Efficient Gene Silencing in Neural Cells by Functionalized Gold Nanoparticles

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Abstract

Neurons are difficult to manipulate experimentally. Most transfection reagents used for gene silencing are neurotoxic, and the postmitotic state of neurons prevents the effective use of viral vectors. This study demonstrated the delivery of antisense DNA against target genes by gold nanoparticle oligonucleotide complexes (GNOs) in primary cultures of rat hippocampal neurons and in a human neuroblastoma cell line. GNOs were designed to knock down expression of metabotropic glutamate receptor 5 (mGluR5), a protein that has been implicated in cell survival, and were evaluated on their cellular uptake and toxicity. Their ability to decrease expression of specific proteins makes them useful experimental tools as well as strong candidates for drug therapies against neural cancers.

Introduction

Unlike most other animal cells, mature neurons do not undergo cell division. After development, they remain in a postmitotic phase in which they perform normal physiological functions but cannot divide. However, changes in neuronal signaling pathways can disrupt this mitotic dormancy, causing erratic growth. Failed cell cycle regulation leads to excessive proliferation of cells that have lost their neuronal characteristics. The proteins involved in these pathways are key targets for disease prevention and therapy. This study focused on one potential target, metabotropic glutamate receptor 5 (mGluR5), which may be involved in the disease pathogenesis of certain types of brain-related cancers.

A better understanding of the role of this receptor in regulating cell survival has been hindered by lack of efficient means of experimentally modifying gene expression in neurons. Gene silencing by antisense DNA or short-interfering RNA (siRNA) has been used to modify the expression of specific proteins in other types of cells. Unfortunately, common methods for delivering these oligonucleotides are inefficient and neurotoxic. To address this, gold nanoparticles oligonucleotides complexes (GNOs) were used to silence mGluR5 in dissociated primary cultures of hippocampal neurons and in a tumoral cell line. Their successful use in other cell types suggests applications in neural cells may prove more efficient than currently available methods.

Background

Metabotropic Glutamate Receptor 5 (mGluR5) and Cell Survival

In the brain, the amino acid glutamate (Glu) is the primary excitatory neurotransmitter. Glu release at the synapses transmits electrical signals from one neuron to another. Receptors on the postsynaptic neuron bind Glu and initiate cellular cascades that trigger intracellular processes. One such receptor, mGluR5, has been implicated in cellular pathways that regulate cell survival; mGluR5 has been linked to various brain-related cancers such as ganglioglioma, neuroblastoma, and glioneuronal tumors.[1–3] The positive effect of mGluR5 on cell survival may be responsible for the robust health of cancer cells in the brain. In the case of a nonneural cancer, the oral squamous cell carcinoma, an increase in mGluR5 expression correlated with decreased patient survival. Correspondingly, mGluR5 agonists increased tumor cell migration and adhesion, indicators of increased malignancy; mGluR5 antagonists reversed this effect.[4] The role of mGluR5 in the pathogenesis of certain cancers may make them important therapeutic targets for certain cancers — hence, the need for further study.

Gene Silencing in Neural Cells

More experimentation is necessary to understand the specific role of mGluR5 in cell survival. Unfortunately, many techniques for gene silencing are ineffective in neural cells. The complex structure and function of neurons, their high sensitivity to toxins, and their inability to divide after development make traditional genetic experimentation more difficult. Pharmacological agents, when available, have been used in a wide range of biological experiments. However, in the case of mGluR5, certain antagonists have been reported to affect other glutamate receptors.[5] To address these issues, a more targeted means of altering protein expression or activity is needed.

Gene knockdown using antisense DNA has proved successful in a variety of cell types. When in the cell, these oligonucleotides bind to complementary mRNA through Watson-Crick base pairing and inhibit the translation of target proteins.[6] However, delivery of antisense DNA to cells commonly requires the use of viral vectors or lipid-based transfection reagents, which have negative side effects in neurons and result in cell death. Once in the cell, the sequences are prone to enzymatic degradation, further decreasing their efficacy.[7] The use of short-interfering RNA (siRNA) has improved attempts at gene knockdown by leveraging the cellular RNA interference machinery.[8] Still, the same challenges of delivery and degradation affect siRNA, with the additional caveat of decreased stability of RNA oligomers.

Oligonucleotide Conjugated Gold Nanoparticles

GNOs are a less toxic and more efficient tool for silencing genes.[9] These 13 nm particles can be reversibly functionalized with biomolecules such as alkylthiol-terminated DNA sequences.[10] When functionalized with antisense DNA, GNOs are readily taken up by a variety of cell types and are resistant to oligonucleotide degradation.[11] By binding target mRNA, the particles prevent translation of the target proteins, thereby decreasing gene expression. Furthermore, a high efficiency of uptake and low cellular toxicity make them ideal tools for use in neurons.

Approach

GNOs have been used experimentally to modify signaling pathways in a wide variety of cell types and as potential therapies against certain cancers. However, their use had not been demonstrated in neural cells. In order to establish that they are viable alternatives to available methods, the particles were characterized on their ease of uptake, cellular toxicity, and knockdown of protein expression in the SH-SY5Y neuroblastoma cell lines and primary hippocampal cultures of neurons and glia. For this experiment, nanoparticles conjugated to either antisense mGluR5 (mGR5-AS) or mGluR5-mutated sequence (mGR5-mut) were generously provided by David A. Giljohann and Chad A. Mirkin, who have pioneered their use in a variety of cell lines. Since mGluR5-mut is not directed against a particular gene, this GNO was used as a control to determine the relative efficiency of mGluR5 knockdown.[12]

Some GNOs were labeled with a Cy5 fluorophore, which emits light when excited by the appropriate wavelength, thereby permitting visualization by epifluorescent microscopy. To measure the cytotoxic effect of GNOs, the nuclear morphology of treated cells was evaluated for distinct changes associated with apoptotic or necrotic cell death. Cells were labeled with an ultraviolet fluorescent dye that binds to nuclear DNA, and the distribution of the area and circularity of the nuclei in culture were compared between different treatment conditions to check for cell toxicity. To evaluate expression of the target gene, proteins were electrophoresed and immunoblotted with specific antibodies.

Results and Discussion

GNO Uptake in Neurons, Microglia, and Astrocytes

Knockdown of the target mRNA requires endocytosis of the GNOs by the cells. Treatment of primary cultures with fluorescently labeled GNOs showed uptake in all present neural cell types (Figure 1). This uptake increased with both increasing concentration and duration of treatment. Cy5-fluorescence was prevalent throughout cells with a clear neuronal morphology, indicating presence of the GNOs in the dendritic branches as well as the cell body. Notably, the appearance of very bright, small, circular cells in the 6 nM treatment suggested uptake by microglia cells, which are neural macrophages responsible for clearing cellular debris and foreign substances. Though not tested in this study, this inferred result can be confirmed by colabeling cultures with a microglia specific marker such as CR3/43. Furthermore, to determine if the particles might induce an immune response in vivo, it would be worthwhile to determine if microglia are activated in the presence of GNOs. Any insult to these cells might limit their ability to protect against infection. For this reason, it is imperative that the effect of GNOs on microglia be studied in detail before they can be proposed for use as therapies against brain-related cancers.

Colabeling for the cell-type specific marker glial fibrillary acidic protein (GFAP) revealed that astrocytes also took up GNOs both in cytoplasmic and perinuclear regions (Figure 2). Interestingly, mGluR5 is

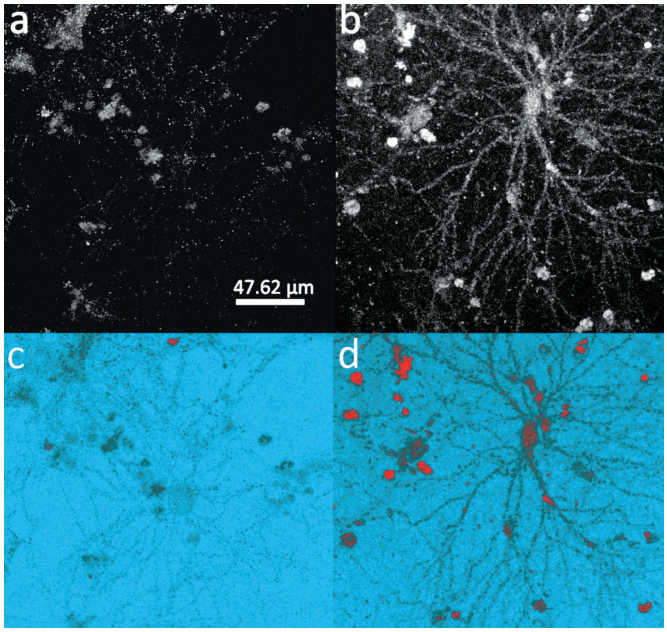

Figure 1. Neural cells readily take up GNOs in a dose-dependent manner. Cultures were treated with cy5-mGluR5-AS GNOs to a concentration of 1 nM (a and c) and 6 nM (b and d). Lower images show a heat map of fluorescence, demonstrating a greater uptake at greater concentrations. There is evidence of uptake in a variety of cell types. Large cells with long extensions are hippocampal neurons; smaller, brighter aggregates are obvious in microglia.

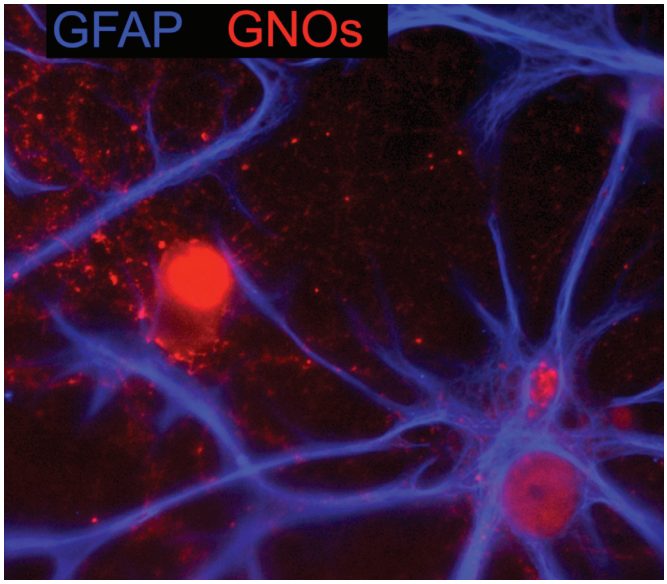

Figure 2. Fluorescent GNOs (red) are taken up by astrocytes, as indicated by localization in a cell expressing GFAP (blue).

known to be expressed in all of these neural cell types. The ubiquitous nature of GNO uptake makes it possible to knockdown expression in any of these neural cells, but because each has its own function in the brain, it is possible that the effect of decreasing mGluR5 expression in one cell type may differ from that in another.

The diversity of cell types in primary cultures used in these experiments is an important representation of living systems in which many different types of cells work together to allow for tissue functioning. While the omnipresence of GNOs in these cultures demonstrates their potential utility as tools for neural experimentation on many different cells, it also presents a unique challenge to in vivo applications. In the case of cancer therapies, the goal is to reduce malignancy without having a deleterious effect on normal cells, but if all cells take up GNOs, the therapy may have unintended effects. One potential solution to this dilemma is to bifunctionalize the particles with specialized proteins that preferentially target certain cell types.

The rapid uptake of these particles at nanomolar concentrations is a stark difference compared with the uptake of the viral vectors sometimes used for gene delivery. Retroviruses, which incorporate their oligonucleotide contents into the genome of the cell, require a mitotically active cell; the postmitotic state of neurons impedes this type of gene modification. Viral vectors derived from the adenovirus or the lentivirus, among others, are generally inefficient for the transfection of short oligonucleotide sequences. However, unlike the GNOs described in this paper, viral vectors can be used to introduce entire plasmids into a cell, thereby permitting the expression of a new gene or the overexpression of a down-regulated gene.

GNO Toxicity in Primary Cultures

A major disadvantage to using lipid-based delivery of oligonucleotides to silence genes is the toxic effect of their transfection reagents on the target cells. For this reason, there is a practical limit to the extent to which a gene's expression can be reduced. In order to demonstrate the viability of these gold nanoparticles as an efficient shuttle for oligonucleotides, the particles must not damage the cell or interfere with its normal functions.

With regard to neurons specifically, it is critical that these particles do not disrupt chemical neurotransmission, the means by which neurons communicate with one another. The proteins responsible for receiving these important chemical signals, including mGluR5, are housed in specialized regions of the plasma membrane called lipid rafts. To ensure that these regions were not altered by GNO treatment, cells were labeled with a fluorescent cholera toxin (Figure 3), which integrates itself into lipid rafts. Even with the duration of treatment increasing to as much as 24 hr, there was no visible change in the morphology of the lipid rafts.

On this timescale, toxic species would likely initiate an apoptotic cellular response, resulting in the controlled degradation of the infected cell from the inside out. Both nuclear condensation and DNA fragmen-

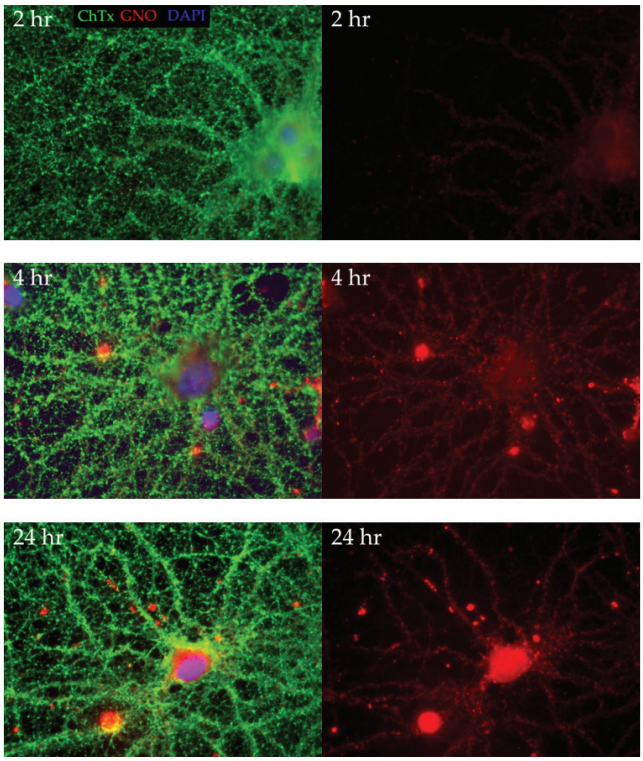

Figure 3. Lipid rafts, the specialized regions of the membrane that house synaptic proteins, are preserved after prolonged treatment with GNOs (red, right). Lipid rafts were labeled by treating cells with fluorescent cholera toxin (green). Nuclei were counterstained with DAPI (blue). Loss of nuclear staining in smaller cells may indicate poor health of microglia after overwhelming uptake of particles.

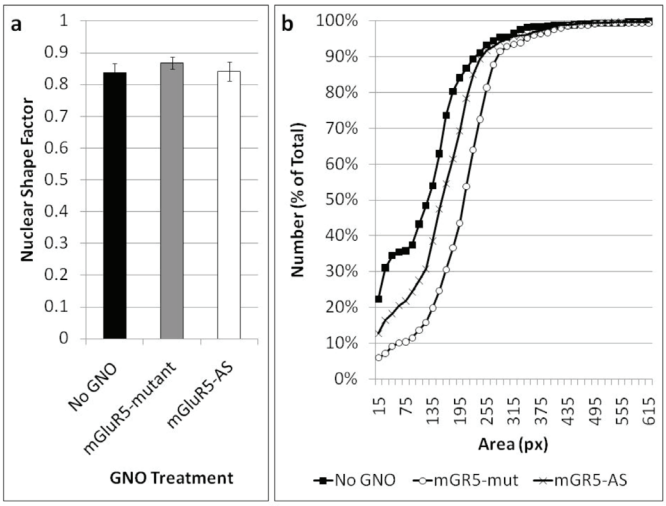

Figure 4. Treatment with GNOs does not significantly alter the morphology of the nucleus in most cells. (a) Most nuclei in each condition were nearly circular. In contrast, cells undergoing apoptosis have visible fragmentation of their DNA, resulting in an irregular shape. The nuclear shape factor varies from 0 (irregular) to 1 (perfect circle) and is proportional to the ratio of the area to the square of the circumference. (b) GNO treatment results in a loss of smaller nuclei. Cells with a smaller average nuclear size, likely to be microglia, make up a smaller percentage of total cells in GNO-treated cells. This could indicate microglial death due to the pronounced GNO uptake seen in other experiments.

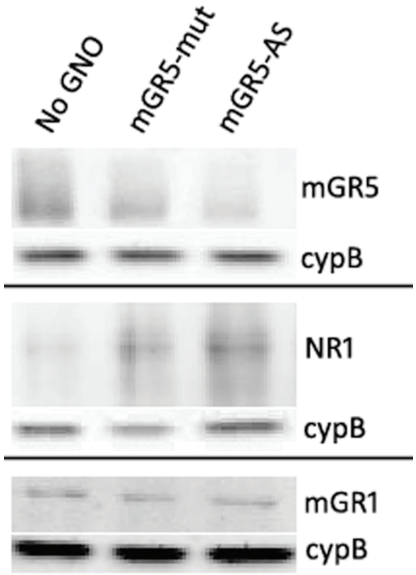

Figure 5. GNOs are capable of reducing expression of mGluR5 (mGR5) in both dimeric and monomeric forms with regard to the control mutated sequence (mut). These particles did not knock down metabotropic glutamate receptor 1 (mGR1), a closely related protein. mGluR5 knockdown corresponded with an increase in expression of the N-methyl-D-aspartate receptor (NR1), another glutamate receptor that has been implicated in learning and memory and calcium excitotoxicity. Cyclophilin B (cypB), a housekeeping gene, was used as loading control.

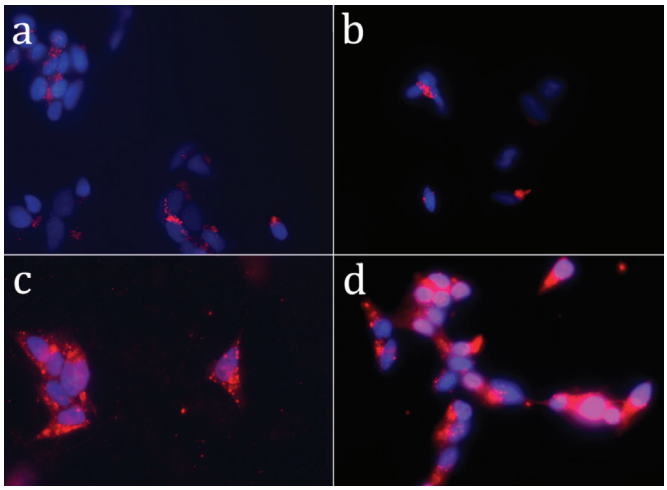

Figure 6. Fluorescent GNOs (red) are readily taken up into SH-SY5Y cells. Uptake increases between 2 hr (a-b) and 6 hr (c-d) treatments. Uptake also increases with higher concentration of treatment (6 nM (b and d) versus 1 nM (a and c). Significantly, cellular uptake was not saturated at lower concentrations or shorter treatment durations. Nuclei were counterstained with DAPI (blue).

tation occur during apoptosis. The morphology of the nucleus was measured to evaluate the cell's health by using a fluorescent DNA-binding dye. As shown in Figure 4, the nuclei in cultures treated with GNOs have a similar distribution of areas and circularities as those in untreated cultures do. If the cells were apoptotic, their DNA would begin to fragment and degrade, causing the nuclei to shrink in size and become more irregular in shape.

There is a notable loss of smaller but not larger nuclei in GNO-treated cells. The lack of an apparent loss of larger cells — namely, neurons and astrocytes — suggests that these particles may affect the function of the smaller cell type, microglia, which may be overwhelmed by the substantial phagocytosis of GNOs seen in other experiments. As previously mentioned, these cells are crucial to the brain's immune response, so any potential effect of these particles on microglial health or activity requires further evaluation before GNOs can be used in vivo.

mGluR5 Knockdown in Primary Cultures

The most critical test of the knockdown efficiency of GNOs in neural cells is a detectable decrease in expression of the target protein. After GNO treatment, proteins were isolated and separated by gel electrophoresis (Figure 5). Protein-specific antibodies were used to detect mGluR5 and two other related glutamate receptor proteins, the N-methyl-D-aspartate receptor subunit 1 (NR1) and metabotropic glutamate receptor 1 (mGluR1). Protein concentrations were normalized in order to permit direct comparison of expression levels in different samples.

To ensure that this effect was sequence specific, cultures were treated with a GNO conjugated to a mutated mGluR5 sequence. With regard to this control, there was a detectable decrease in mGluR5 but not mGluR1 expression after treatment with the mGluR5-AS GNO. This decrease also corresponded with an increase in expression of NR1. The role of mGluR5 in cell survival may involve decreasing NMDAR-associated calcium excitotoxicity by limiting expression of NR1. A partial knockdown of the target protein suggests that these particles can be used to silence genes in neural cells. This knockdown likely can be optimized by varying the conditions of treatment, including duration, concentration, and protein extraction buffer.

Uptake and Knockdown in SH–SY5Y Cells

As described in this study, GNOs may be a valuable experimental tool for studying neural cells, but other researchers have demonstrated their potential as cancer therapies. For this reason, in this study, GNOs were also characterized in the SH-SY5Y human neuroblastoma cell line to determine if they can be used to treat a tumor. Again, because of its implications in cell survival, mGluR5 was used as a target protein. As shown in Figure 6, fluorescently labeled GNOs are taken up into the cells in a dose- and time-dependent manner. Notably, in these cultures, the 6 nM concentration was sufficient to saturate the entire cell population with particles.

Since it was not previously known whether mGluR5 was expressed in undifferentiated SH-SY5Y cells, mGluR5 expression was evaluated by western blot both without treatment and after a 6 hr, 6 nM GNO treatment (Figure 7). Both mGluR5 and mGluR1 were expressed, and there was an observable decrease in mGluR5 immunoreactivity with regard to the no-treatment control; samples were normalized by loading equal amounts of proteins. Additionally, the antisense sequences used in these experiments were designed against the mouse homolog of mGluR5, but the cell line in which they were tested expresses the human homolog. The knockdown will likely be more efficient if the sequences are less divergent. Further work remains to be done to optimize the conditions of this gene silencing.

Conclusions

The knockdown of genes in neurons has been a major impediment to the study of cell signaling in the brain. The use of GNOs, an efficient vehicle for the delivery of antisense DNA, may address this issue. In primary cultures of neurons and glial cells, GNOs showed substantial uptake and low toxicity at nanomolar concentrations. While the conditions for a knockdown of the target protein were not fully optimized, a notable decrease in gene expression was observed. These factors demonstrate the potential utility of GNOs as both scientific tools for studying proteins in cellular models for neural processes and neurological diseases and important therapies to decrease expression of oncogenes in certain neurological cancers.

It is significant that the unique morphological characteristics of neurons still present interesting challenges to gene silencing. Translated proteins are transported from the cell body to various other parts of the cell, and in many cases, the distances are vast when compared with the size of the molecules. This could mean that despite blocking translation of a target protein near the nucleus, protein expression in other parts of the cell may not be affected for some additional time. It may take varying concentration, duration, and number of treatments to optimize the silencing of a particular gene based on the parameters of the experiment. A more complete knockdown of the gene may be possible with the use of siRNA. By leveraging the cells' native RNA-interference machinery, GNOs could not only block translation of mRNA but also trigger the degradation of transcribed sequences. For this reason, siRNA knockdown can be efficient and long-lasting.

Finally, once the parameters of knockdown have been optimized, it will be necessary to reevaluate the role of mGluR5 in the survival of neural cells to determine if mGluR5 is a viable target for anticancer therapies. Ideally, these studies should be conducted on pure cultures containing only one cell type. These cultures can be treated first with GNOs against mGluR5 and later with a known toxic compound; presumably, if mGluR5 is critical for the survival of the cell, those with a decreased expression of the protein will react to the toxic treatment more dramatically.

As has been demonstrated here with mGluR5, GNOs are promising tools for the experimental study of cellular signaling in neurons, astrocytes, and potential neuroblastomas. As such, they could prove useful in the study of a wide array of brain-related topics apart from cancer therapy, including the study of learning and memory, movement, sensory perception, and cognition. The application of this new technology will help neuroscientists overcome some of the challenges inherent in their work and will contribute to a greater understanding of the human mind.

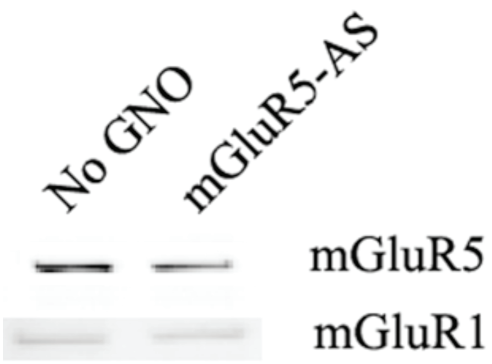

Figure 7. GNO treatment results in a specific decrease in mGluR5 expression in SH-SY5Y neuroblastoma cells. Expression of mGluR5 but not mGluR1 decreases slightly in SH-SY5Y cells after a 6 hr treatment at 6 nM. Protein concentrations were determined, and equal amounts were loaded into the gel.

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