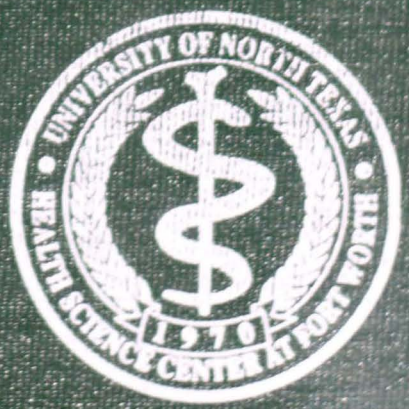
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Mingjun Fang. Modulation of $GABA_A$ Receptor Function by Tyrosine Phosphorylation. Master of Science (Biomedical Sciences), May, 1998. 32 pp., 6 illustrations, bibiography, 42 titles.

The goal of this study was to determine the modulation of $GABA_A$ receptor function by tyrosine kinase phosphorylation, and to detect which subunit is phosphorylated to alter the GABA-induced chloride currents. From previous studies, we suggested that protein tyrosine phosphorylation may maintain $GABA_A$ receptor function. Here we tested the hypothesis that tyrosine phosphorylation modulates other $GABA_A$ receptor subtypes e.g., $\alpha_1\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$, and subsequently attempted to determine which subunit(s) may be phosphorylated. Our results support the hypothesis that PTK phosphorylation may maintain $GABA_A$ receptor function. In addition, we suggest this tyrosine phosphorylation occurs at the γ_2 subunit of the receptor.

MODULATION OF $GABA_A$ RECEPTOR FUNCTION

BY TYROSINE PHOSPHORYLATION

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MODULATION OF $GABA_A$ RECEPTOR FUNCTION BY TYROSINE PHOSPHORYLATION

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

BY

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FORT WORTH, TX

June 1998

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CHAPTER I

Introduction

Importance of $GABA_A$ Receptor in Mammalian Brain

γ-aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian brain. It is estimated that 20-50% of all central synapses use GABA as their transmitter (1, 2). GABA mediates its effects via $GABA_A$, $GABA_B$ and $GABA_C$ receptors. Because they open the chloride channels, $GABA_A$ receptors are responsible for most of the fast inhibitory synaptic transmission in the brain (3, 4). $GABA_A$ receptors are of importance because of the pivotal role that they play in the regulation of brain excitability. With their hypofunctioning, mental disturbances like anxiety, panic, or muscular disorder may occur (5). $GABA_A$ receptors are also important because their function is directly or allosterically regulated by several distinct classes of therapeutic compounds. These include anxiolytic benzodiazepines, barbiturates, neurosteroids, alcohol, and some volatile anesthestics (6, 7, 8).

Review of the structure of $GABA_A$ receptors

The GABA receptors are classified as $GABA_A$ and $GABA_B$ receptors, respectively, according to their respective transduction mechanisms following activation. $GABA_A$ receptors are stimulated by GABA, muscimol and inhibited by the convulsants bicuculline and picrotoxin (9,10). $GABA_A$ receptors are directly associated with a Cl^- ion channel (4,9). While $GABA_B$ receptors are coupled indirectly to Ca or K channels (9,10).

Because of the clinical and pharmacological importance of $GABA_A$ receptors, we focus on studying $GABA_A$ receptors.

Molecular cloning studies have revealed that $GABA_A$ receptors belong to the superfamily of ligand-gated ion channels that includes nicotinic acetylcholine receptors, $5HT_3$ receptors and glycine receptors (11,12, 13). $GABA_A$ receptors have pentameric structures , formed by distinct subunits (13). Each subunit has four tramsmembrane domains and named M1, M2, M3 and M4, numbered from the amino terminal end. The first several hundred amino acids on the amino end and the last dozens amino acids on the carboxyl end are extracellular. There is a large intracellular loop between M3-M4, which contains numerous potential consensus sites for protein phosphorylation by various protein kinases (14, 15). Together the subunits may form a cylindrical chloride ion channel that can exist in both an open and a closed configuration (16, 17, 18).

$GABA_A$ receptor subunits have been identified and named α, β, γ, δ, ε subunits. Some of the subunits have multiple subtypes (α_{1-6}, β_{1-4}, γ_{1-3},δ, ε) (14, 19), so there are more than one hundred $GABA_A$ receptor isoforms that can be formed by the different subunits, theoretically. However, the accurate subunit composition and stoichiometry of native $GABA_A$ receptors are unknown now. The $\alpha_X\beta_2\gamma_2$ subtypes produce the chloride channel that shares many functional characteristics with the native neuronal receptors, therefore the $\alpha_X\beta_2\gamma_2$ is thought to be the prototype $GABA_A$ receptor in mammalian brain. The $\alpha_1\beta_2\gamma_2$ subunit combination is believed to be the most abundant population (15, 20).

Regulation of $GABA_A$ receptor

There are many potential consensus sites for protein phosphorylation in intracellular domain (14, 15). This may suggest that $GABA_A$ receptors may be phosphorylated and regulated by protein kinase. Protein phosphorylation is an important post-translational mechanism for regulation of voltage-gated (21) and ligand-gated (22, 23) ion channels, may be a primary mechanism for the regulation of neurotransmitter function (24). Phosphorylation is a process by which the highly charged phosphate group of ATP is transferred to serine, threonine, or tyrosine residues of a substrate protein, thus altering its functional properties. Several studies showed that $GABA_A$ receptors have been phosphorylated by exogenously protein kinase, e.g., PKA (25, 26, 27, 28), PKC (25, 27, 29), CaM kinase II (29, 30), cGMP-dependent protein kinase (30), tyrosine kinase (31), or an unknown receptor-associated kinase (30). This suggests that $GABA_A$ receptor function may also be regulated by protein phosphorylation. From several previous studies of native $GABA_A$ receptors, it has been proposed that ATP limits rundown of the $GABA_A$ receptor by acting as a substrate for protein phophorylation (32, 33, 34). Rundown, observed from chloride ion channel associating with neuronal $GABA_A$ receptor, is a time-dependent decrease in the responsiveness of $GABA_A$ receptor to GABA with minimal intracellular medium. PKA or PKC may be involved in the phosphorylation of serine or threonine residues and regulates the function of $GABA_A$ receptor (29, 35).

Although much evidence implicates protein phosphorylation in the regulation of $GABA_A$ receptor function, the results obtained from functional studies are conflicting (36). In addition, no direct evidence has showed that phosphorylation of these residues modulates rundown of the $GABA_A$ receptor.

The role of tyrosine phosphorylation on the functional properties of $GABA_A$ receptors has aroused intense interest in recent years. Some studies demonstrated that tyrosine phosphorylation, which is mediated by tyrosine kinase (PTK), enhances activity of neuronal and recombinant $GABA_A$ receptors (30, 31). Evidence suggested that endogenously active PTK may be an important modulator of $GABA_A$ receptors (31). However, it is still not known if the activity of PTK is integral to maintaining function of the $GABA_A$ receptor.

Because of the important role of $GABA_A$ receptors in brain function and dysfunction and the ubiquitous signaling pathways using PTKs and PTPs in the brain, we wished to study the modulation of the $GABA_A$ receptor. There are dozens of different $GABA_A$ receptor subtypes mixed together in the brain and it is impossible to view how exactly each kind of receptor subtype responds to transmitter and is regulated. In order to simplify and make sure of the exact response from individual $GABA_A$ receptor subtypes, we use recombinant $GABA_A$ receptor expressed in HEK 293 cells. The human embryonic kidney cell line HEK 293 is used widely for the expression of cloned channels, partly because this cell line expresses few endogenous channels (36). The characteristics of recombinant $GABA_A$ receptors expressed in this system are similar to those seen in native

receptors (38). Recombinant receptor preparations have several advantages over the native neuronal preparations to study long-term receptor function. Most importantly, their use eliminates possible interaction among unknown receptors and channels, and in addition, allow determination whether rundown is influenced by receptor subunit composition. This in turn gives us information about regulatory sites that exist on the receptor subunits.

Proposed model for modulation of $GABA_A$ receptor function

Previous investigations, provide little information about maintenance of the function of $GABA_A$ receptor by protein kinase. In this paper, we studied the role of the tyrosine phosphorylation on maintaining recombinant $GABA_A$ receptor function.

We set out to study what effects phosphorylation of $GABA_A$ receptor has on rundown of the receptors. Rundown, observed from chloride ion channel associating with neuronal $GABA_A$ receptor, is a time-dependent decrease in the responsiveness of $GABA_A$ receptor to GABA. Several labs have reported that ATP (32, 33, 34) may regulate rundown of the channel. We increased the concentration of ATP from 1 to 4 mM, and the current did not run down. But no one explained what the mechanism that ATP influences rundown.

In previous studies of recombinant $\alpha3\beta2\gamma2$ receptors, researchers have characterized rundown and the role of ATP in this process. Based on the results, they proposed that ATP maintains the long-term receptor function by promoting activation of

an endogenous protein tyrosine kinase, stimulation of which enhances $GABA_A$ receptor-related channel function.

A limitation in previous studies, however, is that the examination of the role of PTK to maintain $GABA_A$ receptor function was done using genistein. Genistein has proved to be a useful molecular tool for probing the biochemical pathways and biological processes in which PTKs may be implicated (38). However, genistein is not a very selective inhibitor of PTK, it also inhibits other kinases (39). In opposite, Lavendustin A is a high selective inhibitor of PTK (40), it only inhibits the activity of PTK and does not inhibit PKC and PKA activity (protein inhibitor), thus it is an ideal reagent for us to get evidence to support our hypothesis.

We proposed our hypothesis that tyrosine phosphorylation by endogenous PTK maintains the function of $GABA_A$ receptor based on recombinant $\alpha3\beta2\gamma2s$ $GABA_A$ receptors. In the present study, we tried to examine this hypothesis on recombinant $\alpha1\beta2\gamma2s$, $\alpha6\beta2\gamma2s$ $GABA_A$ receptors; Furthermore, we sought to determine which subunit is phosphorylated by PTK, $\beta2$ or $\gamma2$.

Significance of this research:

$GABA_A$ receptors are critical in mediating fast synaptic inhibition; such a regulation by tyrosine kinases may therefore have profound effects on the control of neuronal excitation. Results of these experiments will provide insight into modulation of GABA-mediated inhibition in the CNS.

CHAPTER II

METHODS

Preparation of Cloned $GABA_A$ receptors.

cDNAs encoding rat α1, α6, β2, and γ2 $GABA_A$ receptors subunits have been stably transfected in human embryonic kidney cell lines (HEK293) as descibed previously (41), and form different recombinant $GABA_A$ receptor subtypes as α6β2γ2, α1β2γ2, α1β2 and α1γ2 $GABA_A$ receptors. Briefly, the cells were transfected with plasmids containing cDNA and a plasmid encoding G418 resistance. After 2 weeks of selection in 1 mg/ml G418, resistant cells were assayed by Northern blotting for the ability to synthesize $GABA_A$ receptor mRNAs. Cells that have been shown to express all subunits were used for electrophysiology. All studies were conducted on cells expressing the above receptor configurations.

Electrophysiological recordings.

Electrophysiological experiments with recombinant $GABA_A$ receptors stably expressed in HEK were conducted 3-5 days when these cells grew on a coverslip in a dish filled with media. We used the conventional whole-cell configuration of the patch clamp technique to study GABA-induced Cl^- currents. Patch pipettes were pulled from thin-walled borosilicate glass using a horizontal micropipette puller (Sutter Instrument, P-87) and a resistance of 1-3 MΩ when filled with the following modified pipette solution

(in mM): CsCl, 140; EGTA, 10; Mg^{2+}-ATP, 4; N-[2-hydroxyethyl] piperazine-N'-{2-ethanesulfonic acid] (i.e. HEPES), 10; pH 7.2.

Coverslips containing the cultured cell were transferred to a small chamber (1ml) on the stage of an inverted light microscope (Olympus, IMT-2) and superfused continuously (5-8 ml/min) with the following external solution (in mM): NaCl, 125; KCl, 5.5; $CaCl_2$, 3.0; $MgCl_2$, 0.8; HEPES-Na, 20; glucose, 25; pH, 7.3.

Whole-cell currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage. GABA-induced Cl^- currents were monitored simultaneously on a storage oscilloscope and a thermal-head pen recorder, and stored on a computer using an on-line data acquisition system (pClamp; Axon Instruments). To minimize the possibility that changes in access resistance could affect current recordings over time, we measured and stored on our digital oscilloscope, at the initiation of each recording, the current response to a 5 mV voltage pulse. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data was not included in the analysis. All recordings were made at room temperature, and, with the exception of collection of current-voltage recordings, all cells were voltage-clamped at -60 mV.

Data analysis

All data were reported as mean ± standard error of the mean (S.E.M.). Significance was assessed by Student's t-test (unpaired). Peak current amplitudes were measured directly from the computer screen using pClamp software.

Current amplitude was expressed as I_t/I_o, where I_t was the current amplitude recorded at t min after initial recording, and I_o was the initial recording. In experiments examining the potential rundown of currents with time, the initial current amplitude in response to GABA was assigned a value of 100%. All subsequent currents were expressed as a percentage of this current.

To construct concentration-response curves, the data were normalized relative to the value obtained at 2 mM GABA (I_{max}) and fitted with the equation $I/I_{max}=c^n/(c^n+EC_{50}^n)$. In this equation I/I_{max} is normalized current, c is GABA concentration, EC_{50} is the half-maximal effective GABA concentration, and n is the Hill coefficient.

Pharmacological agents

Drugs used in the experiments were: γ-Aminobutyric acid (GABA) (from Sigma) was freshly prepared as stock solution in saline. lavendustin A and genistein (all from Calbiochem-Novabiochem Co., La Jolla, CA). A concentrated stock solution of lavendustin A was prepared in dimethylsulphoxide (DMSO), and for each experiment, lavendustin A was freshly diluted in pipette solution.

Experimental Protocol

GABA was dissolved in the external solution and applied 5 seconds to the target cell through a Y-tube positioned within 100 μM of the cell. In experiments where the drug effect is concerned, the drug genistein or lavindustein A were dissolved in internal pipette solution. We studied GABA-induced Cl- currents in response to low and high

concentrations of GABA with or without the presence of lavendustein A, a highly selective inhibitor of PTK, to examine the effect of drug to the currents over 30 minutes. Low concentration of GABA for recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor is approximately 5 uM, similar to EC_{20} for this receptor subtype. 500 μM GABA as high concentration and it may induce a maximal GABA response; Low concentration of GABA for recombinant $\alpha_1\beta_2$ $GABA_A$ receptor is approximately 1 μM, similar to EC_{20} for this receptor subtype, and 100 μM GABA as high concentration and it may induce a maximal GABA response.

CHAPTER III

RESULTS

Different $GABA_A$ receptor subtypes like recombinant $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ and $\alpha_1\beta_2$ have different concentration of GABA that evokes a half-maximal response (EC_{50})

Four different $GABA_A$ receptor subtypes were expressed by transfection of HEK 293 cells: $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_2$ and $\alpha_1\gamma_2$ $GABA_A$ receptor subtypes. After expression in the HEK 293 cells, $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ and $\alpha_1\beta_2$ $GABA_A$ receptor subtypes tested in this study produced inward chloride currents in response to the application of GABA as described in Methods.

Fig. 1A shows the typical chloride current amplitudes evoked by low to high concentration of GABA in recombinant $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptor subtypes, it shows the current amplitude enhanced with the increase of the concentration of GABA. The maximal amplitude at each concentration for each subtype is plotted below (fig. 1B). These three smooth curves in fig. 1B are fitted to the data points according to the logistic equation. The EC_{50} for recombinant $\alpha_1\beta_2\gamma_2$ receptor subtype is 20.7 μM, and the Hill number (n) is 1.4. The EC_{50} for recombinant $\alpha_6\beta_2\gamma_2$ receptor subtype is 1.6 μM and the Hill number (n) is 1.5. And the EC_{50} for recombinant $\alpha_1\beta_2$ receptor subtype is 2.0 μM and the Hill number (n) is 1.4. According to our experiment results, we did not

successfully observe recombinant $\alpha_1\gamma_2$ receptor subtype respond to GABA, so there is no EC_{50} for recombinant $\alpha_1\gamma_2$ receptor subtype.

Genistein inhibits the recombinant $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor

Genistein has proved to be a useful molecular tool for probing the biochemical pathways and biological processes in which PTKs may be implicated. (40). Here we use genistein as an inhibitor of protein tyrosine kinase to confirm the hypothesis that PTK phosphorylates tyrosine residue and modulates the function of the $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor. For this and all subsequent experiments, cells are voltage-clamped to -60 mV in external solution at room temperature during whole-cell recordings, and follow the protocol in Methods. Genistein or later lavendustin A is all applied intracellularly via recording pipettes during the whole-cell patch recording in individual cells.

Other researchers from this lab characterized the rundown of the $\alpha_3\beta_2\gamma_2$ $GABA_A$ receptors expressed in HEK 293 cells. They modified the pipette solution and increased the concentration of ATP to 4 mM, and they found the current amplitude kept constant or even a little run-up. Thus they suggested this is because tyrosine residue is phosphorylated and the phosphorylation maintains the function of the $GABA_A$ receptor. In this and the present experiments on $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ and $\alpha_1\beta_2$ $GABA_A$ receptors, we used the modified pipette solution. If the hypothesis is true, when we apply genistein or later Lavendustin A, the tyrosine phosphorylation would be inhibited and the chloride current amplitude would run down over time. We applied GABA at 10 minutes interval, and each application of GABA was 5 seconds in duration. The typical current curve with

1μM GABA in Fig. 2A shows the recorded current amplitude of $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor runs up over time. In comparison, the recorded current amplitude in response to GABA plus genestein runs down over time. Figure 2B demonstrates the average peak whole-cell currents obtained during the control recording and genistein recording. Genistein treatment significantly decreased the current amplitude of recombinant $\alpha_6\beta_2\gamma_2$ receptor subtype. The unpaired Student's test gives the significant differences between the control and genistein treatment data ($p<0.05$). This result further confirms our hypothesis that tyrosine residue is phosphorylated by endogenously active tyrosine kinase, and the tyrosine phosphorylation maintains the function of $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor.

Lavendustin A inhibits the recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype

As we know, genistein is not a very selective inhibitor of protein tyrosine kinase, it also inhibits other protein kinase (38). In our experiment, we want to detect the role of tyrosine kinase, thus we choose a very selective inhibitor of PTK, lavendustin A, which would not inhibit PKC or PKA activity (39). $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor is the major subtype in the brain, therefore it is very important to determine if tyrosine phosphorylation modulates the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype. We examined the effect of Lavendustin A on the response to low and high concentration of GABA as described in Methods. In the cell in Fig. 3A, The typical current curve of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor with 5 or 500 μM GABA as control shows a little run-up over time, We explain this is because tyrosine residue is phosphorylated and the phosphorylation maintains the function of the $GABA_A$ receptor. To confirm, we intracellularly applied Lavendustin A.

If the explanation is true, the current amplitude should run down over time. The recorded current amplitude responded to GABA plus Lavendustin A in fig. 3B do run down over time.

We repeat several times, accumulate the data and average the results. Fig. 4A demonstrates the average peak whole-cell currents to 5μM GABA obtained during the GABA and Lavendustin A treatment recording. Lavendustin A treatment significantly decreases the current amplitude of recombinant $\alpha_1\beta_2\gamma_2$ receptor subtype in low concentration of GABA. The unpaired Student's test gives the significant differences between the control and Lavendustin A treatment data ($p<0.05$) after 20 minutes. Fig. 4B shows the average peak whole-cell currents to 500μM GABA obtained during the control recording and Lavendustin A treatment recording. The results are similar to low concentration of GABA. The unpaired Student's test gives the significant differences between the control and Lavendustin A treatment data ($p<0.05$) after 20 minutes, either. These results confirm our hypothesis that tyrosine residue is phosphorylated by endogenously active tyrosine kinase, and the tyrosine phosphorylation maintains the function of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor.

Lavendustin A does not affect the recombinant $\alpha_1\beta_2$ $GABA_A$ receptor subtype

As we mention before, $\alpha_X\beta_2\gamma_2$ $GABA_A$ receptor subtypes are thought to be the prototype $GABA_A$ receptors in mammalian brain (15). We have tested our hypothesis that tyrosine residue is phosphorylated by endogenously PTK, and the tyrosine phosphorylation maintains the function of $GABA_A$ receptor on $\alpha_1\beta_2\gamma_2$, $\alpha_6\beta_2\gamma_2$ and previously on $\alpha_3\beta_2\gamma_2$ $GABA_A$ receptor (A paper from our lab which is in press). Now

arise the question in which subunit the tyrosine residue is phosphorylated. Previous results from our lab suggested it was not α subunit. Some reported it may be phosphorylated on β_2 subunit (31) and other reported on γ_2 subunit (29). To test the hypothesis that β_2 subunit may be phosphorylated, we study the effect of lavendustin A on receptor composed of $\alpha_1\gamma_2$; While in order to test the hypothesis that γ_2 subunit may be phosphorylated, we study the effect of lavendustin A on receptor composed of $\alpha_1\beta_2$.

Fig. 5A demonstrates the typical peak whole-cell currents of $\alpha_1\beta_2$ receptor subtype to 1μM and 100μM GABA as control. Fig. 5B shows the typical peak whole-cell currents of $\alpha_1\beta_2$ receptor subtype to 1μM and 100μM GABA obtained during the control recording and Lavendustin A treatment recording. Lavendustin A treatment does not change the current amplitude of this receptor subtype.

Fig. 6A demonstrates the average peak whole-cell currents of $\alpha_1\beta_2$ receptor to 1μM GABA obtained during the control recording and Lavendustin A treatment recording. Both the average peak whole-cell currents of control and Lavendustin A treatment keep constant over the time. The unpaired Student's test gives no differences between the control and Lavidustein A treatment data ($p>0.05$). Fig. 6B shows the normalized peak whole-cell currents to 100 μM GABA obtained during the control recording and Lavendustin A treatment recording. Both the average peak whole-cell currents of control and Lavendustin A treatment keep constant over the time. The unpaired Student's test gives no differences between the control and Lavendustin A

treatment data ($p>0.05$), either. This result suggests the function of $\alpha_1\beta_2$ $GABA_A$ receptor does not change with or without the inhibitor of PTK.

The results of these experiments are consistent with the hypothesis that the tyrosine residue which is phosphorylated by endogenously PTK is from γ_2 subunit.

We did not successfully observe the GABA-induced Cl^- currents on $\alpha_1\gamma_2$ receptor subtype. Thus we can not see the effect of lavendustin A on $\alpha_1\gamma_2$ receptor subtype.

CHAPTER IV

DISCUSSION

The function of recombinant $GABA_A$ receptor is maintained partly by endogenous Protein Tyrosine Kinase

In previous study we have characterized the rundown in the recombinant $\alpha_3\beta_2\gamma_2$ $GABA_A$ receptors stably expressed in HEK 293 cells, and we use rundown as a mark to determine the function of $GABA_A$ receptors. According to previous study in our lab, rundown is prevented by enhanced concentration of ATP in pipette solution. Previous study in our lab also suggested that $GABA_A$ receptor-mediated currents were inhibited by pipette application of genistein. In addition, another drug, daidzein, (an inactive analogue to genistein) which is structurally similar to genistein but has no effect on PTK activity, did not affect the GABA current. From all of these results, the researchers in our lab concluded that the endogenous PTKs may play an important role in maintaining the function of recombinant $GABA_A$ receptors.

There were some reports that $GABA_A$ receptors are phosphorylated by cAMP-dependent protein A, Protein kinase C, or the type II calcium/calmodulin-dependent protein kinase (25, 26, 27, 28, 29, 30). In previous study from this lab, the researchers detected no significant effect from PKA, which is consistent with stelzer et al (32). In addition, they found that PKC did not contribute to maintaining the normal function of recombinant $\alpha_3\beta_2\gamma_2$ $GABA_A$ either.

To confirm hypothesis that protein tyrosine phosphorylation maintains the function of $GABA_A$ receptor, we selected another drug, Lavendustin A. Compared to genistein, this drug is a more specific inhibitor of protein tyrosine kinase (40). The results from present study were consistent to those of genistein. Thus we strongly suggested that the function of $GABA_A$ receptor is maintained by endogenous protein tyrosine kinase.

Significance of differential phosphorylation of $GABA_A$ receptor subtypes

Pharmacological analysis of different subunit combinations expressed in recombinant systems have demonstrated that different subunits give distinct properties to the $GABA_A$ receptors (42). The α subunits and β_2, γ_2 subunits are important determinants of the pharmacology of γ-aminobutyric $acid_A$ ($GABA_A$) receptors with respect to agonists, antagonists, and modulatory compounds. (42). Thus several different functional $GABA_A$ receptors were formed when expressed α_1, α_3, or α_6 with $\beta_2\gamma_2$. In present study, we tested on recombinant $\alpha_1\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors, we observed the same results with $\alpha_3\beta_2\gamma_2$ $GABA_A$ receptor. We suggested that tyrosine phosphorylation by PTK maintained the function of $\alpha_1\beta_2\gamma_2$ and $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors.

γ_2 subunit is phosphorylated to maintain the function of $GABA_A$ receptor

Tyrosine residues exist in most of the $GABA_A$ receptor subunits. (14, 15). According to previous results in this lab and some other reports, α subunit variant is not involved in tyrosine phosphorylation. However, there were some controversal reports regarding which subunit is phosphorylated. In 1995 Moss et al (29) have reported that the γ_2 subunit is phosphorylated; While in 1997 Wan et al (31) have reported that β subunit is phosphorylated. Our present results may argue for a contribution of γ_2 subunit

phosphorylation to the functional maintaining of the receptor. This conclusion is consistent to Moss et al (1995) (29). Moss et al have reported that the γ_2, but not β, subunit that is fully accountable for the functional modulation of the receptor function by protein tyrosine phosphorylation. In contrast, Wan et al (1997) (31) have reported that β subunits are phosphorylated by protein typosine kinase and the phosphorylation maintains the function of $GABA_A$ receptor. They found genistein was capable of decreasing GABA current in HEK 293 cell stably expressing functional $GABA_A$ receptors containing the β_2 subunit. The GABA currents in cells expressing $\alpha_1\beta_2$ or $\alpha_1\beta_2\gamma_2$ all decreased with the application of genistein. We suggest Wan et al's paper in 1997 (31), which concluded β_2 subunit is tyrosine phosphorylated , is not convincing.

In the present work we have demonstrated that the GABA current in cells expressing $\alpha_1\beta_2$ receptor does not change with the pipette application of Lavendustin A. As we mentioned before, genistein is not a very selective inhibitor of PTK, it also inhibits other kinases (39). In conpasion, Lavendustin A is a high selective inhibitor of PTK, it only specifically inhibits PTK (40). This is the major different from Wan's paper in 1997 (31). We explain there is phosphorylation on β_2 subunit. The phosphorylation on β_2 subunit can be inhibited by genistein but not by lavidustein A. Thus we suggest it is not tyrosine residue which is phosphorylated on β_2 subunit. From our data, we strongly suggest that γ_2 subunit is phosphorylated.

From our data, moreover, we also try on cells expressing $\alpha_1\gamma_2$ receptor subtype, which is lack of β_2, and we observe no GABA current is produced. This is in agreement

with that of Moss et al (1995) (29) have reported that both β and γ_2 subunits are tyrosine phosphorylated in cells cotransfected with the v-scr cDNA, however, phosphorylation of the γ_2 subunits alone affects receptor function. (29). In our present study, we strongly support the importance of γ_2 subunits in the maintaining of the function of recombinant $GABA_A$ receptor. β_2 is phosphorylated but not tyrosine phosphorylated to modulate the function of the $GABA_A$ receptor simply because that GABA current from cells expressing $\alpha_1\beta_2$ was unchanged with or without Lavendustin.

In conclusion, our results indicate that tyrosine phosphorylation is an important modulater of $GABA_A$ receptor function. In additon, we demonstrate the effect is due to phosphorylation at the γ_2 subunits. Our results support the hypothesis that the subunit itself is phosphorylated. However, we can not rule out the possibilities of an associate protein that requites the presence of γ_2 to elicit its effect. Also, we do not know how exactly the tyrosine phosphorylation regulates $GABA_A$ receptor function. Tyrosine phosphorylation makes the $GABA_A$ receptor more functional. It may increase the channel opening time, or make the channel easier to open, in other words, the number of the channel open per time increases, or the channel conductance may be enhanced by phosphorylation. To reveal how exactly the tyrosine phosphorylation acts, it needs additional investigation.

CHAPTER V

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Figure Legends

Figure 1. GABA concentration-response curve during whole-cell recording with 4mM ATP and 10 mM EGTA in the pipette solution for recombinant $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors. ***A***, raw GABA dose-response curves for $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors. ***B***, Normalized GABA dose-response curve for $\alpha_6\beta_2\gamma_2$, $\alpha_1\beta_2\gamma_2$ and $\alpha_1\beta_2$ receptors. EC_{50} for $\alpha_6\beta_2\gamma_2$ receptor is 1.6 μM (n=1.5); EC_{50} for $\alpha_1\beta_2\gamma_2$ receptor is 20.7 μM (n=1.4); and EC_{50} for $\alpha_1\beta_2$ is 20 μM(n=1.4).

Figure 2. Prevention of PTK-induced phosphorylation on $\alpha_6\beta_2\gamma_2$ causes rundown of GABA response during whole-cell patch recording. ***A,*** Typical current traces of $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptor subtype to 1 μM GABA recorded at 5 min interval after formation of a stable whole-cell patch with modified pipette solution or pipette solution plus genistein (5μM), an inhibitor of PTK. ***B,*** Mean responses to 1μM GABA over 30 min using whole-cell patch recording technique (n=5). Compare the difference with t-test, all have significance at 20, 25, and 30 minutes (all $p<0.05$).

Figure 3. Prevention of PTK-induced phosphorylation on recombinant $\alpha_1\beta_2\gamma_2$ causes rundown of GABA response during whole-cell patch recording. ***A***, Typical current traces of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype responded to 5 and 500 μM GABA recorded at 10 min interval after formation of a stable whole-cell patch with modified pipette solution; ***B***, Typical current traces of $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor subtype to 5 and 500 μM GABA recorded at 10 min interval after formation of a stable whole-cell patch with modified pipette plus Lavendustin A (5μM), a specific inhibitor of PTK.

Figure 4. Mean results for experiment illustrated in fig. 3 ***A*** Mean responses to 5μM and 500 μM GABA over 30 min using whole-cell patch recording technique (n=5) with modified pipette solution. ***B***, Mean responses to 5μM and 500 μM GABA over 30 min using whole-cell patch recording technique (n=6) with modified pipette solution plus Lavendustin A. * means significance ($p<0.05$).

Figure 5. Effect of Lavendustin A on recombinant $\alpha_1\beta_2$ to GABA response during whole-cell patch recording ***A***, Typical current traces of $\alpha_1\beta_2$ $GABA_A$ receptor subtype to 1 and 100 μM GABA recorded at 10 min interval after formation of a stable whole-cell patch with modified pipette solution; ***B***, Typical current traces of $\alpha_1\beta_2$ $GABA_A$ receptor subtype to 1 and 100 μM GABA recorded at 10 min interval after formation of a stable whole-cell patch with modified pipette plus Lavendustin A, a specific inhibitor of PTK.

Figure 6. Mean results for experiment illustrated in fig. 5, Mean responses to 1μM and 100 μM GABA over 30 min using whole-cell patch recording technique (n=5) with modified pipette solution. ***B***, Mean responses to 1μM and 100 μM GABA over 30 min using whole-cell patch recording technique (n=5) with modified pipette solution plus Lavendustin A.

Fig. 1

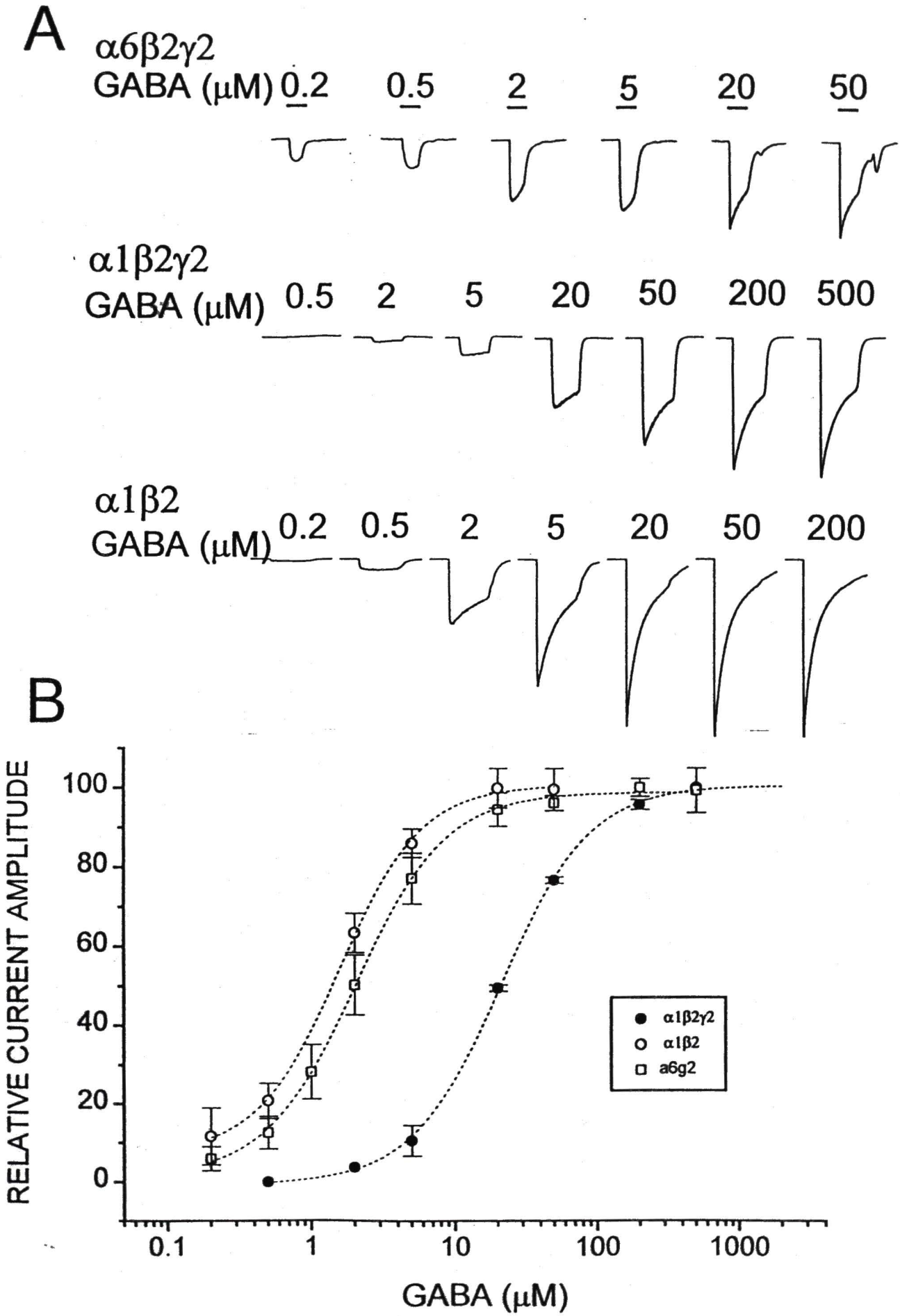
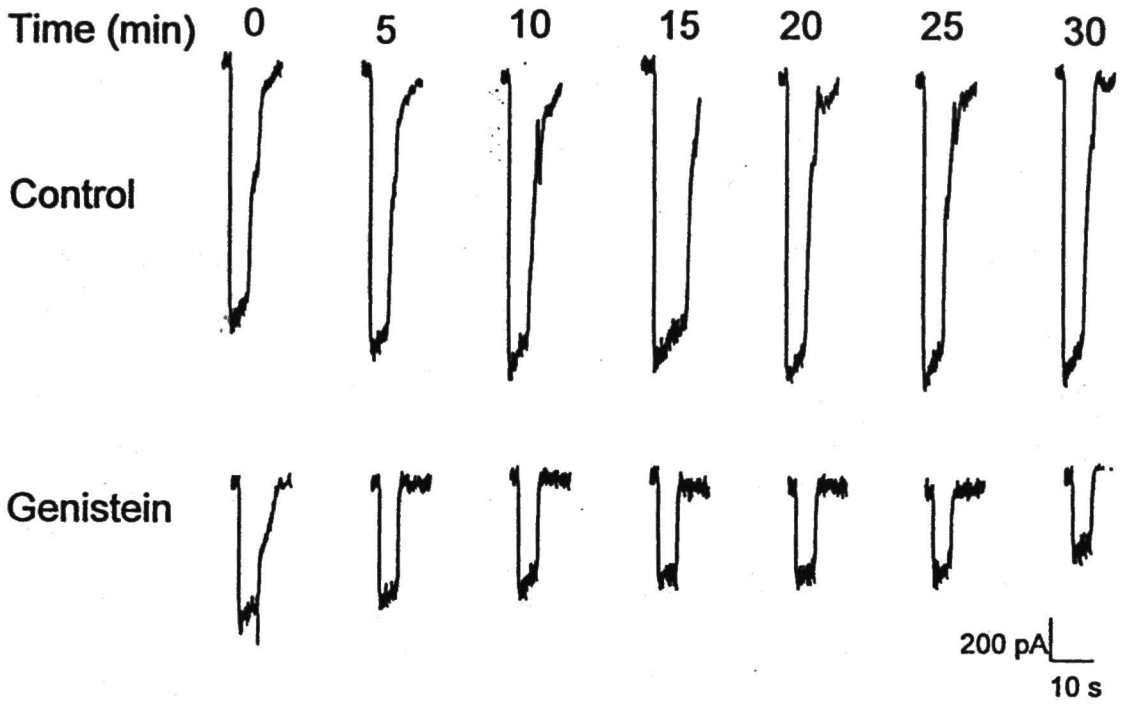
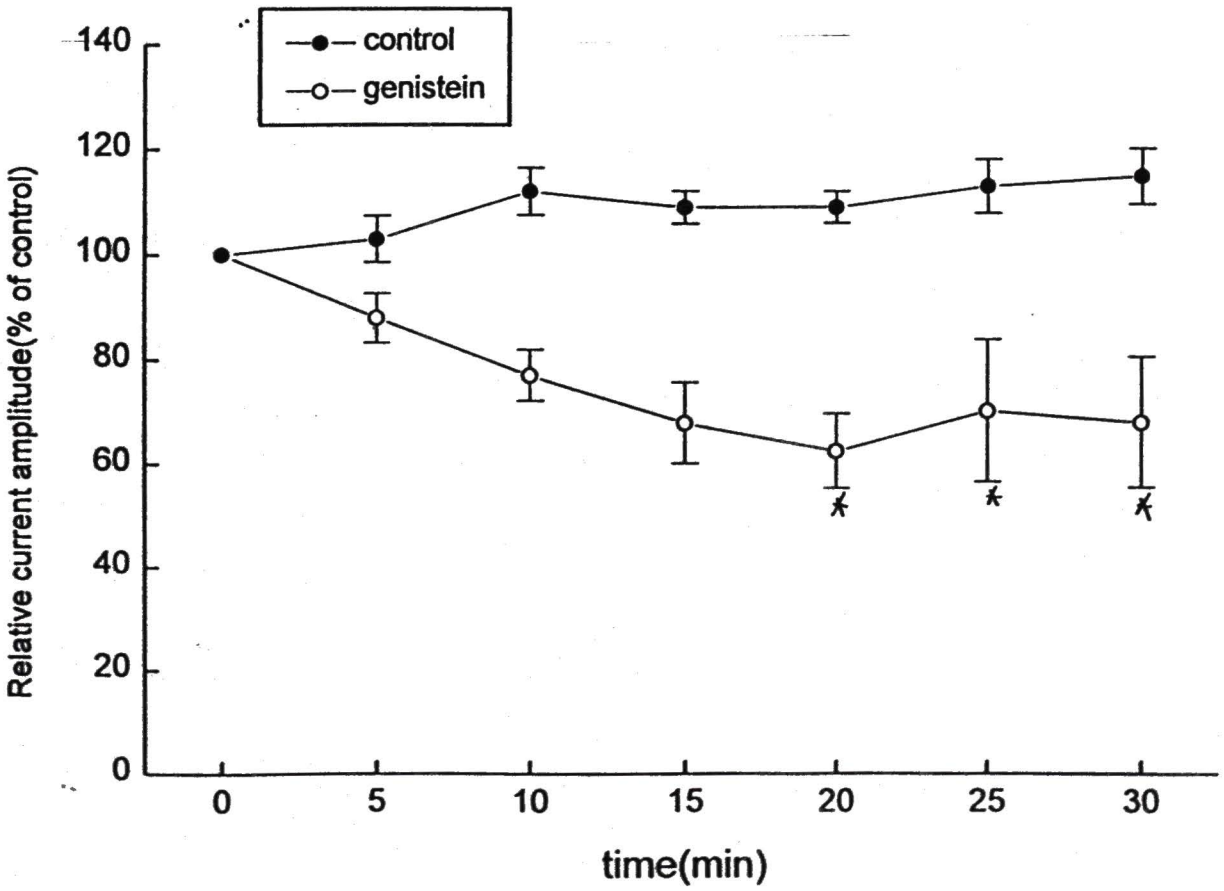

A
α6β2γ2
GABA (μM) 0.2 0.5 2 5 20 50
α1β2γ2
GABA (μM) 0.5 2 5 20 50 200 500
α1β2
GABA (μM) 0.2 0.5 2 5 20 50 200
B
RELATIVE CURRENT AMPLITUDE
100
80
60
40
20
0
0.1
1
10
100
1000
GABA (μM)
α1β2γ2
α1β2
a6g2

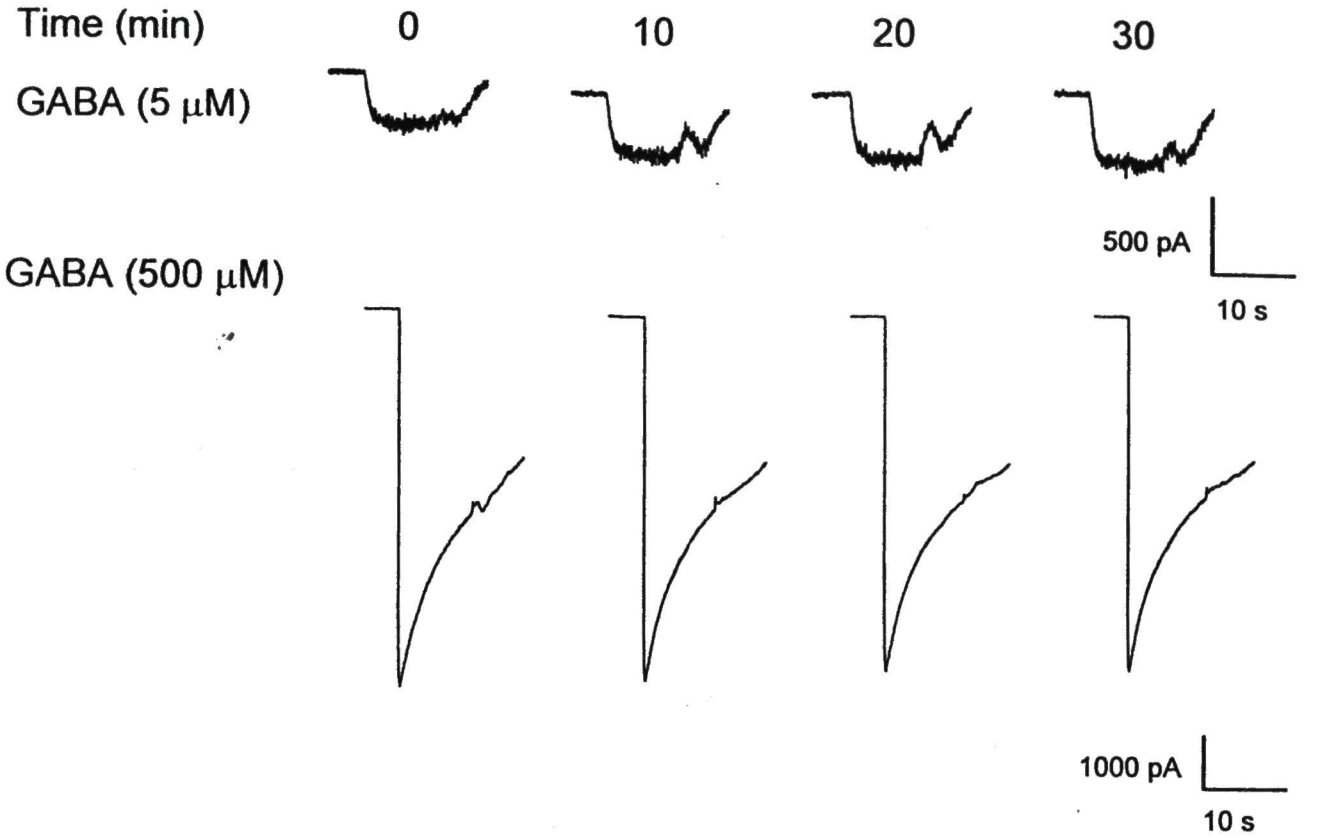
Fig. 2


Time (min)
0
5
10
15
20
25
30
Control
Genistein
200 pA
10 s


control
genistein
Relative current amplitude(% of control)
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
time(min)

α1β2γ2

Control



Lavendustin A

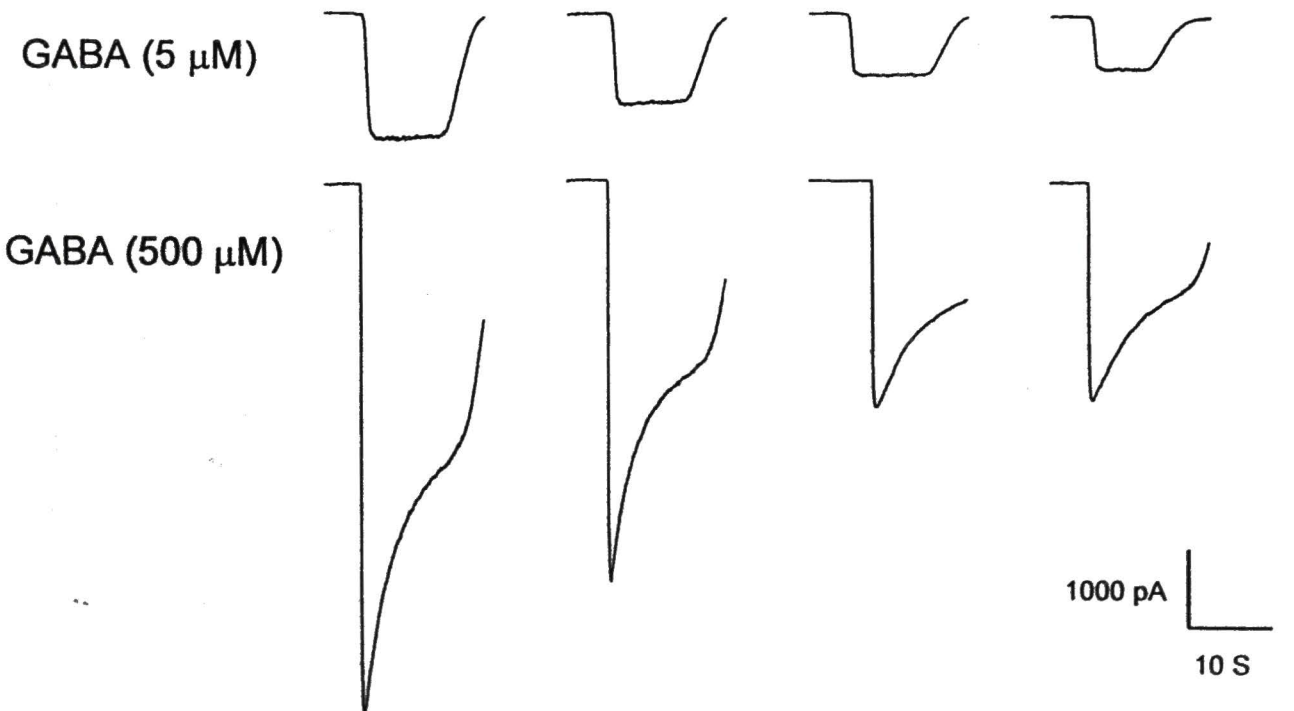
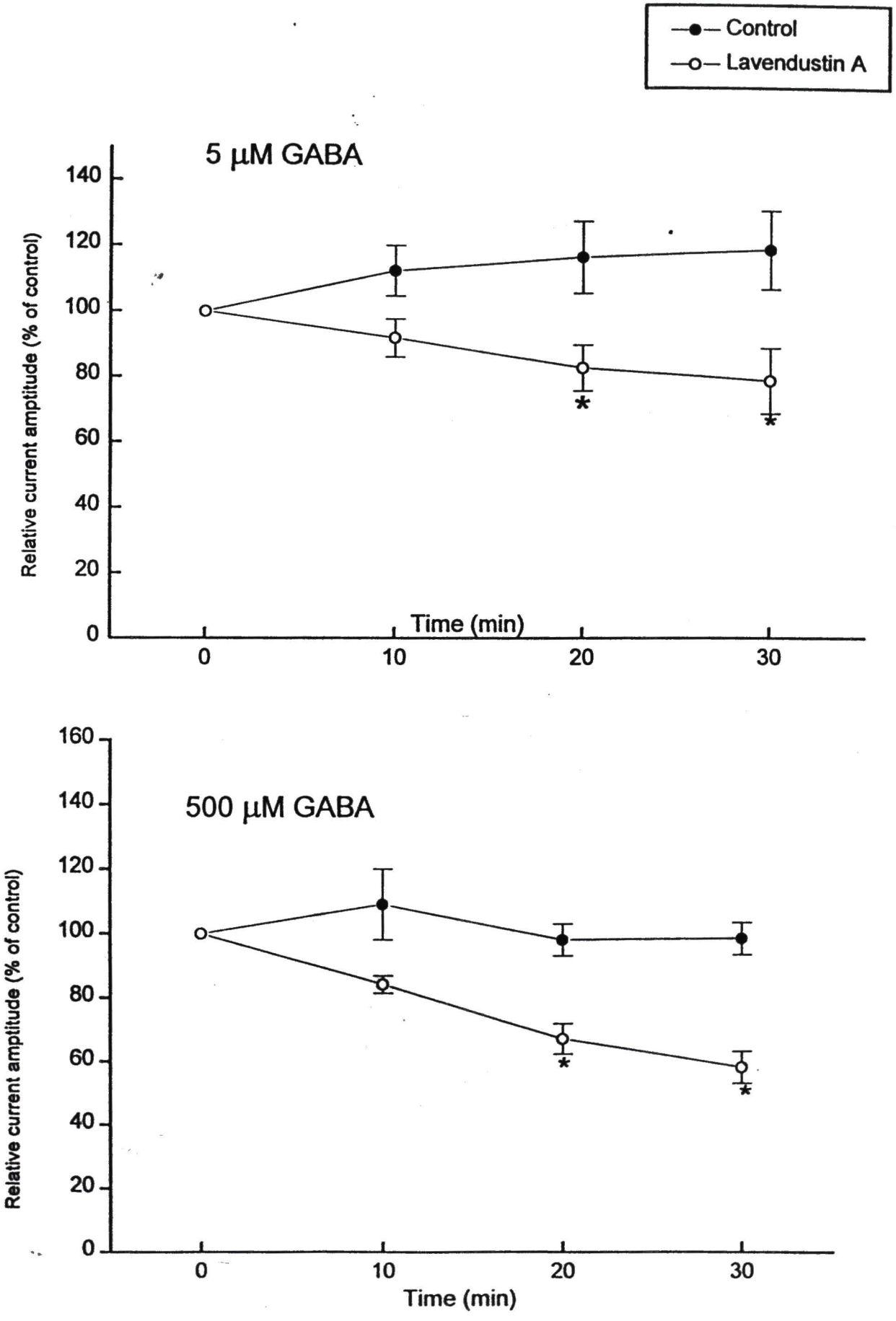


Fig.4


Control
Lavendustin A
5 μM GABA
Relative current amplitude (% of control)
140
120
100
80
60
40
20
0
Time (min)
0
10
20
30
*
*
500 μM GABA
Relative current amplitude (% of control)
160
140
120
100
80
60
40
20
0
Time (min)
0
10
20
30
*
*

α1β2

Fig. 5

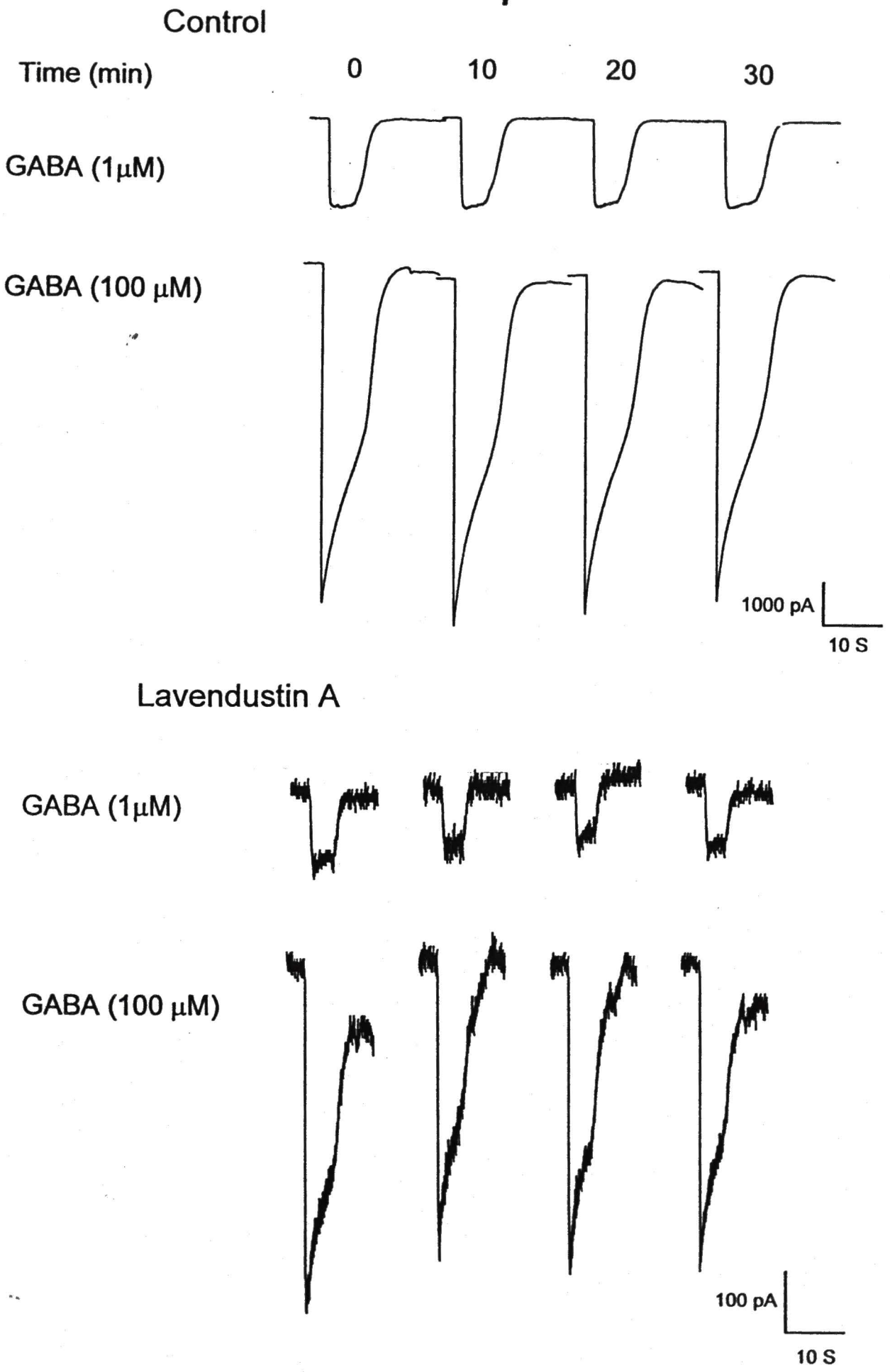
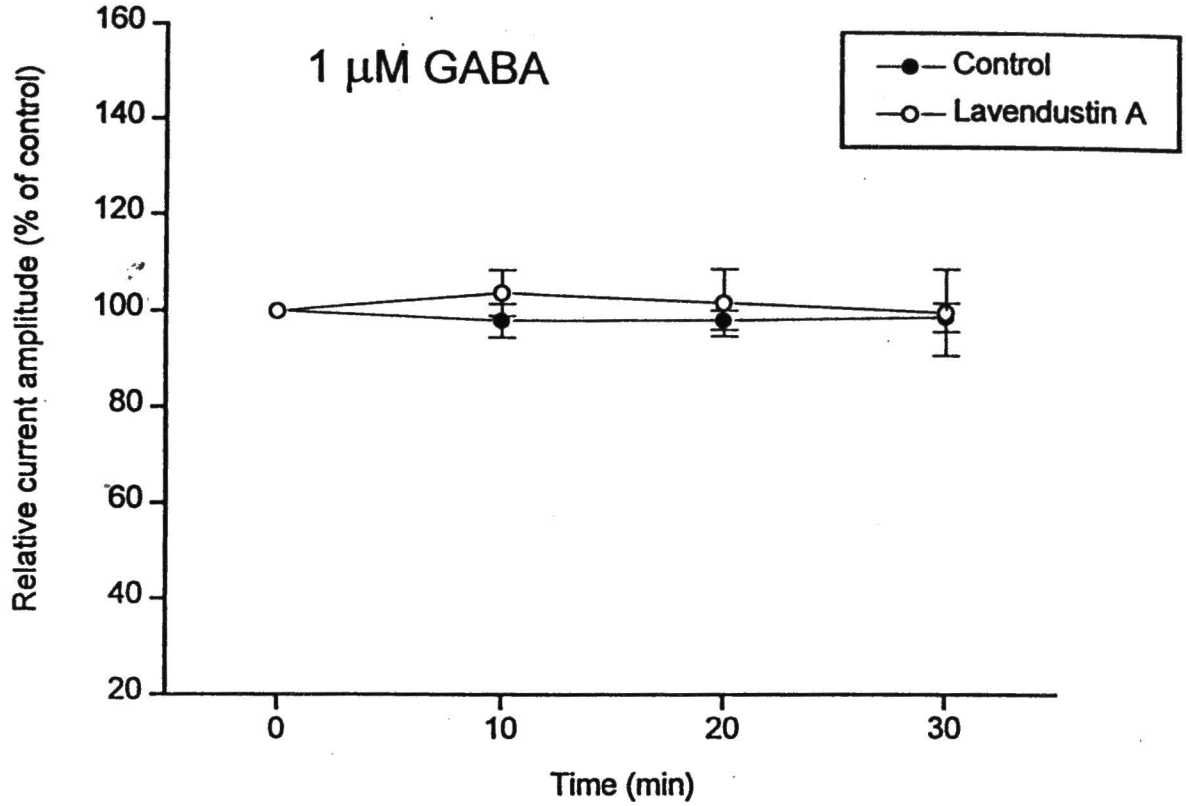
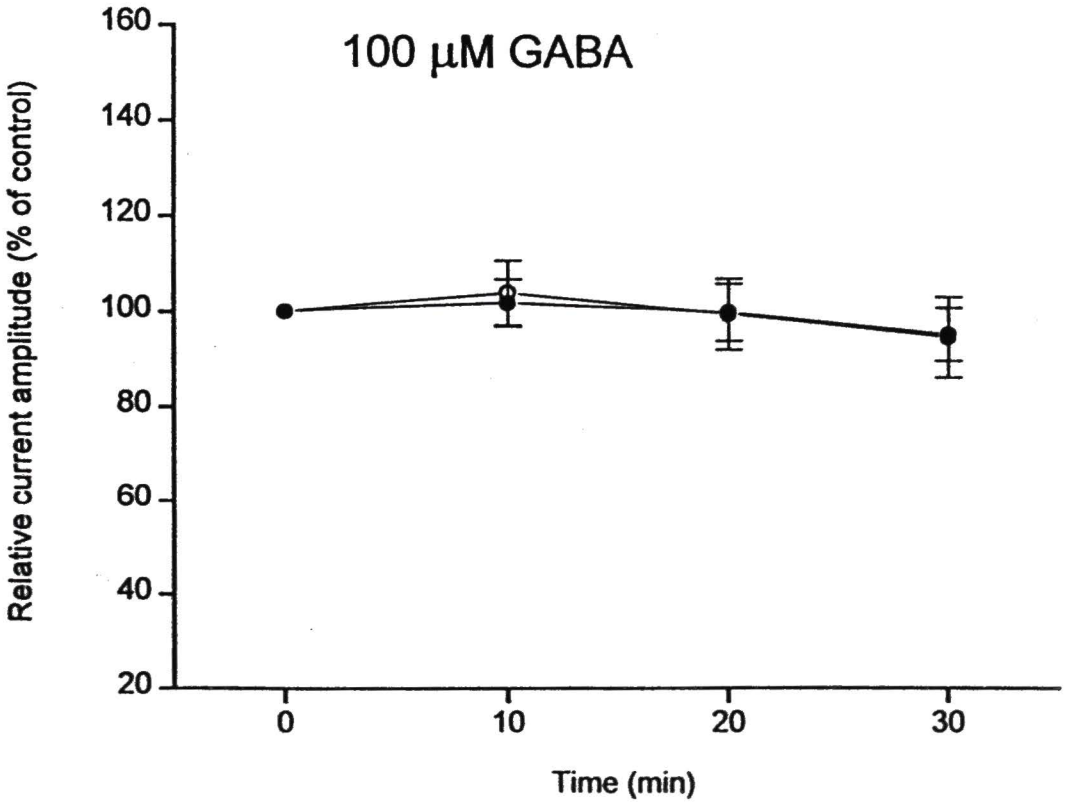

Control
Time (min)
0
10
20
30
GABA (1μM)
GABA (100 μM)
1000 pA
10 S
Lavendustin A
GABA (1μM)
GABA (100 μM)
100 pA
10 S

Fig. 6


1 μM GABA
Control
Lavendustin A
Relative current amplitude (% of control)
160
140
120
100
80
60
40
20
0
10
20
30
Time (min)


100 μM GABA
Relative current amplitude (% of control)
160
140
120
100
80
60
40
20
0
10
20
30
Time (min)