippocampal cells were examined using conventional immunofluorescence microscopy and counted from three 40× magnification fields on one slide using conventional immunofluorescence microscopy and counted from three 40× magnification fields on one slide for each experimental condition. Each experiment was repeated using a different culture preparation.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

RT-PCR was performed using a sensitive two-step PCR protocol according to [59] with some minor modifications. Total RNA was isolated from 3-day-old rat primary cultured hippocampal neurons (from two different experiments) and from rat striatum (P14) by using the Qiagen (Mississauga, On, Canada) RNeasy midi-kit in conjunction with the RNase-free DNase set according to the manufacturer’s protocol. First strand cDNA was generated from 1 µg total RNA in a 20 µl reaction containing: 2.5 µM random hexamers (Applied Biosystems, Foster City, CA, USA), 10 mM DTT (Sigma), 20 U Ribonuclease Inhibitor (Takara Biomedicals, Otsu, Japan), 0.5 mM dNTP, 1X First strand buffer, and 100 U SuperScript II RNase H- Reverse Transcriptase (all from Invitrogen). Following an overnight incubation at 42°C, the enzyme was denatured at 70°C and the RNA complementary to the cDNA was hydrolysed with 2U RNaseH (Takara Biomedicals) for 20 min at 37°C. Reactions in which the reverse transcriptase was omitted were run in parallel as controls for any residual genomic DNA.

In the first step PCR, cDNAs for dopamine receptor subtypes D₁ to D₅ were amplified simultaneously from 2 µl of each reverse transcription reactions in 20 cycle multiplex reactions (mPCR). This was followed by a second round of 35 cycles PCR in which individual cDNAs (D₁ to D₅) were amplified separately in reactions using 2% of the first round products as substrate. All PCR amplifications (94°C, 30 s; 60°C, 30 s; 72°C, 35 s) were performed in a 96-well thermocycler (GeneAmp 9700, Applied Biosystems). The final reaction volume for each amplification reaction was 100 µl and contained 1× PCR buffer, 2 mM MgCl₂, 200 µM dNTP, 1 U Platinum Taq DNA polymerase (all from Invitrogen), and 10 pmols of each selected forward and reverse primers. Primer pairs (custom-synthesized by Invitrogen) for D₂-like dopamine receptor subtypes D₂, D₃, and D₄ were designed to flank at least one intron according to the NCBI GenBank sequence database and to lie outside regions of significant homology. Likewise, primer pairs amplifying sequences from intronless coding regions of D₁-like (D₁ and D₃) receptor subtypes were derived from regions of low homology. Primer positions for D₂ or D₃ were chosen in the vicinity of those used by [60] to detect alternative splicing isoforms.

The following oligonucleotide primers were used (the predicted size for PCR products are given in parentheses): receptor D₁, forward 5’-CATCACCTTCGATGTGGCGTTGT-GTG-3’ and reverse 5’-GCTTATCCAGCTCCTTCATT-3’ (300 bp); receptor D₂, forward 5’-GCCAACCCT-GCTTGTGCTGCT-3’ and reverse 5’-GCTTCTGCGGTCT-CATCGTCT-3’ (538 bp and 451 bp); receptor D₃, forward 5’-GCCCTGTATGTGCTGTGC1-3’ and reverse 5’-GCTTTCTTCTTGCCTTCTTCA-3’ (523 bp and 410 bp); receptor D₄, forward 5’-GCGCTATGGTGTGCTGTGCT-3’ and reverse 5’-GCTTCTTCTTGCCTTCTTCA-3’ (324 bp); receptor D₅, forward 5’-GCTACCCGAGGTTGGCGGTG-3’ and reverse 5’-GCTGACACAG-GGAGCCAGCTC-3’ (403 bp).

Fifteen µl of each second round PCR were analyzed on a 2% agarose gel with 1 µg of molecular size standards (Invitrogen). Discrimination between potential amplification of genomic DNA sequences and RT-PCR on mRNA was based on the size of the PCR product (in the case of D₂, D₃, and D₅ receptors) and on the absence of a PCR product when reverse transcriptase was omitted (for all 5 sub-
Toxicity induced by growth medium deprivation
At day 3 of plating, the medium was removed and cells were incubated at 37°C in D-MEM medium supplemented with 15 mM HEPES and 5 µg/ml insulin and devoid of putrescine, progesterone, transferrin, selenium and KCl. Cells were then treated with either vehicle or different drugs. Neuronal viability was determined 3 days later using the MTT and neutral red (NR) colorimetric assays (see below).

Assessment of neuronal survival
Neuronal survival was estimated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and NR [3-amino-7-dimethy lamino-2-methylphenazine hydrochloride] dyes, which are respectively indicators of mitochondrial activity and lysosomal uptake of living cells. Cell survival was spectrophotometrically determined at 570 nm (for MTT assay) and 540 nm (for NR assay) using a micro-plate reader (Bio-Tek Instruments Inc., Ville St-Laurent, Québec, Canada) [58].

Statistical analyses
Optical density (OD) reflecting MTT reduction and NR intake into intact cells, was proportional to the number of viable cells. The OD of the control group (CT, i.e. the group of non-treated cells deprived during 3 days with growth medium) was regarded as 100%. The rate of surviving cells treated with various drugs during 3 days was expressed as percent of control groups. Statistical analysis was performed using one-way ANOVA followed by a Newman Keuls’ multiple comparison test with p < 0.05 being considered statistically significant. An unpaired t-test was used to compare reactive oxygen species production (as estimated by the DCF assay) between control group and groups treated with drugs, survival of cells treated with clozapine alone and cells treated with ralclopride and clozapine (Table 2), and survival of non-treated cells and cells treated with caspases (Table 3).

Authors’ contributions
SB carried out the cell cultures experiments and related experiments, performed the statistical analysis, and drafted the manuscript. MD carried out the immunohistochemistry and molecular biology (RT-PCR) studies and helped to draft the manuscript. FM carried out the preliminary RT-PCR studies. SW helped to draft the manuscript. RQ conceived the study, participated in its design and coordination and helped to draft the final manuscript. All author(s) read and approved the final manuscript.

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