# Expression of NMDA Receptors in Multipotent Stromal Cells of Human Adipose Tissue under Conditions of Retinoic Acid-Induced Differentiation

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The system of NMDA glutamate receptors in human adipose tissue multipotent stromal cells and SH-SY5Y human neuroblastoma cells was used as a model for studies of NMDA receptor expression during neurodifferntiation. Glutamate NMDA receptors were detected in multipotent stromal cells of human adipose tissue. The expression of NR1 subunits of NMDA receptors increased significantly after 6-day incubation of multipotent stromal cells of human adipose tissue with 10 μM retinoic acid. Only NR1 subunits of NMDA receptors were expressed in SH-SY5Y neuroblastoma cells. Incubation with retinoic acid did not promote the appearance of mRNA of other subunits (NR2A-D, NR3). The results indicate that expression of NMDA receptors can serve as an indicator of neuronal differentiation of cells and as a marker of the efficiency of neuronal differentiation protocol.

**Key Words:** *multipotent stromal cells of human adipose tissue; SH-SY5Y neuroblastoma; retinoic acid; NMDA receptors*

The glutamatergic system responsible for conduction of excitatory stimuli in human brain is presented by neurotransmitter glutamate and glutamate receptors differing by their structure and function. Glutamate receptors are divided into 2 subtypes: ionotropic (linked with ionic channels) and metabotropic (modulating intracellular signaling systems). NMDA receptors belonging to ionotropic glutamate receptors are involved in the realization of various functions of the brain and in the realization of pathological states induced by oxidative stress [1,3,4]. Recent studies showed limited expression of NMDA glutamate receptors in some other tissues [5], but NMDA receptors are still considered as markers of the nervous system cells.

Structurally NMDA receptors are heteromeric complexes consisting of subunits of several types. NR1 subunit of NMDA receptor is more amply presented and cooperates with one or several subunits of the second family, including NR2A, NR2B, NR2C, and NR2D. Functionally active NMDA receptors require the presence of NR1 and NR2 subunits, because NR1 subunit contains glycine-binding domain and NR2 subunits contains glutamate-binding site [6,8]. The possibility of differentiation of multipotent stromal cells (MSC) of human adipose tissue into nervous tissue cells was previously described, but was not confirmed by other authorities [7,12,13]. Neurodifferentiation is usually characterized by such markers as nestin, β-tubulin-3, NSE, NeuN, and GFAP.

Despite numerous data on the pharmacology and functions of NMDA receptors, their studies in cell cultures are limited. Just few attempts at deri-

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vation of cells phenotypically close to neurons from stem cells are known [14]. Some human cell strains were tested for glutamate receptors [10,15]. The presence of NMDA receptors in SH-SY5Y neuroblastoma cells after long (2 weeks) incubation with retinoic acid were described, but these data are fragmentary [11,12]. On the other hand, the presence of these receptors in differentiating stem cells can serve as an evidence of acquisition of the neuronal phenotype by these stem cells. We describe the appearance of NMDA receptor NR1 subunits in human SH-SY5Y neuroblastoma and human adipose tissue MSC under conditions of their differentiation under the effect of retinoic acid.

## MATERIALS AND METHODS

**Culturing of SH-SY5Y human neuroblastoma cells.** The culture of SH-SY5Y human neuroblastoma cells (ATCC) was maintained in EMEM:F12 (1:1) with 10% fetal bovine serum and 1% antibiotics (streptomycin and penicillin; 100 U/ml each). The cells were cultured in Petri dishes (10 or 5 cm in diameter) at 37°C and 5% $CO_2$. The medium was replaced every 4-6 days; experiments were carried out on days 8-11 after inoculation.

This cell culture is a mixture of floating cells and cells adhering to the dish surface, and hence, in order to collect all cells before the experiment, the medium with floating cells was centrifuged for 1 min at 3000 rpm and precipitated cells were used.

**Isolation of adipose tissue MSC.** Samples of fat from the anterior abdominal wall were obtained during cosmetic liposuction ($n$=3) or other surgical interventions ($n$=7). The age of donors was 26-55 years. The tissue was washed 3-4 times in PBS (pH 7.4) and suspended in an equal volume of the same buffer with 1% bovine serum and 0.1% collagenase-1 (Worthington Biochemical Corp.) The tissue was disaggregated on a magnetic stirrer at 37°C for 1 h and centrifuged for 5 min at 300-500$g$. The supernatant containing mature adipocytes was discarded, while cell precipitate (stromal vascular fraction) was used for culturing.

The cells were inoculated in plastic Petri dishes (Corning) 90 mm in diameter in DMEM/F12 growth medium with 10% FCS (HyClone). The cultures were incubated at 37°C and 5% $CO_2$ for 48 h, after cell adhesion the medium was replaced. After attaining 75-90% confluence, the cells were removed with trypsin and reinoculated into new dishes. Inoculation density for all passages was $1.5\times10^3$ cell/cm$^2$.

Adipose tissue MSC after 4-5 passages (which corresponded to 20-25 generations in culture) were studied. At this culturing term, the stromal fraction can be most completely purified from admixture cells (adipocytes and vascular endotheliocytes) present during earlier passages. "Stromal" markers expressed at a high level on adipose tissue MSC (CD13, CD29, CD44, CD49a, CD63, CD73, CD90, CD146, CD166) were selected for characterization of the isolated population.

**Detection of nucleotide sequences by PCR.** mRNA was isolated using QIAGEN RNeasy Mini Kit (Qiagen) according to manufacturer's instruction. The final concentration of mRNA was measured on a Biorad SmartSpec 3000 spectrophotometer at $\lambda$=260 nm ($OD_{260}$=1 at RNA concentration of 40 μg/ml).

Reverse transcription reaction was carried out using PROMEGA M-MLV RT Kit (Promega).

The PCR was carried out using an MBI Fermentas PCR kit on an Eppendorf Master Cycler Gradient device according to the following protocol: 5 min at 95°C (denaturing initiation), 30 sec at 94°C (denaturation), 1 min at 60°C (primer annealing), and 2 min at 72°C (amplification). The following primers (shown for 5'→3' position) to NMDA receptor subunits were used:

NR1: sense AACCTGCAGAACCGCAAG, antisense GCTTGATGAGCAGGTCTATGC; NR2A: sense TCCATTCTTCTGTCATCCTGC, antisense AAGACCGTCTCTCACTCTTGC; NR2B: sense TG CACAATTACTCCTCGACG, antisense TCCGATT CTTCTTCTGAGCC; NR2C: sense TTGAGGACA ACGTGGACACC, antisense TCCAGTCGTATTCC TCCAGC; NR2D: sense GCACTTGCATCAGAGA CTCG, antisense CTCACCAATCATGCCATTCC.

PCR products were analyzed by electrophoresis in 1.7% agarose gel, the results were analyzed in infrared light on a Biostep transilluminator, and digital photos were made.

**NMDA receptor NR1 subunits** were detected on a FACS Calibur flow cytometer (BD Biosciences) using specific antibodies and second FITC-labeled antibodies (GeneTex). The mean values of fluorescence were measured and cell counts were evaluated using gates for analysis of cell populations. The results were analyzed using WinMdi 2.7 software.

**Cell differentiation.** For induction of differentiation the cells of all types were incubated with 10 μM retinoic acid for 5-6 days.

## RESULTS

The data on the expression of NMDA receptor subunits in SH-SY5Y neuroblastoma cells used as a model for evaluation of their expression in the course of neurodifferentiation are presented in Figure 1. Initially only NR1 subunits of NMDA receptor were present in these cells. After 5-day incubation with

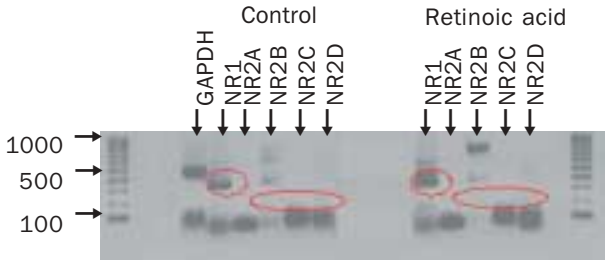


**Fig. 1.** NMDA receptor subunits in human SH-SY5Y neuroblastoma cells in control and after incubation with retinoic acid. Expected detection of subunits by PCR is shown with a red oval. GAPDH: 469 b. p.; NR2B: 222 b. p.; NR1: 333 b. p.; NR2C: 204 b. p.; NR2A: 224 b. p.; NR2D: 224 b. p.

10 μM retinoic acid, the expression of NR1 subunits increased, but no other subunits (NR2A-D, NR3) were detected.

In our experiments, the initial stages of differentiation observed on day 5 of incubation with retinoic acid correlated with the appearance of NMDA receptor NR1 subunits (an initiating factor for the formation of mature NMDA receptors). Further selection of differentiation conditions should lead not only to the creation of a convenient model for studies of glutamate receptors, but also to the formation of a cell culture with characteristics maximally close to the properties of real neurons.

Our data indicate that adipose tissue MSC contain NMDA glutamate receptors (previously unknown fact). The presence of NMDA receptors in adipose tissue cells is surprising, though the stationary level of expression of NMDA receptor NR1 subunits is not high. Six-day incubation with retinoic acid significantly increased fluorescence of cells expressing these receptors (Fig. 2). The appearance of NMDA receptors as a result of differentiation of these cells is important for characterization of cell culture. We think that NMDA recep-

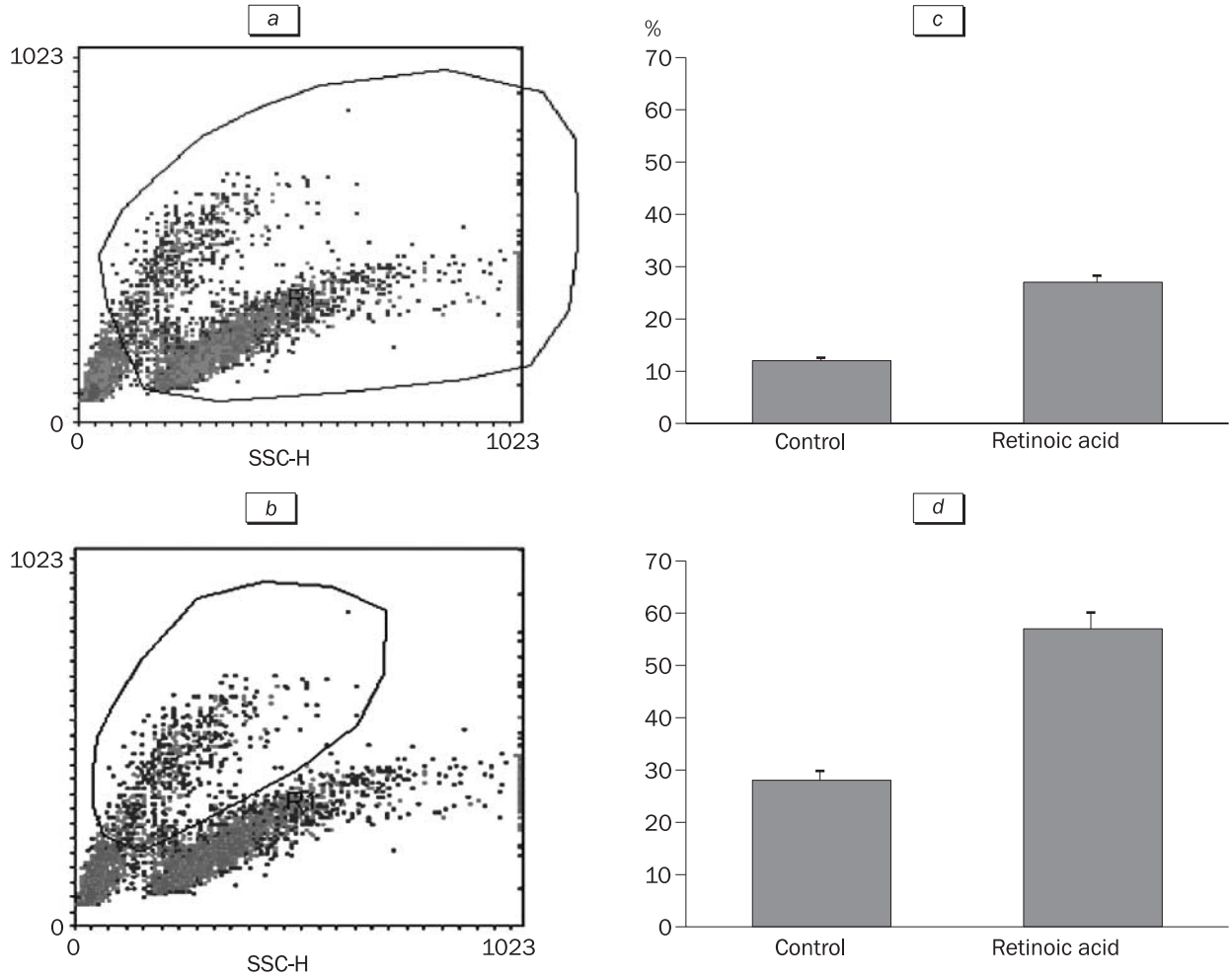


**Fig. 2.** Fluorescence signals of NMDA receptor NR1 subunits of adipose tissue MSC in the control and after incubation with retinoic acid. *a*) gate used for analysis is open for all living cells in the population; *b*) chosen gate is open for the population of large agranular cells; *c, d*) fluorescence signal in samples with antibodies (% of unlabeled cells) in the population of all living cells (*a*) and large agranular cells (*b*), respectively.

**TABLE 1.** Analysis of Immunophenotype of Human Adipose Tissue MSC

| Antigen | Index, % ($n$=14) |
|---|---|
| CD13 | 97.28±2.46 |
| CD29 | 97.20±3.27 |
| CD44 | 84.30±22.35 |
| CD49a | 38.38±28.86 |
| CD63 | 26.61±12.01 |
| CD73 | 96.50±2.25 |
| CD146 | 7.00±5.79 |
| CD166 | 56.92±28.82 |
| CD90 | 100 |

tors can be used as a marker of the neuronal phenotype of cell culture (other known markers of neuronal tissue are nestin, β-tubulin-3, NF-09, and GFAP).

Cell cultures used in the experiment were characterized by expression of a specific set of antigens detected in adipose tissue MSC (Table 1).

Analysis of adipose tissue cells on a flow cytometer distinguished two subpopulations (cases when adipose tissue MSC were presented by a single population are not discussed) containing large (agranular) and small (granular) cells. Figure 2 presents data characterizing fluorescence of cells labeled with antibodies to NMDA receptor NR1 subunit in the control and in samples incubated with retinoic acid. The results were analyzed using gates for both populations and for each of them separately. Analysis of the population of large nongranular cells is presented. The number of cells in both subpopulations of control and experimental samples changed negligibly in the course of the experiment.

Measurement of the mean fluorescent signal in the entire adipose tissue MSC population showed that in the control this value was 9-11% of that in unlabeled cells, while in samples incubated with retinoic acid it reached 25-28%. Analysis of each population showed that the major part of cells reacting with NR1 antibodies is the population of large agranular cells. If the gate was open for this population alone, the fluorescence signal of FITC labeled antibodies in experimental samples reached 55-60% (after incubation with retinoic acid; Fig. 2). Hence, it seems that just one of two populations of adipose tissue MSC expresses NMDA receptors during differentiation induction.

Our results permit the following conclusions.

Expression of NMDA receptor NR1 subunits was detected in human adipose tissue MSC; this expression increased significantly after incubation of cells with retinoic acid. Flow cytometry showed that adipose tissue cells consisted of two populations, the greatest increment in the expression of NMDA receptors as a result of differentiation was observed in the population of large agranular cells.

NMDA receptor NR1 subunit, but not NR2A-D and NR-3 subunits, is expressed in SH-SY5Y neuroblastoma cells. Five-day incubation of cells with 10 μM retinoic acid stimulated the expression of NR1 subunit, but did not lead to the appearance of other subunits.

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