Title: Haloperidol's Effect on The Expressions Of TGFB, NT-3 And BDNF Genes in Cultured Rat Microglia

Running title: Haloperidol's effect on TGFB, NT-3 and BDNF genes

Authors: Elham Namjoo¹, Mohammad Shekari², Aliyar Pirouzi³, Hossein Forouzandeh³, Davod Khalafkhany ⁴, Abdolvahid Vahedi ², Iraj Ahmadi⁴

1. Department of biology, faculty of Science, Islamic Azad University, Arsenjan branch, Fars, Iran.
2. Genetics and Molecular Biology, School of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran.
3. Gerash Cellular and Molecular Research Center, Gerash University of Medical Sciences, Gerash, Iran.
4. Molecular biology And Genetic Department, Bogazic University, Istanbul, Turkey.
5. Department of physiology, faculty of medicine, Ilam University of Medical Sciences, Ilam, Iran.

Corresponding author
Hossien Forouzandeh
Email: hosainforouzandeh@yahoo.com
Tel:0098-9382109998
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Abstract

Microglia, small glia cells, which are mesodermal in origin and found in the brain and spinal cord, play a key role in maintenance of neurons and immune defense. Haloperidol, an antipsychotic drug, is used as a treatment for many neurological and neurodegenerative disorders while its mechanism is not totally understood but it is considered that haloperidol may result in WNT signaling pathway activation. The aim of this study was activating the WNT signaling pathway using haloperidol and determining the effect of GSK3 inhibition on the expression of TGFB, NT-3 and BDNF genes in cultured rat microglia. For this purpose, microglia isolation was done, and immunohistochemistry technique was performed to confirm microglia purity. RNA extraction was followed by cDNA synthesis. Real time RT-PCR was used to evaluate any significant changes in the expressions' level of these genes. These three genes' expressions in microglia were proportional to the different concentrations of drug. More concentration of drug resulted in higher levels of expressions of these genes. It is notable that haloperidol had no effect on the expression of Beta actin gene as the reference gene. The result confirmed the benefit of using haloperidol in targeted microglia therapy. This study can be a breakthrough in neurology research.

Key words: Microglia, Haloperidol, TGFB, NT-3, BDNF.
1. Introduction

Microglial cells which are among the non-neural cells of brain are known as the unique defense agents of brain (Lull and Block, 2010). Origin of microglia has been studied and discussed for years. Recent studies indicate that microglia arise from progenitors in the embryonic yolk sac and, significantly, appear to persist there into adulthood (Frick et al., 2013). Approximately 10% to 15% of the brain is made up of Microglia which shows the importance of microglia presence (Reemst et al., 2016). New studies show that microglial cells not only function as the first immune sentinels but also have fundamental roles in the control of neuronal proliferation and differentiation (Ginhoux et al., 2015). The number and activity of microglia are strictly controlled because extra number and activity of microglia have the potential to damage brain tissue (Graeber and Streit, 2010). Microglia dysfunctions have been identified in several neuropsychiatric conditions, but it remains unclear whether microglia abnormalities were the cause or the effect of those conditions (Perry et al., 2010, Prewitt et al., 1997). It was found that the number and function of microglia remains steady under physiological conditions, however in response to neurodegeneration microglia multiply and adopt an activated state. Microglial cells detect foreign particles, dead cells and cellular derbies and swallow them in a healthy brain. Microglial cells, the resident macrophages of the CNS, swallow foreign particles and represent foreign antigens on their surface and attract helper T-cells. These cells release cytokines and have role in inflammation (Prewitt et al., 1997, Wohleb, 2016). Identification of microglial cells could be identified in tissue by using known Microglial markers. There are some established markers for microglial cells and more recently identified markers including GLUT5, CD163 and CCR2 (Borda et al., 2008, Graeber and Streit, 2010, Roberts et al., 2004). Wnt/β-catenin signaling is validated as a potent pro-inflammatory regulatory signaling cascade in microglia. Wnt signaling which is related to numerous diseases plays crucial roles in several essential cellular processes such as: cell proliferation, differentiation, migration and synaptic activity (Halleskog and Schulte, 2013, Yao et al., 2015). 19 human Wnt proteins have been discovered so far. Wnt signaling inhibits constitutive β-catenin phosphorylation by GSK-3 and allows for β-catenin accumulation, nuclear import and the regulation of gene transcription. Defects in this pathway has been linked to many diseases, including Alzheimer disease. more recently studies have shown neuro-protective effects of Wnt pathway activation in neurodegenerative diseases and promoting the differentiation of neural stem cells (Halleskog and Schulte, 2013, Inestrosa and Varela-Nallar, 2014, Yao et al., 2015). Activation of Wnt signaling pathway can inhibit
GSK3 which is a growth factor inhibitor and somehow affects the expressions of transcription factors of growth proteins such as TGFB, NT-3 and BDNF (Tsai et al., 2014). TGFB proteins are members of a large family which is comprised of more than 30 members in human species. Members of Transforming growth factor beta superfamily are expressed in different tissues and function at early stages of development of animals and their lifetime as well. TGFB pathway has key role in regulating cell growth, differentiation and migration (Butovsky et al., 2014, Weiss and Attisano, 2013). NT-3 is a member of neurotrophin family and has a critical role in controlling survival and differentiation of mammalian neurons. The protein encoded by this gene has effect on the development of embryonic neurons and is involved in maintenance of adult nervous system (Joo et al., 2014, Silva-Vargas and Doetsch, 2014). BDNF encodes the brain-derived neurotrophic factor, a neurotrophin family member. BDNF is found in both central and peripheral nervous system and supports the survival of neurons and results in growth and differentiation of new neurons and synapses (Wei et al., 2015). BDNF and NT-3 are members of neorotrophin family and help to stimulate and control neurogenesis. Recent studies were focused on using NT-3 and BDNF gene therapy to improve central and peripheral nerve functions (Wei et al., 2015, Yalvac et al., 2016). We aimed to investigate the effect of haloperidol on the expression of BDNF, NT-3 and TGFB genes through activating Wnt signaling pathway.

2. Material and Methods

3.1. Microglia isolation

All procedures were in complete accord with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Ethics committee of the university. Microglia cells were obtained from 4 newborn Wistar rats of 1-2 days age. At the first step rats were washed using 70% alcohol. Under sterile condition brain was removed from the skull and soaked in Hank's buffer. After removing all extra parts such as olfactory lobes and capillary networks, cortex was located in a sterile plate containing DMEM medium supplemented with 10% FBS and was microdissected using Pasture pipet. Trituration was done to separate cells completely. Isolated cells were transferred to 50ml flask containing DMEM medium plus 10% FBS. Medium was changed the other day to remove unstuck cells.

2.2. Cellular passage
After 7-8 days as microglial cells proliferated and grew until 80% confluent, cell passaging was needed so that cells receive enough place and nutrients. For this purpose, medium was removed from the flask and cells were washed twice with PBS. To detach the cells from the flask appropriate amount of EDTA-trypsin was added to the flask. After observing separated cells under the microscope trypsin was blocked by adequate amount of DMEM medium with 10% FBS. It takes 4-5 hours for cells to fix to the surface again.

2.3. Purification of microglia in cultured medium

To have pure microglia, EDTA-Trypsin was used. In this method detection of microglia depends on the different resistance of glia cells to Trypsin. Microglial cells are more resistant to Trypsin in comparison with the other glia cells. Notably neurons' sticking to the surface is more time consuming than microglia cells sticking. Considering these differences microglia purification was accomplished.

2.4. Immunohistochemistry assay

GFAP, a specific antibody was used to detect CD163 in microglia. Cells were cultured on the coverslips, washed with PBS and fixed by 4% paraformaldehyde for 15 minutes at 4c and then rinsed in PBS and 0.05%Tween20 for 5 minutes at room temperature. TritonX-100 was used to make cells permeable. After rinsing cells in PBS and 0.05%Tween20, cells were incubated in a mixture of goat serum, PBS and 0.05%Tween20 for 45 minutes at room temperature. Primary antibody diluted in 0.5% BSA was added to cells and after incubation at 37c for an hour; cells were washed with PBS and 0.05%Tween20. Samples were then exposed to FITC-conjugated secondary IgG antibody and incubated for 20 minutes at 37c. Finally, cells were washed with PBS and 0.05%Tween20 for ten minutes in dark and were observed under a fluorescent microscope.

2.4. Haloperidol treatment

Equivalent volumes of cell suspension were transferred to 4 flasks containing appropriate medium. 2, 4 and 8 mmol of haloperidol solution was added to flask number 1, 2 and 3
respectively. Flask number 4 was considered as the control. All flasks were placed in CO2-containing incubator at 37c for 48 hours. Then phase contrast microscope was used to observe the morphology of the cells from all flasks.

2.5. Reverse transcriptase (RT)-PCR and quantitative real-time PCR

After 48 hours of treating cells with haloperidol and monitoring cells' morphology, cells were exposed to trypsin. Then medium containing fluent cells was transferred to a 50ml falcon and centrifuged in 2000RPM. Liquid phase was removed and RNA extraction was performed using Roche kit according to the manufactures' instruction. Finally the RNA pellet was resuspended in 50ml of DEPC-treated RNase-free water. Microtubes containing RNA were incubated for 10 minutes at 58C. RNA concentration and purity were checked by nanodrop1000 spectrophotometer and 18s and 28S bands were detected on agarose gel as an evidence for RNA integrity. Fermentase kit was used for cDNA synthesis. RNA was reverse transcribed with Revert AidTM M-Mulv Reverse transcriptase using random hexamer according to the protocol. Proper primers were designed for three genes and one inner control gene. cDNA was amplified in a 10 microliter PCR reaction mixture with specific primer for TGFB, NT-3, BDNF and B-actin (Tab.1.) . RT-PCR amplification products of each sample were subjected to ethidium bromide gel electrophoresis and photographs were captured under UV illumination. Real time RT-PCR using SYBER green I fluorescent dye and Light Cycler was performed according to the instruction to evaluate the genes' expressions. Pfaffle mathematical model was recruited to calculate the relative expression ratio.

2.6. Standard curves of TGFB, BDNF, NT-3 and beta actin genes

Real time PCR Efficiencies were calculated according to the given slopes. Real time PCR Efficiencies rates for TGFB, NT-3, BDNF and reference gene were 2, 2.08, 2.08 and 1.92 respectively (Fig.4).

2.7. Data analyzing
The relative quantification of the target genes in the comparison to the reference gene was determined using Pfaffle formula which calculates the relative expression rates based on efficiencies and C\(_T\) of target and reference genes from treated and controlled samples (Pfaffl, 2001). The assay precision was investigated in three repeats within one light cycler run. Data were analyzed by one-way ANOVA. There were meaningful differences between different dosages effect on the expression of the genes (Fig.5).

\[
\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{T \text{ target}}(\text{control}\text{--treated})}}{(E_{\text{ref}})^{\Delta C_{T \text{ ref}}(\text{control}\text{--treated})}}
\]

3. Result

We chose to study the effect of haloperidol on the cortex-derived microglia from Wistar rats to identify if this drug has the potential to activate neurogenesis factors or not. Microglial cells were grown on proper medium and after treatment with haloperidol, morphology of the cells and genes' expressions were investigated.

3.1. Microglial cells' purity

Microglial cells were derived from Wistar rats' brain and cultured in DMEM medium. The different resistances to Trypsin helped microglia purification and Immunohistochemistry assay was carried out to verify microglial cells' purity. Purified cells exposed to specific antibody were detected bright green which confirmed presence of microglia specific marker, CD163 (Fig.1A,B). Control cells which had not been exposed to the specific antibody had no color under florescent microscope (Fig.1C,D). CD163 is a specific marker for microglia therefore after purification of microglia through the procedures that mentioned previously, exposure to the specific antibody visualized microglia under fluorescent microscope. Microglial cells were observed under phase-contrast microscope, these small cells had spindle-shaped nucleus and a few appendages.
3.2. Haloperidol affects cellular morphology

Cells were treated with different dosages of haloperidol for 48 hours and then were detected under microscope. The number of cells and cells' appendages was increased significantly in flasks number 1, 2 and 3 which were treated respectively with 2, 4 and 8 mmol of haloperidol (Fig.2B,C,D). No significant change was seen in flask number 4 as the negative control (Fig.2A). Indeed, morphological changes in the control cells were negligible in comparison with the changes in the treated cells. In a word, higher dosages of drug resulted in greater changes in cells' proliferation. Haloperidol increased proliferation of cells and the number of appendages in treated cells that means it can affect key factors and pathways.

3.3. Haloperidol intensifies gene expression

To investigate the effects of haloperidol on microglia's protective functions, expressions of TGFB1, BDNF and NT-3 genes were checked in microglial cells. RT-PCR verified the expressions of these genes and specificity of primers subsequently the effect of different dosages on the expressions of studied genes was visible on the Ethidium bromide stained agarose gel (Fig.3). Then Real-time RT-PCR quantified the alternations of expressions. Alterations in TGFB1, BDNF and NT-3 expressions were directly proportional to the concentration of haloperidol in each flask. It simply means higher concentrations of haloperidol resulted in higher expressions of these genes; however, no change was seen in the expression of B-Actin gene as the reference gene.

4. Discussion

Neurodegenerative diseases which are becoming a major health problem and usually intensify by aging have been the subject of many studies recently(Korecka et al., 2016). Finding a way to stop diseases' progress or even a stable cure is not impossible in close future due to last considerable success in neuroscience and molecular genetics. Several studies have confirmed the fact that most of neurodegenerative disease show similar pathological phenotypes (D Skaper et al., 2014, Frick et al., 2013, Norden et al., 2015) which somehow leads to a common treatment or at least persuades scientist to look into molecular pathways carefully. Numerous studies showed that Microglia play multiple roles in human neurodegenerative disease. Defects in microglia can impair the performance of some physiological functions such as derbies phagocytosis or enhance neurotoxic factors'
secretion (Cartier et al., 2014, Von Bernhardi et al., 2015). It is established that these impairments are associated with several disorders such as amyotrophic lateral sclerosis, Alzheimer’s disease, Huntington’s disease, multiple sclerosis, X-linked adrenoleukodystrophy (X-ALD) and lysosomal storage diseases (LSD) and recently activated microglia have been implicated in psychiatric disorders, such as schizophrenia and mood disorders (Wolf et al., 2017). Microglia are dynamic cells that move gently through their surrounding microenvironment just like guards to ensure the absence of any imperfection under resting conditions. Through this journey microglia are primed to detect any injury, homeostatic imbalances or pathology within the CNS. Presence of each defect results in microglia activation (27). Microglia are prepared to delay or aggravate neurodegeneration relying on the balance between the production of trophic versus toxic factors. Aged microglia just like impaired ones are not efficient enough to clear derbies and fail in responding to defects immediately (Monji et al., 2013, Monji et al., 2014). Microglia replacement is becoming a critical approach in treating many CNS diseases which currently lack efficacious treatments (Ajami et al., 2007). Wnt signaling pathway is essential for microglia to act correctly and in a report Activating Wnt signaling pathway was confirmed as a probable treat for Alzheimer disease (Rosso et al., 2005). Low levels of BDNF, NT-3 and TGFβ have been associated with pathogenesis of several neuropsychiatric and neurodegenerative diseases. Wnt signaling pathway regulates the expressions of BDNF and NT-3 genes and also has crosstalk with TGFβ pathway (Patapoutian et al., 1999). There are also reports which verified cooperation of BDNF and Wnt signaling (Hiester et al., 2013). Indeed, BDNF appears to be a direct target of Wnt signaling in glia cells. It was shown that NTs can regulate Wnt signaling pathway through phosphorylation of GSK-3 (Arevalo and Chao, 2005). There are evidences for Multiple levels of cooperation between the TGFβ and Wnt signaling pathways in regulating gene expression (Warner et al., 2005). Considering the importance of microglia in maintenance of the brain environment (Pierre et al., 2017) and role of Wnt signaling in appropriate function of Microglia and also key role of neurotrophic factors in neurogenesis the necessity of study these pathways in brain is accentuated. Based on these consecutive findings; investigating the effect of a known drug that influences Wnt signaling pathway was highly recommended. Haloperidol is a well-known antipsychotic drug which used to have critical role in treating patients with known psychosis or other behavioral problems. There are numerous studies on investigating haloperidol effects on neurodegenerative disease (Keilhoff et al., 2010, Shin and Song, 2014). According to the Previous studies Dvl is an important transducer of Wnt signaling for both the canonical pathway as well as the PCP pathway (Qu).
Previous studies have shown that Dvl is activated by haloperidol which is sufficient to activate Wnt signaling pathway (Sutton et al., 2007) therefore we assumed that Haloperidol can have a key role in activating NTs and TGFB. The results of the present study focused on investigating the effects of haloperidol on the expressions of NTs and TGFB in rat cultured microglia verified our hypothesis that this drug can increase these genes expression. Changes in Number of microglial cells and their appendages were visible under microscope that is a good indication of direct effect of haloperidol on the proliferation of microglia and suggest more comprehensive studies on different effects of this drug on glial cells to clear if haloperidol can be used as a safe treatment in proper disorder or not. BDNF, brain-derived neurotrophic factor, NT-3, key factors of neurogenesis (Pandya et al., 2013) and TGFB showed significant increase in their levels of expression. Presence and function of these factors seem to be a chance to heal the wounds in neuropsychiatric and neurodegenerative disease. The present study fulfilled our aim to clarify the effect of haloperidol on microglial cells and neurotrophic factors’ expression. Obviously the result of our study is limited to in vitro conditions and not surprisingly different result will be obtained from in vivo experiments due to interactions of cells with each other, effects of the drug on other glia cells and also possible effects of surrounding microenvironment on the cells and gene expression. A study reported decrease in the expression level of BDNF in occipital and frontal cortex of rat after treating with haloperidol(Angelucci et al., 2000).

5. Conclusion

According to our findings which are in consistent with previous studies, it is not exaggerating to postulate accomplishment of this study as a significant step in neuroscience studies. Using haloperidol to treat diseases with microglia's involvement in a targeted therapy can be effective. To find out more in this field and to be more satisfied with our findings we suggest more precise and comprehensive investigation including in vivo experiments.

Conflict of interest

The Authors of this study declares that they have no conflict of interest

Acknowledgment

We would like to greatly appreciate who patiently collaborated on this project.

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Tab.1. Primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer seq.</th>
</tr>
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<tbody>
<tr>
<td>TGFB</td>
<td>Forward Primer: 5'-CCT GGA AAG GGC TCA ACA C-3</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: 5'- CAG TTC TTC GTG GAG CTG A-3</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>NT-3</td>
<td>Forward Primer: 5'- AGT GGGCAG CTT TTG CTC-3</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: 5'- GTA GAA AGT GGG GGG GAT-3</td>
</tr>
<tr>
<td>BDNF</td>
<td>Forward Primer: 5'- GTACTCTGGAGAGGCTGAATGG-3</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer: 5'- ACTACTGAGCATCACCCCTGGA-3</td>
</tr>
</tbody>
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**Fig. 1.** Immunohistochemistry assay of microglia. A and B, in the presence of specific antibody for CD163, specific marker for microglia, microglial cells are visible. C and D, Microglial cells in the absence of specific antibody are invisible.
**Fig. 2.** Effects of haloperidol on the morphology of microglial cells. A, control microglial cells. B, Microglial cells treated with 2mmol of haloperidol. C, Microglial cells treated with 4mmol of haloperidol. D, Microglial cells treated with 8mmol of haloperidol. Higher dosage of haloperidol cause more significant increase in number of the cells.

**Fig. 3.** Effect of haloperidol on the expression of neurotrophic factors. As it is clear on the gel, higher concentrations of haloperidol resulted in more considerable change in the expressions of these genes. Expressions of these genes in Control cells which had not experienced exposure to haloperidol were unchanged compared to treated cells.
Fig. 4. NT-3, BDNF, TGFB and B-Actin genes' standard curves. A, Standard curve of B-actin gene, reference gene, with a slope of -3.5. B and C, NT-3 and TGFB standard curves with slopes of -3.3 and -3.1. D and E show standard curve of BDNF with slope of -3.3.
Fig. 5. Relative expression rates of TGFB, NT-3 and BDNF in treated microglial cells.