Effect of High Concentration of Glucose on Dopamine Release From Pheochromocytoma-12 Cells

Kunio Koshimura, Junko Tanaka, Yoshio Murakami, and Yuzuru Kato

To investigate the mechanism of the action of high concentration of glucose on transmitter release from neuronal cells, we examined the effect of high concentration of glucose on dopamine release from pheochromocytoma-12 (PC12) cells. When the cells were incubated with 9.0 or 13.5 mg/mL glucose (2- or 3-fold of the optimum glucose concentration for PC12 cells), dopamine release was increased in a dose-related manner. Glucose-induced increase in dopamine release was blunted by nicardipine, a Ca^{2+} channel blocker. Following addition of 13.5 mg/mL glucose, intracellular Ca^{2+} concentration was increased, which was eliminated by nicardipine. Administration of 9.0 or 13.5 mg/mL glucose induced membrane depolarization in a dose-related manner. Glucose-induced dopamine release was inhibited by pinacidil or diazoxide, adenosine triphosphate (ATP)-sensitive K^+ channel (KATP channel) openers. These results suggest that a high concentration of glucose induced ATP production, which blocked the KATP channel to induce membrane depolarization, and increased intracellular Ca^{2+} concentration and dopamine release. When the cells were cultured with 9.0 or 13.5 mg/mL glucose for 7 days, high potassium chloride (KCl)-induced dopamine release and $^{45}Ca^{2+}$ uptake were increased. These results suggest that long-term incubation with a high concentration of glucose increased the capacity of Ca^{2+} uptake to enhance depolarization-induced dopamine release from PC12 cells. These data taken together suggest that a high concentration of glucose induced activation of the Ca^{2+} channel to stimulate dopamine release from PC12 cells.

HYPERGLYCEMIA is a major pathological condition of diabetes mellitus (DM). The effect of hyperglycemia on neurons as diabetic neuropathy is considered to be caused by insufficiency of arterial blood flow due to hyperglycemia-induced atherosclerosis.[1,3,7] Recently, it was reported that a high concentration of glucose itself has diverse effects on neuronal cells. Depending on the neuronal types, a high concentration of glucose causes depolarization or hyperpolarization.[8,13,15,17] These results suggest that various signal transduction pathways are involved in neuronal responses to high glucose concentrations.

In β cells, glucose stimulates adenosine triphosphate (ATP) production, which blocks ATP-sensitive K^+ channels (KATP channels) to induce membrane depolarization, Ca^{2+} channel activation, and insulin secretion.[5,21] Thus, KATP channels play a critical role for insulin secretion. Sulfonylurea (SU), a potent hypoglycemic agent, acts on its specific binding site in KATP channels to induce insulin secretion from pancreatic β cells.[4,18] Recently, binding sites of SU were determined in the neuronal cells.[6,19] SU was observed to stimulate dopamine release from pheochromocytoma-12 (PC12) cells, an established cell line for neuronal model[23] or acetylcholine release from rat striatum.[12] These findings raised the possibility that glucose modulates neurotransmitter release from these neuronal cells in a similar manner to its action on insulin secretion from pancreatic β cells.

In the present study, we focused on the effect of a high concentration of glucose on PC12 cells in order to investigate the mechanism of the action of high glucose concentration on neurotransmitter release. We demonstrate that a high concentration of glucose stimulated Ca^{2+} channel activity, resulting in enhancement of dopamine release from PC12 cells.

MATERIALS AND METHODS

Drugs

Mouse nerve growth factor (NGF, 7.0S) and bis-(1,3-dimethylthiobarbituric acid)-trimethine oxonol [$DiSBaC_2(3)$] were purchased from Funakoshi (Tokyo, Japan). Fura-2 acetoxymethylester (fura-2/AM) was purchased from Dojin (Kumamoto, Japan). Nicardipine and dopamine were purchased from Sigma Chemical Co (St Louis, MO). Pinacidil and diazoxide were purchased from Wako Pure Chemical Industries (Osaka, Japan). $^{45}Ca^{2+}$ was purchased from New England Nuclear Research Product (Boston, MA). All other chemicals are of the purest grade available from regular commercial sources.

Cell Culture

PC12 cells were obtained from Riken Cell Bank (Tsukuba, Japan), and maintained and subcultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 10% horse serum, 4 mmol/L glutamine, 500 U/mL penicillin, and 500 mg/mL streptomycin. The concentration of glucose in DMEM was 4.5 mg/mL. At 90% of confluency (2×10^6/mL), cells were plated at 10^5/mL on culture plates and were cultured for 3 to 5 days. At this point, confluency of the cells was about 60%. Then, the cells were differentiated by administration of 100 ng/mL NGF at 48-hour intervals to DMEM, which was supplemented with 0.5% fetal calf serum, 0.5% horse serum, 4 mmol/L glutamine, 500 U/mL penicillin, and 500 mg/mL streptomycin. Following 5 to 7 days of culture for differentiation, when the cells were grown at about 70% of confluency, the cells were used for experiments.

Dopamine Release Assay

Dopamine release was examined as described recently.[9] Nicardipine, pinacidil, and diazoxide were added to the incubation medium during the preincubation period (10 minutes) and the subsequent dopamine release period (20 minutes). Glucose and potassium chloride (KCl)

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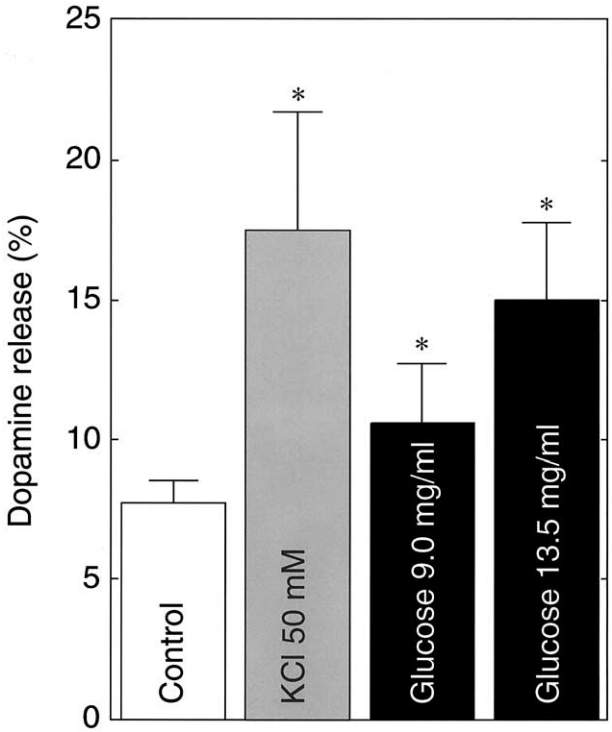

Fig 1. Effect of glucose on dopamine release from PC12 cells. After preincubation at 37°C for 10 minutes, the cells were incubated with test drugs at 37°C for 20 minutes. Then, dopamine in the incubation medium and the cells was determined with high-performance liquid chromatography (HPLC) and electrochemical detection. Dopamine release was expressed as (released dopamine/released dopamine and cellular dopamine) × 100 (%). Results are expressed as means ± SEM of 6 determinations. *$P < .05$ *v* control.

were added during the dopamine release period alone. Dopamine release was expressed as (released dopamine/released dopamine and cellular dopamine) × 100 (%).

Measurement of Membrane Depolarization

Membrane depolarization was estimated using voltage-sensitive fluorescent dye, $DiSBaC_2(3)$.[11] The cells were collected and suspended in Krebs-HEPES solution (NaCl 140 [mmol/L], KCl 4.7, $CaCl_2$ 1, $MgCl_2$ 1.2, KH_2PO_4 1.2, glucose 11, HEPES 5, pH 7.4) at 10^6 cells/mL. Aliquots of 2 mL of the cells were placed in a cuvette of spectrofluorophotometer (Shimadzu RF-5000, Kyoto, Japan) for measurement of fluorescence (excitation at 535 nm and emission at 559 nm). During the measurements, cell suspension was stirred with magnetic stirrer. After 30 to 60 seconds, 50 μL of 6 μmol/L $DiSBaC_2(3)$ was added into the cuvette by microsyringe. After a stable baseline was attained, glucose was added to the cell suspension. Administered drugs were present in the incubation mixture until the end of the experiments. At the end of each experiment, cells were depolarized with 50 mmol/L KCl to estimate the cell viability.

Measurement of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} concentration was measured with fura-2 as described previously.[16] At the end of each experiment, cells were depolarized with 50 mmol/L KCl to estimate the cell viability. Maximum fluorescence (F_{max}) was obtained by lysing cells with Triton X-100 (0.1%) and minimum fluorescence (F_{min}) by adding EGTA (5 mmol/L). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the relationship; $[Ca^{2+}]_i = Kd \times (F - F_{min})/(F_{max} - F)$, using 220 nmol/L as the Kd (dissociation constant) for the calcium complex of fura-2.

$^{45}Ca^{2+}$ Uptake Assay

$^{45}Ca^{2+}$ uptake into PC12 cells were estimated as reported previously.[22] Following the preincubation period (10 minutes), cells were incubated with $^{45}Ca^{2+}$ for 1 minute. $^{45}Ca^{2+}$ uptake into the cells was expressed as nmol $^{45}Ca^{2+}$/mg protein.

Protein Content

Protein content was measured using Bio-Rad (Hercules, CA) protein assay with bovine serum albumin as the standard.[2]

Statistical Analysis

All results were expressed as means ± SEM. The significance of difference was evaluated with analysis of variance (ANOVA) and Fisher's test. A probability level of $P < .05$ was considered statistically significant.

RESULTS

First, the effect of glucose on dopamine release was examined after short-term exposure of glucose. When the cells were incubated with Krebs-HEPES buffer containing 9.0 or 13.5 mg/mL glucose at 37°C for 20 minutes, dopamine release was increased. At 13.5 mg/mL, glucose stimulated dopamine release to a similar extent as the maximal release induced by 50 mmol/L KCl (Fig 1). Then, the mechanism of the effect of glucose on dopamine release was investigated. Membrane potential was monitored using fluorescent dye $DiSBaC_2(3)$. Following addition of 9.0 or 13.5 mg/mL glucose to the cell suspension, membrane potential was depolarized in a dose-related manner (Fig 2). Intracellular Ca^{2+} concentration as measured with fura-2 was increased by 13.5 mg/mL glucose, which was eliminated by 1 μmol/L nicardipine, a Ca^{2+} channel

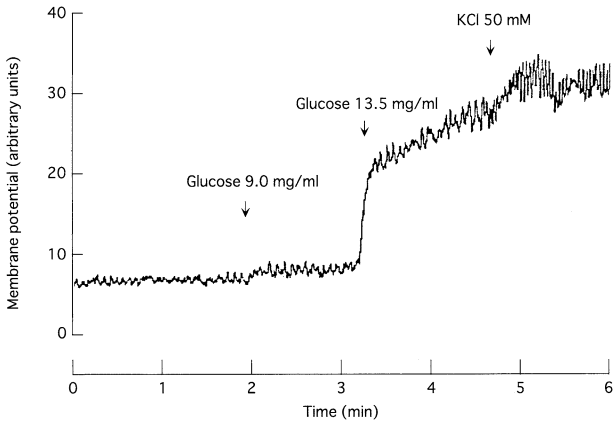

Fig 2. The effect of glucose on the membrane potential of PC12 cells. The cells were loaded with voltage-sensitive fluorescent dye, $DiSBaC_2(3)$, and the membrane potential was monitored. Drugs were added to the cell suspension at the final concentrations as shown. A representative result is shown. Similar results were obtained from 6 independent experiments.

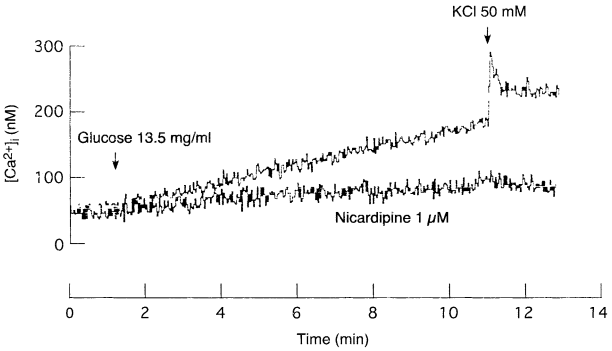

Fig 3. The effect of glucose on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of PC12 cells. The cells were loaded with fluorescent dye fura-2 and the intracellular Ca^{2+} concentration was monitored. Drugs were added to the cell suspension at the final concentrations shown. A representative result is shown. Similar results were obtained from 6 independent experiments.

blocker (Fig 3). Next, the involvement of Ca^{2+} channels in the effect of glucose on dopamine release was examined. When the cells were incubated with 13.5 mg/mL glucose and 1 μmol/L nicardipine at 37°C for 20 minutes, glucose-induced increase in dopamine release was blunted (Fig 4A). Since dopamine release from PC12 cells was reported to be stimulated by KATP channels,[23] the effect of glucose on dopamine release was studied in the presence of KATP channel openers. Under conditions where KATP channel was kept open by 10 μmol/L pinacidil or 10 μmol/L diazoxide, KATP channel openers, glucose-induced dopamine release was eliminated (Fig 4B).

Then, the effect of high concentrations of glucose on dopamine release was investigated after 7-day culture with 9.0 or 13.5 mg/mL glucose. Basal dopamine release was not significantly increased after 7-day culture with 9.0 or 13.5 mg/mL glucose. In contrast, 50 mmol/L KCl-induced dopamine release was enhanced after 7-day culture with 9.0 or 13.5 mg/mL glucose (Fig 5A). Ca^{2+} uptake was measured using $^{45}Ca^{2+}$ after 7-day culture with 9.0 or 13.5 mg/mL glucose. As observed in dopamine release, basal $^{45}Ca^{2+}$ uptake was unchanged but KCl-induced $^{45}Ca^{2+}$ uptake was stimulated by 7-day culture with high concentrations of glucose (Fig 5B).

DISCUSSION

We have demonstrated in the present study that glucose stimulated dopamine release from PC12 cells.

As reported previously, the optimal glucose concentration for PC12 cells is 4.5 mg/mL.[10] Thus, we chose 9.0 and 13.5 mg/mL glucose as high concentrations of glucose. These glucose concentrations corresponded to 2- and 3-fold of the optimal glucose concentration for PC12 cells.

Short-term exposure to high concentrations of glucose increased dopamine release from PC12 cells. This effect was not observed when L-glucose was used in the experiments (data not shown). Thus the effect of high concentrations of glucose was

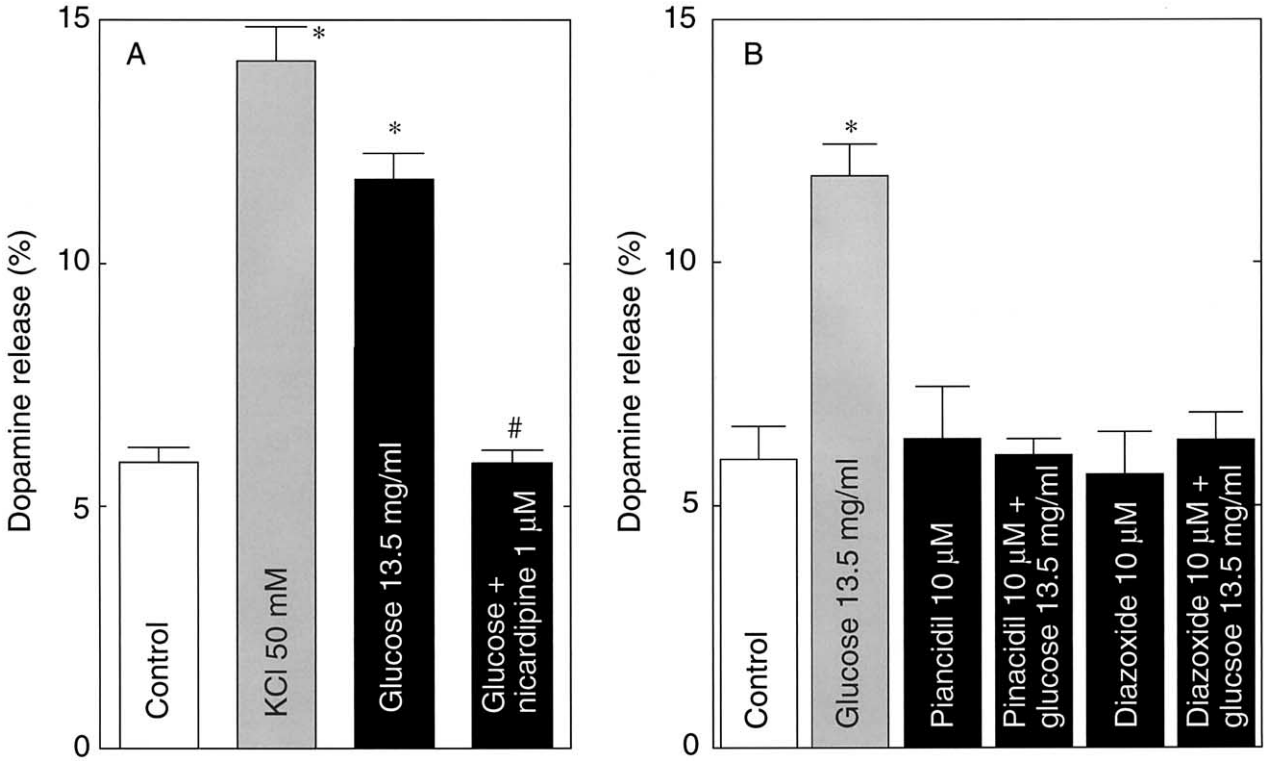

Fig 4. The effect of (A) the Ca^{2+} channel blocker, nicardipine, or (B) KATP channel openers, pinacidil and diazoxide, on glucose-induced dopamine release from PC12 cells. Results are expressed as means ± SEM of 6 determinations. *$P < .05$ *v* control. #$P < .05$ *v* the cells loaded with 13.5 mg/mL glucose.

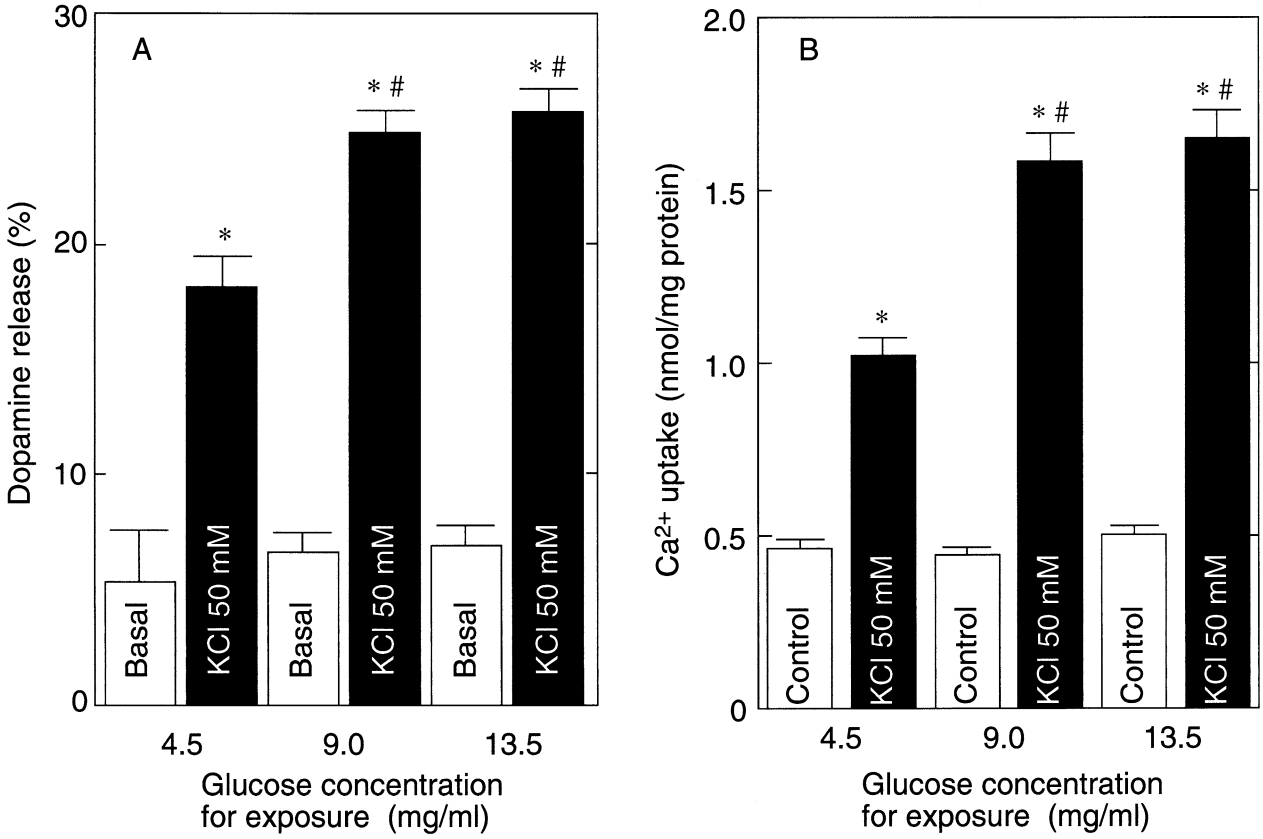

Fig 5. The effect of 7-day culture with 9.0 or 13.5 mg/mL glucose on (A) dopamine release from PC12 cells and (B) Ca^{2+} uptake into PC12 cells. Results are expressed as means ± SEM of 6 determinations. *$P < .05$ *v* corresponding basal or control value. #$P < .05$ *v* 50 mmol/L KCl-induced dopamine release from the cells loaded with 4.5 mg/mL glucose.

not due to the osmotic effects on PC12 cells. The effect of high concentrations of glucose on PC12 cells is assumed to be mediated by glucose metabolism. Since the effect of glucose was observed in 20 minutes, the short-term effect of glucose on dopamine release may be due to stimulation of an exocytotic mechanism, rather than enhancement of dopamine synthesis. High concentrations of glucose induced membrane depolarization, which activated voltage-gated Ca^{2+} channels. The stimulating effect of higher concentration (13.5 mg/mL) of glucose on membrane potential may be due to the action of the higher concentration of glucose and/or delayed effect of lower concentration (9.0 mg/mL) of glucose. Intracellular Ca^{2+} concentration was increased by glucose and the increase was sensitive to a Ca^{2+} channel blocker. These data taken together suggest that high concentrations of glucose induced membrane depolarization and Ca^{2+} channel activation. The resulting increase in the intracellular Ca^{2+} concentration may trigger exocytotic dopamine release. These speculations were supported by the finding that dopamine release induced by a high concentration of glucose was eliminated by the Ca^{2+} channel blocker.

It is known that glucose stimulates insulin secretion from pancreatic β cells by blocking the KATP channels.[5] Membrane depolarization was induced by blocking the KATP channels. SU, a potent hypoglycemic agent, stimulates insulin secretion from pancreatic β cells by blocking the KATP channels.[18] The binding site for SU was identified in neuronal cells,[6,19] and SU was observed to stimulate transmitter release from neuronal cells,[12,23] These findings raised the possibility that high concentrations of glucose block the KATP channel to induce membrane depolarization in PC12 cells. Our observation was supported by the fact that the dopamine release induced by a high concentration of glucose was eliminated by KATP channel openers. These data taken together lead to the hypothesis that short-term exposure of high concentrations of glucose blocks KATP channel to induce membrane depolarization and Ca^{2+} channel activation, triggering dopamine exocytosis from PC12 cells.

When the cells were incubated with high concentrations of glucose for 7 days, high KCl-induced dopamine release and $^{45}Ca^{2+}$ uptake were enhanced. These results suggest that 7-day culture with high concentrations of glucose induced an increase in Ca^{2+} uptake capacity, such as induction of Ca^{2+} channel molecule or increase in Ca^{2+} channel conductance. Thus these results taken together indicate that high concentrations of glucose stimulated dopamine release from PC12 cells by activating the Ca^{2+} channel or modulating the Ca^{2+} channel molecule.

In the early stage of DM, a high concentration of blood glucose stimulates insulin secretion from pancreatic β cells,

resulting in hyperinsulinemia.[14,21] Long-lasting oversecretion of insulin causes deterioration of β-cell functions.[20,24] Taking account for the mechanism of hyperglycemia-induced β-cell dysfunction, it is possible that hypersecretion of neurotransmitters from neuronal cells induced by high concentrations of blood glucose in the early stage of DM may induce collapse of neurotransmitter release mechanism and desensitization of the neurotransmitter receptors in the advanced stage of DM.

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