0026-895X/91/050599-05$03.00/0

MOLECULAR PHARMACOLOGY, 39:599-603

ACCELERATED COMMUNICATION

Regulation of γ-Aminobutyric Acid$_A$ Receptor Subunit Expression by Activation of *N*-Methyl-D-aspartate-Selective Glutamate Receptors

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Received January 8, 1991; Accepted February 20, 1991

SUMMARY

Exposure of primary cultures of rat cerebellar granule cells to specific antagonists of the *N*-methyl-D-aspartate (NMDA)-selective glutamate receptor reduces the steady state levels of mRNAs encoding various γ-aminobutyric acid$_A$ (GABA$_A$) receptor subunits. These neurons are glutamatergic and require a depolarizing concentration of K^+ (25 mM) for optimal development and survival. When the neuronal differentiation rate is retarded by lowering of the extracellular [K^+] (to 12.5 mM), a persistent stimulation of the same glutamate receptors with nonneurotoxic doses of NMDA increases the expression of these GABA$_A$ receptor subunits. This suggests that the lowered K^+ concentration reduces neuronal depolarization and the consequent release of glutamate from the cells. These results show that the neuronal content of selected GABA$_A$ receptor subunit mRNAs is optimized by certain levels of glutamate in the culture medium, suggesting a neurotrophic action of this neurotransmitter at certain developmental stages of granule cells in culture.

Central nervous system development, function, and plasticity depend on the coordinated transcriptional modifications of sets of genes encoding proteins that are relevant to particular neuronal operations. *In vitro* studies with homogeneous populations of neuronal cells in primary culture provide experimental avenues for the elucidation of the regulatory mechanisms for the genetic programs that are operative in various aspects of neuronal function.

Primary cultures of cerebellar neurons enriched in glutamatergic granule cells require 25 mM extracellular K^+ for their differentiation and long term survival (1, 2). The differentiation-promoting effects of such depolarizing K^+ concentrations may be mediated, *inter alia*, via oscillations of neuroplasmic ionized Ca^{2+} content, which are promoted by glutamate release and its action on NMDA-selective glutamate receptors. Thus, such stimulation of glutamate receptors may play an important role at certain stages of granule cell development by regulating neuronal transcriptional programs (3–5).

The type A GABA receptor is a heteropolymeric protein complex with an intrinsic Cl^- channel that contains sites for the action of various positive and negative endogenous modulators, such as neurosteroids (reviewed in Ref. 6) and various peptides (reviewed in Ref. 7). These sites are clinically important, because drugs such as barbiturates and benzodiazepines elicit their actions by binding to these recognition sites (8). The genes encoding the GABA$_A$ receptor subunits constitute a relatively large multigene family (reviewed in Ref. 9), individual members of which are expressed differentially in the developing central nervous system. In rat, there are multiple molecular forms of the α (10–13) and β (14) subunit cDNAs, and several additional receptor subunit cDNAs have been recently identified, including those encoding the γ_2 and δ subunits (15). Transient transfection experiments with combinations of vectors expressing α, β, and γ cDNAs have shown that the efficacy, potency, and type (either positive or negative) of modulation of GABA action by drugs acting on the allosteric regulatory sites of these receptors are dependent on the specific subunits present in the assembled receptor (16–19). This relevance of the structure of the GABA$_A$ receptor to the information processing triggered by the GABA signal suggests that the expression of the various GABA$_A$ receptor subunits might be regulated by afferent synaptic signals projecting to neurons containing GABA$_A$ receptors.

Because granule cells contain both GABA$_A$ receptors and various subtypes of glutamate receptors, we tested whether stimulation of various receptor types could alter the expression

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; CPP, 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; GABA, γ-aminobutyric acid; MK 801, dibenzocycloheptenimine; PCR, polymerase chain reaction; bp, base pairs.

of mRNAs encoding various $GABA_A$ receptor subunits. Preliminary experiments indicated that neither GABA nor muscimol affected the receptor subunit mRNA levels, as measured by a PCR-derived technique. In contrast, glutamate may have a role in regulating the expression of genes encoding the various $GABA_A$ receptor subunits.

Materials and Methods

Cell culture. Cerebellar granule cells were prepared, as described (20), from 8-day-old Sprague Dawley rats. Cells were plated on 100-mm-diameter Nunc dishes precoated with poly-L-lysine and were cultured in basal Eagle's medium supplemented with 10% heat-inactivated bovine calf serum, 2 mM glutamine, and 50 μg/ml gentamicin. Unless specified, the final concentration of KCl was 25 mM. Cytosine arabinoside (10 μM) was added to all cultures 24 hr after seeding. Cell density was $3 \times 10^5/cm^2$.

RNA preparation and PCR. mRNA was extracted as described (21) and reverse transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of ^{32}P, as recommended by the manufacturer (BRL). The resulting cDNA was quantitated by determining the amount of radioactivity incorporated into trichloroacetic acid-precipitable nucleic acids, as described (22). PCR was performed with *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus) in 100 μl of standard buffer (10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001%, w/v, gelatin) containing 1 μM subunit-specific oligonucleotide primers (Table 1) and 10 μl of reverse transcribed (0.5 ng) cDNA. Thirty cycles of amplification were performed with a DNA thermal cycler (Perkin-Elmer Cetus) and a step program (94°, 30 sec; 60°, 30 sec; 72°, 60 sec), followed by a 15-min final extension.

After amplification, 10 μl of the reaction mixture were separated by electrophoresis [1.2% agarose gel containing 0.5× 0.045 M Tris-borate, 0.001 M EDTA buffer (20) and ethidium bromide (0.5 μg/ml)], transferred to nitrocellulose, and incubated at 80° for 2 hr. The blot was then hybridized at 42° in 6× (5 gm Ficoll, 5 gm polyvinylpyrrolidone, 5 gm bovine serum albumin) SSPE [saline, sodium, phosphate, EDTA (22)], 5× (5 gm Ficoll, 5 gm polyvinylpyrrolidone, 5 gm bovine serum albumin) Denhardt's solution (22), 0.5% sodium dodecyl sulfate, 100 μg/ml salmon sperm DNA, with a third ^{32}P-5′ end-labeled subunit-specific oligonucleotide probe for 24 hr, washed for 15 min under high stringency conditions (0.5× SSPE 0.1% sodium dodecyl sulfate, at 42°), and exposed to X-ray film (Kodak) for various times. The third subunit-specific oligonucleotide corresponded to a sequence presenting the lowest homology to other $GABA_A$ receptor subunits, which was contained within the amplified target DNA sequence. The specificity of the amplification was assessed by both the size of the amplified DNA product and the hybridization signal obtained after stringent washing of the heterologous oligonucleotide specific for the subunit of interest. The results from a representative experiment, in which the PCR primers were specific for the α_1 $GABA_A$ receptor subunit, are shown in Fig. 1, *left inset*. In this case, the blot was hybridized to an oligonucleotide that corresponds to nucleotides 1386 to 1424 of the published rat α_1 $GABA_A$ receptor subunit cDNA (10), i.e., 5′-TTAAACAGAGAGCCTCAGCTAAAAGCCCCCACACCCCAT-3′.

In parallel, PCR was performed, as described above, with the addition of trace amounts of [^{32}P]dCTP. The amplified DNA was separated by electrophoresis, the ethidium bromide-stained DNA band was removed, and its radioactivity content was determined by scintillation counting. Under defined experimental conditions, the amount of incorporated radioactivity was proportional to the amount of original template for each of the various transcripts measured. The results of a representative incorporation experiment, with α_1-specific primers, are shown in Fig. 1, *right inset*. Using this approach, we have found that the amount of PCR-amplified product is proportional to the relative abundance of the specific mRNA encoding a given $GABA_A$ receptor subunit.

TABLE 1
Primer sequences

Subunit		Primer Sequences	G+C %	Ref.
α_1	PCR-1	5′-AGCTATACCCCTAACTTAGCCAGG-3′	50	10
	PCR-2	5′-AGAAAGCGATTCTCAGTGCAGAGG-3′	50	
	Sequences amplified: from 1178 bp to 1482 bp; Δ = 304 bp			
α_4	PCR-1	5′-CAAGAAGGCCTTGGAAGCAGCTAA-3′	50	10
	PCR-2	5′-GGTTTCCTGTCTTACTTTGGAGAG-3′	46	
	Sequences amplified: from 1188 bp to 1535 bp; Δ = 347 bp			
β_1	PCR-1	5′-CCTGGAAATCAGGAATGAGACCAG-3′	50	14
	PCR-2	5′-GGAGTCTAAACCGAACCATGAGAC-3′	50	
	Sequences amplified: from 1190 bp to 1531 bp; Δ = 341 bp			
β_2	PCR-1	5′-TGAGATGGCCACATCAGAAGCAGT-3′	50	14
	PCR-2	5′-TCATGGGAGGCTGGAGTTTAGTTC-3′	50	
	Sequences amplified: from 1201 bp to 1518 bp; Δ = 317 bp			
β_3	PCR-1	5′-GAAATGAATGAGGTTGCAGGCAGC-3′	46	14
	PCR-2	5′-CAGGCAGGGTAATATTTCACTCAG-3′	46	
	Sequences amplified: from 1199 bp to 1554 bp; Δ = 355 bp			
γ_2	PCR-1	5′-TGTGAGCAACCGGAAACCAAGCAA-3′	50	15
	PCR-2	5′-CGTGTGATTCAGCGAATAAGACCC-3′	50	
	Sequences amplified: from 1116 bp to 1490 bp; Δ = 374 bp			
δ	PCR-1	5′-TGAGGAACGCCATTGTCCTCTTCT-3′	50	15
	PCR-2	5′-ACCACCGCACGTGGTACATGTAAA-3′	50	
	Sequences amplified: from 1097 bp to 1430 bp; Δ = 333 bp			

Results

Using a PCR-derived method, we found that stimulation of granule cells maintained in medium containing 25 mM KCl with 10 μM glutamate for 5 days *in vitro* failed to affect the expression of $GABA_A$ receptor subunit mRNAs, as determined either by Southern blot analysis or by quantitation of the amount of the PCR-amplified product (Fig. 1). In contrast, when the cultures were exposed for 5 days to 1 μM MK-801, a selective, noncompetitive, allosteric antagonist of the NMDA-selective glutamate receptor (23), a significant reduction in the steady state concentrations of mRNAs encoding the α_1, β_1, β_2, and γ_2 receptor subunits was observed. The amount of amplified DNA sequence obtained by PCR with primers specific for the α_1 and β_1 receptor subunits was decreased by 75% and 60%, respectively, relative to the untreated cultures. The reduction in the yield of PCR product obtained with primers specific for the β_2 and γ_2 subunits was less pronounced (decreases of 25 and 23%, respectively). These findings demonstrate a differential sensitivity of various $GABA_A$ receptor subunit mRNAs to the signal transduction blockade effected by MK-801. That is, the mRNA content of β_2 and γ_2 receptor subunits is affected to a smaller degree than that of the α_1 and β_1 subunits, whereas that for the β_3 and δ subunits is virtually unaffected by MK-801. The possibility that this effect does not result from a generalized down-regulation of RNA synthesis and is specific for mRNAs encoding certain $GABA_A$ receptor subunits is supported by results showing that the amount of mRNA encoding the structural protein β-actin was unaffected by the MK-801 treatment (control, β-actin = 2.1 ± 0.2 pmol/25 amplification cycles, versus 10 μM MK-801, β-actin = 2.1 ± 0.2 pmol/25 amplification cycles; three experiments each). Moreover, exposure of granule cells for 5 days *in vitro* did not change the total RNA content (control, 18 ± 2.0 μg/10-cm plate, three experiments, versus 10 μM MK-801, 16 ± 2.0 μg/10-cm plate, three experiments, total protein content (control, 55 ± 5.0 μg/10-cm plate, three experiments, versus 10 μM MK-801, 61 ± 6.0 μg/10-cm plate, three experiments), or the intensity of neurofibrillary immunohistochemical staining relative to the untreated cultures (data not shown).

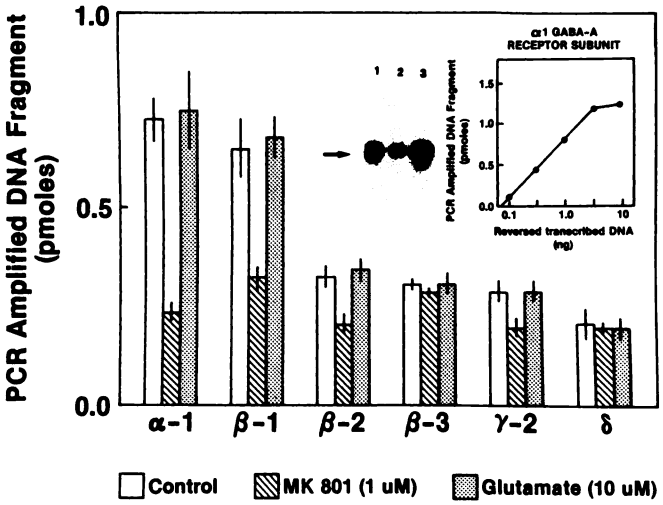

Fig. 1. Relative abundance of various GABA$_A$ receptor subunit mRNAs in cerebellar granule cells exposed for 5 days to glutamate or MK-801. Cells were either untreated (□) or treated with 1 μM MK-801 (▨) or 10 μM glutamate (▩) daily from day 2 to day 7 *in vitro*. At the end of this period, total RNA was isolated, reverse transcribed, and analyzed by PCR (see Materials and Methods). *Vertical axis*, amount of amplification product obtained after PCR using primers specific for the various GABA$_A$ receptor subunits (Table 1). Values are mean ± standard error. α_1, control versus MK-801, $p < 0.001$; β_1, control versus MK-801, $p < 0.001$; β_2, control versus MK-801, $p < 0.001$; β_3, control versus MK-801, not significant; γ_2, control versus MK-801, $p < 0.005$; δ, control versus MK-801, not significant. *Left inset*, a representative Southern analysis of the products obtained after PCR amplification with α_1-specific primers. *Lane 1*, untreated cells; *lane 2*, MK-801-treated cells; *lane 3*, glutamate-treated cells. *Arrow*, expected size of the DNA sequence. *Right inset*, the relation between the amount of template and the amount of PCR product. PCR was performed using α_1-specific primers, with 20 amplification cycles in the presence of [^{32}P]dCTP.

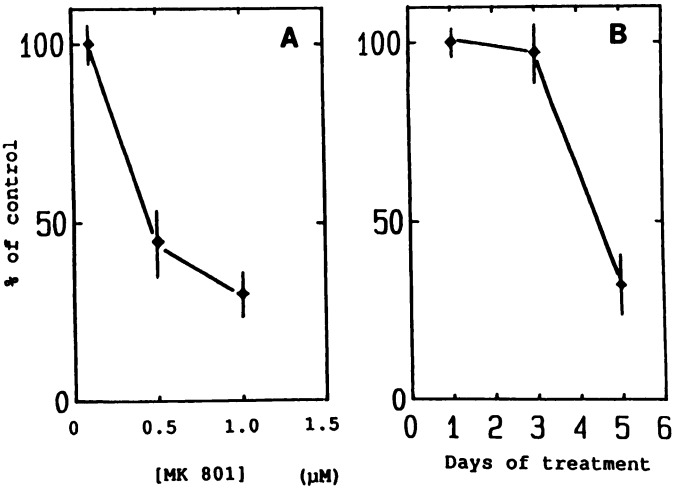

Fig. 2. Relative abundance of the α_1 subunit mRNAs in granule cells exposed to MK-801. A, Dose-response relation. Cells were treated daily with different concentrations of MK-801 from day 2 to day 7 *in vitro*, after which mRNA was extracted. B, Time course. Cells were treated with 1 μM MK-801 for various times starting at day 2 *in vitro*. PCR was primed using α_1-specific oligonucleotides. The amount of PCR-amplified DNA fragment in control cells was 0.75 ± 0.070 pmol. Values are the means ± standard errors of two separate cultures of at least three dishes each.

TABLE 2

Effect of MK-801, CPP, and CNQX on the relative abundance of various GABA$_A$ receptor subunit mRNAs

Cells were treated daily for 5 days (from day 2 to day 7 *in vitro*) with 1 μM MK-801, 10 μM CPP, or 1 μM CNQX, after which mRNA was extracted. Values are the means ± standard errors of at least two separate cultures, each with at least three dishes. The amounts of PCR-amplified DNA fragments in control cells were as follows: α_1, 750 ± 50 fmol; β_1, 650 ± 55 fmol; β_2, 320 ± 35 fmol; β_3, 390 ± 45 fmol; γ_2, 220 ± 35 fmol; and δ 180 ± 30 fmol.

Subunit	Amount of amplified DNA product		
	MK 801	CPP	CNQX
	% of control		
α_1	28 ± 2.5	55 ± 6.4	100 ± 8.8
α_4		50 ± 5.0	
β_1	40 ± 4.0	48 ± 4.9	100 ± 7.6
β_2	75 ± 4.5	55 ± 4.5	100 ± 7.5
γ_2	77 ± 4.0	65 ± 4.9	98 ± 8.5
δ	100 ± 4.0	100 ± 5.5	100 ± 9.0

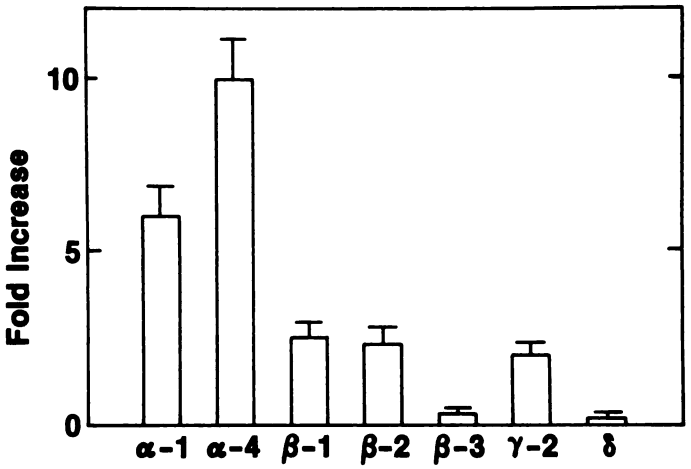

Fig. 3. Relative abundance of various GABA$_A$ receptor subunit mRNAs in NMDA-treated granule cells. Cells were cultured in a medium containing 12.5 mM K^+ for 6 days; NMDA (10 μM) was added daily from day 2 to day 6. The relative amounts of the indicated subunits were determined by PCR and are expressed as a ratio to the amount of each subunit RNA present in the control (untreated) cultures.

The mRNA down-regulation mediated by MK-801 was dependent on both dose and time. The concentration of MK-801 required to reduce by 50% the steady state concentration of mRNA encoding the α_1 subunit was about 400 nM (Fig. 2A). Similar results were obtained by measuring the steady state concentrations of mRNAs encoding the β_1, β_2, and γ_2 receptor subunits (data not shown). Experiments designed to establish the time required for the MK-801 treatment to exhibit its maximal effects indicated that a 5-day period between days 3 and 7 *in vitro* was optimal (Fig. 2B). Exposure of granule cells to 1 μM MK-801 for either 1 or 3 days (Fig. 2B) failed to significantly affect the steady state concentration of mRNA encoding the α_1 receptor subunit. A similar period of latency was observed for the effect of MK-801 on the steady state concentrations of mRNAs encoding the β_1, β_2, and γ_2 receptor subunits (data not shown).

Treatment of the neuronal granule cell cultures with CNQX, in doses that antagonize the non-NMDA-selective glutamate receptors (24), failed to affect the steady state concentrations of various mRNAs encoding GABA$_A$ receptor subunits (Table 2). Therefore, we suggest that glutamate is acting at the NMDA-selective glutamate receptor to regulate GABA$_A$ receptor subunit mRNA concentrations. This is also supported by

experiments showing that a 4-day exposure of granule cells to 1 μM CPP, a competitive antagonist of the NMDA-sensitive glutamate receptor (25), induced a pattern of changes in the concentrations of the $GABA_A$ receptor subunit mRNAs that was qualitatively comparable to that observed with MK-801 (Table 2). CPP treatment also decreased the steady state concentration of the mRNA encoding the α_4 $GABA_A$ receptor subunit.

When the granule cell cultures were maintained in 25 mM KCl, the addition of glutamate failed to alter the mRNA levels for each of the receptor subunits tested (Fig. 1, and data not shown). In order to reduce the effect that chronic depolarization might have on the release of endogenous glutamate from these neurons, we lowered the extracellular K^+ concentration to 12.5 mM. Exposure of granule cells cultured under these conditions for 4 days (from day 2 to day 6 *in vitro*) to 10 μM NMDA increased the expression of the mRNAs encoding the α_1, α_4, β_1, β_2, and γ_2 $GABA_A$ receptor subunits (Fig. 3). The concentrations of mRNAs encoding the β_3 and δ subunits were not affected by NMDA. The enhancement in the yield of PCR products obtained with primers specific for the α_1, α_4, β_1, β_2, and γ_2 $GABA_A$ receptor subunits was not uniform. The α_4 and α_1 receptor subunit mRNAs appeared to be the most sensitive (10- and 6-fold increases over the corresponding basal concentration, respectively).

Discussion

Treatment of granule cell cultures with either a competitive (CPP) or a noncompetitive (MK-801) antagonist of the NMDA-sensitive glutamate receptor effects a down-regulation of mRNAs encoding various $GABA_A$ receptor subunits. In addition, NMDA stimulation of granule cells maintained in low K^+-containing medium results in elevated levels of some of the receptor subunits. The differential response of the various $GABA_A$ receptor subunit mRNAs to either the MK-801 or NMDA treatment may reflect a differential regulation of the mRNA content for the various receptor subunits. These results indicate that regulation of $GABA_A$ receptor subunit mRNAs may involve a complex interplay between continuously expressed neuronal genetic programs and signals generated through heterologous receptor stimulation. However, it remains a possibility that these treatments regulate the receptor mRNA levels by a posttranscriptional mechanism.

Several studies indicate that bioelectrical activity markedly influences neuronal survival during certain stages of development (26–29). In particular, the differentiation and survival of cerebellar granule cells in culture appear to be greatly dependent on neuronal activity (1, 2). The chronic depolarization that is required for their optimal development in culture is usually effected by raising the concentration of K^+ in the culture medium. The effect of high K^+ concentrations on these cells might be mediated through the following chain of events: neuronal depolarization, glutamate release, activation by glutamate of specific cation channels, elevation of neuroplasmic Ca^{2+} concentration, activation of Ca^{2+}-dependent protein kinases, and other processes yet to be defined. These experimental conditions may, however, mask possible effects resulting from the stimulation of glutamate receptors induced by exogenously added agonists. This may be why the addition of exogenous glutamate to the granule cell cultures maintained in high K^+ does not increase the $GABA_A$ receptor subunit mRNA levels.

NMDA also promotes the survival and differentiation of cerebellar granule cell cultures maintained in low K^+-containing medium (30, 31). To establish whether this differentiation-promoting effect of NMDA involved also an altered expression of genes encoding various $GABA_A$ receptor subunits, we analyzed the receptor subunit mRNAs in an experimental condition in which the K^+ concentration was reduced from 25 to 12.5 mM. Evaluation of cell survival as a function of medium K^+ concentration has shown that, up to 6 days *in vitro*, K^+ concentrations in the range of 5 to 50 mM had little, if any, influence on cell number and survival (1, 2, 30). However, measurement of neural growth markers, such as neural cell adhesion molecule, D-3 protein, and synapsin, indicates that reducing the K^+ concentration in the medium markedly reduces the differentiation rate (30). Our data also suggest that stimulation of NMDA-selective glutamate receptors, possibly by facilitating Ca^{2+} influx into the granule cells, could participate in the cell differentiation process. One component of this process might be a change in either the total number or the subtypes of $GABA_A$ receptors expressed.

It has previously been established that stimulation of NMDA-selective glutamate receptors that are present in primary cultures of cerebellar granule cells results in the induction of a series of immediate early genes, including c-*fos*, c-*jun*, *jun*-B, and *zif*/268 (3–5). Immunohistochemical analysis, Western blotting, and 'gel shift' experiments indicate that the protein products of these genes accumulate in the nuclei of the granule cells following the glutamate pulse. These transcription factors have been postulated to function as nuclear third messengers in coupling receptor stimulation to long term phenotypic changes in neurons. Our data are consistent with the hypothesis that the $GABA_A$ receptor subunit genes represent one class of target genes that are regulated by changes in the levels of AP-1 complex present in these neurons, which is, in part, determined by the level of excitatory input.

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