# GABAergic Antagonists Block the Inhibitory Effects of Serotonin in the Lateral Amygdala: A Mechanism for Modulation of Sensory Inputs Related to Fear Conditioning

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Neurons in the lateral amygdala (LA) receive glutamatergic sensory input from the auditory thalamus and auditory cortex, and these inputs can be modulated by serotonin (5-HT). In the present study, we examined whether serotonergic inhibition of glutamatatergic excitation in the LA occurs via activation of GABAergic interneurons. Single-unit extracellular activity in the LA was recorded in response to iontophoretically applied glutamate. Concurrent application of 5-HT reduced the number of glutamate-evoked action potentials in the majority of neurons tested. GABA antagonists were then iontophoresed with both glutamate and 5-HT. Of the neurons that were inhibited by 5-HT, concurrent application of the GABA antagonists significantly reversed this effect. Application of the GABA antagonists alone had little or no effect on basal neuronal activity. We conclude that the 5-HT-induced inhibition of glutamatergic activity occurs in part through activation of serotonergic receptors on GABAergic interneurons.

*Key words: electrophysiology; iontophoresis; rat; glutamate; anxiety; sensory modulation*

Fear conditioning is widely used as a model system for understanding how the brain forms and stores information about aversive emotional experiences. Research on the neural basis of fear conditioning points to the amygdala as a key interface between harmful stimuli in the environment and motor systems that produce defense responses (Davis et al., 1994; Fanselow, 1994; LeDoux, 1994). Sensory information about stimuli that are harmful enter the amygdala by way of the lateral nucleus (LA) (LeDoux et al., 1990). The LA, in turn, projects to other amygdala areas (Pitkanen et al., 1997), which control defense responses (see Davis, 1992).

The LA receives dense serotonergic inputs from the dorsal raphe (Sadikot and Parent, 1990); several serotonin (5-HT) receptor subtypes are present in the LA (Radja et al., 1991); and 5-HT levels increase in the LA during amygdala-dependent processes such as fear conditioning (Inoue et al., 1993; Kawahara et al., 1993). Given that serotonin is known to be a widespread modulator of neuronal activity, we recently examined the contribution of serotonin to the modulation of sensory processing within the LA (Stutzmann et al., 1998). Iontophoresis of 5-HT inhibited the excitation elicited by concurrent iontophoresis of glutamate or by electrical stimulation of glutamatergic inputs from the auditory thalamus or auditory cortex. 5-HT-induced inhibition of synaptic activity could be attributable to a direct inhibitory effect of 5-HT on excitatory neurons postsynaptic to the sensory afferents or could be attributable to activation of GABAergic cells through excitatory 5-HT receptors in the LA, which then inhibit excitatory cells. In support of the latter possibility is the observation that 5-$HT_3$ and possibly 5-$HT_2$ receptor subtypes are present on GABA-containing cells in the LA (Sheldon and Aghajanian, 1990; Radja et al., 1991; Morales and Bloom, 1997). In this study, we used microiontophoresis to locally apply drugs very close to the recording site to examine the role of GABA interneurons in serotonin-induced inhibition of glutamate activity. If 5-HT inhibits glutamatergic excitation by way of GABA interneurons, blockade of GABA receptors should eliminate the inhibition by 5-HT.

## MATERIALS AND METHODS

Experiments were conducted on male Sprague Dawley rats weighing 250–375 gm. Animals were housed on an alternating 12 hr light/dark cycle (lights on at 7 A.M., lights off at 7 P.M.). Procedures were performed in accordance with National Institutes of Health guidelines.

Rats were anesthetized with urethane (1.6 mg/kg) and placed in a stereotaxic frame. The cranium above the LA, medial nucleus of the medial geniculate/posterior–intralaminar nucleus (MGm/PIN), and secondary auditory cortex (TE3) regions were exposed, and the dura was retracted. Electrodes were then placed into the LA using a hydraulic microdrive, and bipolar stimulating electrodes were manually lowered into the MGm/PIN region [inserted at a 10° angle in the anteroposterior (AP) plane] and the TE3 region (inserted in a 20° angle in the AP plane).

As an additional physiological measure to confirm the location of the recording electrode, sensory afferents known to project to the LA were stimulated (Romanski and LeDoux, 1993), and evoked action potentials were recorded. The TE3 and MGm/PIN regions were stimulated with

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single pulses (100–800 μA, 0.3 Hz, 300 μsec duration) from a Grass Instruments (Quincy, MA) 88 constant current stimulator delivered through a bipolar stimulating electrode ($r$ = 30–40 KΩ). Electrode stereotaxic coordinates from the interaural line (in millimeters) were as follows: recording electrode, AP, 5.8–6.2; mediolateral (ML), 5.2; and dorsoventral (DV), 6.0–7.0; MGm/PIN stimulating electrode, AP, 3.8; ML, 3.0; and DV, 6.1; and TE3 stimulating electrode, AP, 4.0; ML, 6.8; and DV 3.3 (Paxinos and Watson, 1986).

Single-unit recordings were obtained from glass micropipettes (1–3 μm tip diameter, 10–20 MΩ impedance) filled with 2.5% Pontamine sky blue in 0.5 M sodium acetate. Single-unit activity was amplified, filtered, and discriminated. Undiscriminated output was viewed on a Tektronix (Wilsonville, OR) storage oscilloscope, and discriminated output was digitized for the construction of poststimulus histograms using a Cambridge Electronic Design (Cambridge, UK) 1401 computer interface.

The technique of microiontophoresis was used as a way to effectively deliver various compounds within the immediate area of the recording site. With this technique, only very small quantities of drug are released from the electrode tip via current ejection. This decreases the possibility that compounds will diffuse far from the neuron recorded from or into neighboring nuclei (as is seen with local infusion methods) but does not allow for accurate dose–response analysis. Microiontophoresis was accomplished by gluing a five-barrel micropipette (10–20 μm tip diameter) adjacent to the single-barrel recording electrode with a light curing dental fixative (Silux, 3M, St. Paul, MN). The tip of the recording pipette extended 15–35 μm beyond the tip of the iontophoretic pipette. The center barrel was filled with 0.9% saline for automatic current balancing. The remaining barrels were filled with glutamate (L-glutamic acid, 10 mM, pH 8.0, −5.0 to −30 nA ejection current, 10 nA holding current), serotonin (serotonin creatinine sulfate, 20 mM, pH 4.0, 10–80 nA ejection current, −10 nA holding current), bicuculline methiodide (5 mM, pH 3.5, 20–80 nA ejection current), and 2-OH-saclofen (20 mM, pH 3.5, 20–80 nA ejection current). Bicuculline methiodide and 2-OH-saclofen were placed in the same electrode barrel. Drugs were purchased from Research Biochemicals (Natick, MA).

Placements of recording sites were marked by iontophoretically depositing Pontamine sky blue or locating the electrode tracks in the LA. Animals were perfused with 10% formalin and post-fixed, and brains were cut on a sliding microtome into 50-μm-thick sections. Sections were Nissl-stained and coverslipped, and the location of the dye spot or electrode tracks was determined under light microscopic examination.

## RESULTS

Neurons in the LA were either silent or had low firing rates (<1 Hz), as is typical for this region (Clugnet et al., 1990). LA neurons were therefore identified via synaptic stimulation from the MGm/PIN and TE3. Iontophoretic application of glutamate was then used to evoke action potentials in these LA neurons that were postsynaptic to the MGm/PIN and/or TE3. A representative distribution of the recording sites and relevant amygdala nuclei are shown in a Gisma-stained coronal section in Figure 1.

The effects of iontophoretically applied 5-HT were tested on neurons activated via glutamate application. After accumulating spikes during this control period, 5-HT was concurrently applied during these conditions, and the total number of spikes was again analyzed and compared with the control period. GABA antagonists were then iontophoresed in conjunction with glutamate and 5-HT, and the number of spikes evoked was analyzed during this phase and compared with the original firing rates during glutamate alone and the presence of 5-HT and glutamate together.

Of the 37 neurons activated by glutamate, 32 were inhibited by the concurrent application of 5-HT. The criterion for inhibition was set at 33% reduction in the number of spikes relative to before 5-HT application. In the majority of these neurons, the inhibitory effects of 5-HT were attenuated by the coadministration of GABAergic antagonists, whereas six neurons had no response. Representative histograms demonstrating typical responses of single neurons are shown in Figure 2. Figure 3 shows the group data for the average number of spikes evoked during each drug condition over a 1 min period. A one-way ANOVA demonstrates a significant difference in the number of spikes evoked among drug application groups ($F_{(2)} = 6.14$; $p < 0.01$). Newman–Keuls *post hoc* analysis demonstrated that the 5-HT group differs significantly from the glutamate ($p < 0.01$) and glutamate–5-HT–GABA antagonist group ($p < 0.01$), whereas the glutamate and glutamate–5-HT–GABA antagonist groups are not significantly different.

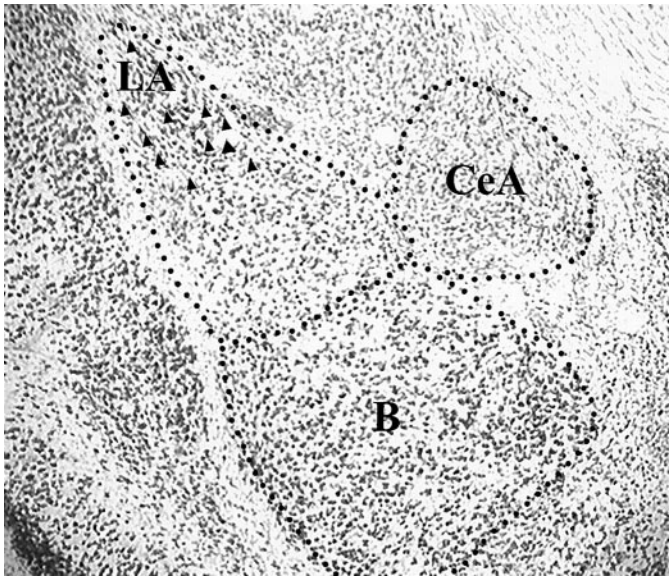


*Figure 1.* Gisma-stained coronal section of the rat brain, showing the region containing the lateral amygdala and representative electrode recording sites. Also outlined are the basal (*B*) and central nuclei of the amygdala (*CeA*).

In several of the neurons that showed a reversal of 5-HT inhibition, glutamate and the GABA antagonists were iontophoresed together to ensure that blockade of the GABA receptors was not enhancing excitatory activity on its own ($n$ = 11). A two-tailed paired $t$ test indicates these conditions are not significantly different ($t_{(10)} = 1.18$; $p > 0.05$). In several of these neurons, the GABA antagonists were iontophoresed alone ($n$ = 13) to ensure that spontaneous activity was not affected. Consistent with the results of Li et al. (1996), there was no change from the normally quiescent state of these neurons with blockade of the GABA receptors.

## DISCUSSION

The purpose of this study was to examine interactions among glutamatergic sensory afferents, GABAergic interneurons, and serotonergic inputs to LA cells. The results suggest that glutamatergic inputs to the LA can be modulated by 5-HT through activation of GABAergic interneurons. 5-HT, when locally applied to glutamate-activated neurons, inhibits this excitatory input, and blockade of GABA receptors reverses this effect in many of the neurons tested. This modulation can potentially have significant effects on the overall flow of information conducted through the amygdala and thereby influence the ability of the amygdala to control autonomic, endocrine, and behavioral responses normally elicited by threatening stimuli.

### GABA antagonists reverse the inhibitory effects of 5-HT

The amygdala is highly modulated by GABA. It contains both $GABA_A$ and $GABA_B$ receptors (Bowery et al., 1987), GABAer-

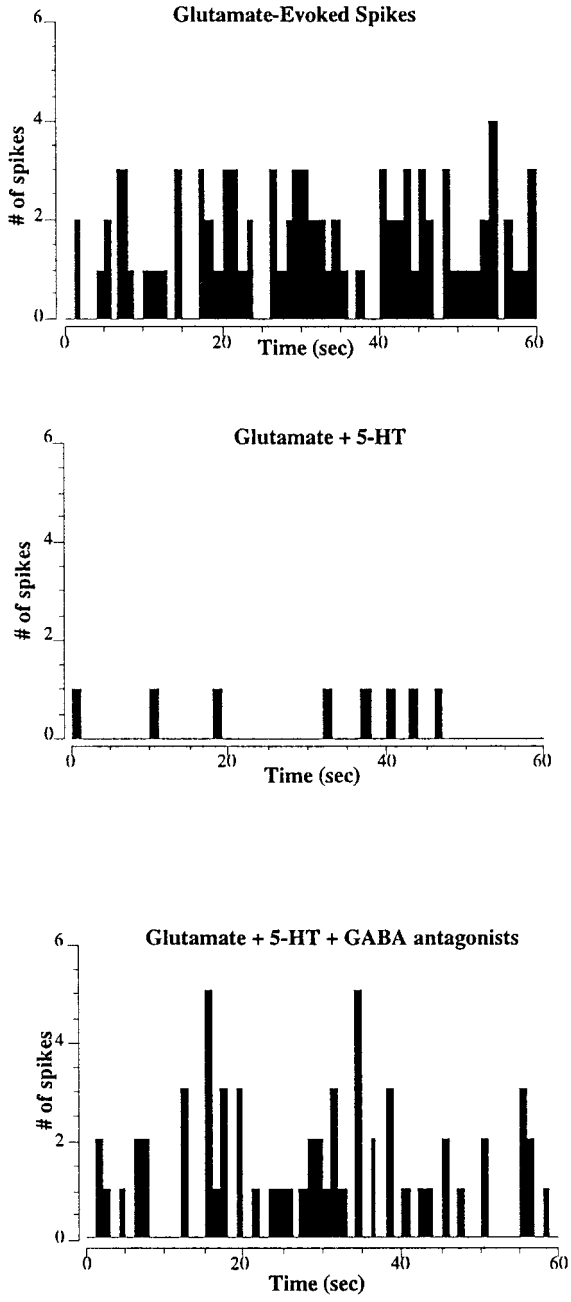


*Figure 2.* Time histograms of glutamate-evoked spikes over a 1 min period, inhibition by 5-HT when released with glutamate, and the reversal of this effect with the addition of GABA antagonists.

gic interneurons (McDonald, 1985) and 5-HT receptors anatomically and physiologically involved in GABAergic activation (Gellman and Aghajanian, 1994; Morales and Bloom, 1997). Previous work has demonstrated that glutamate activation of LA neurons can be inhibited by local serotonin release and that this depends on adrenal glucocorticoid hormones (Stutzmann et al., 1998). The present study implicates GABAergic neurons in this inhibition. Iontophoretic application of GABA antagonists attenuated the inhibitory effects of 5-HT, returning glutamate-evoked neuronal activity to within baseline levels in many LA neurons. It should be noted that other regulatory mechanisms are likely used in the LA, because not all neurons inhibited by 5-HT were reversed by GABA blockade.

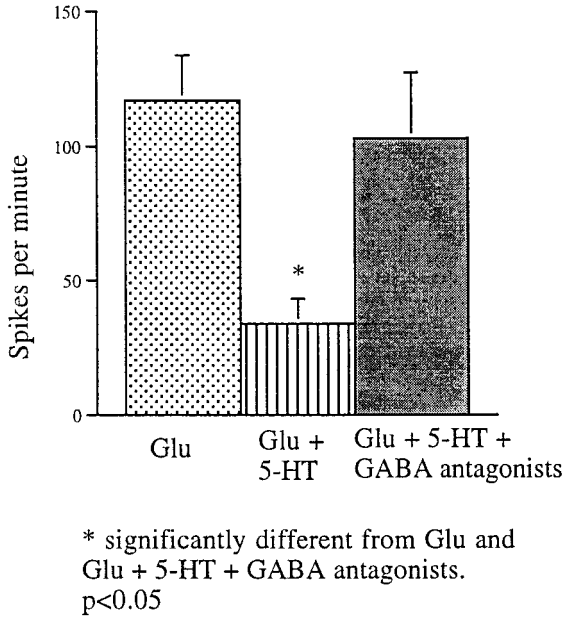


*Figure 3.* Bar graph demonstrating number of spikes evoked during various iontophoretically applied compounds. Glutamate (*Glu*) evoked action potentials in normally quiescent lateral amygdala neurons, and coapplication of 5-HT inhibited this effect. The additional application of $GABA_A$ and $GABA_B$ antagonists reversed the inhibitory effects of 5-HT.

## 5-HT/GABAergic modulation in other brain regions

Several recent studies examined 5-HT modulation of glutamate transmission elsewhere in the brain and have proposed a similar mechanism for the modulation of sensory input to the LA. The hippocampus has been examined in detail. Median raphe serotonergic neurons heavily innervate GABAergic interneurons in the CA1 region of the hippocampus (Moore and Halaris, 1975), and in these GABAergic neurons, serotonin facilitates inhibitory transmission through activation of the $5\text{-}HT_3$ receptor subtype, suppressing the firing of CA1 pyramidal cells (Ropert and Guy, 1991). In addition, neurons of the cerebellar nuclei receive a serotonergic input, which suppresses excitatory amino acid-induced activity, as well as potentiating the inhibitory effects of GABA (Kitzman and Bishop, 1997).

## Proposed circuitry model

The results of this study suggest that excitatory serotonin receptors activate GABAergic neurons, which locally regulate LA neurons receiving glutamatergic sensory afferents. A schematic of the proposed connections is presented in Figure 4. Stimuli (including environmental stressors) that increase 5-HT levels in the amygdala would activate 5-HT receptors on GABAergic neurons, facilitating GABAergic release onto LA projection neurons.

## Clinical implications

Dysfunctions in the 5-HT system have been linked to multiple anxiety and stress disorders (Eison and Eison, 1994), and this connection is consistent with malfunctioning of serotonergic modulation in the amygdala (Gargiulo et al., 1996; Liang, 1998). Decreased serotonergic functioning might result in deficient GABAergic modulation of excitatory sensory afferents, perhaps allowing innocuous sensory signals to be processed through the LA as emotionally stimulating events. Overall, the net effect of 5-HT acting through GABAergic mechanisms in the LA appears

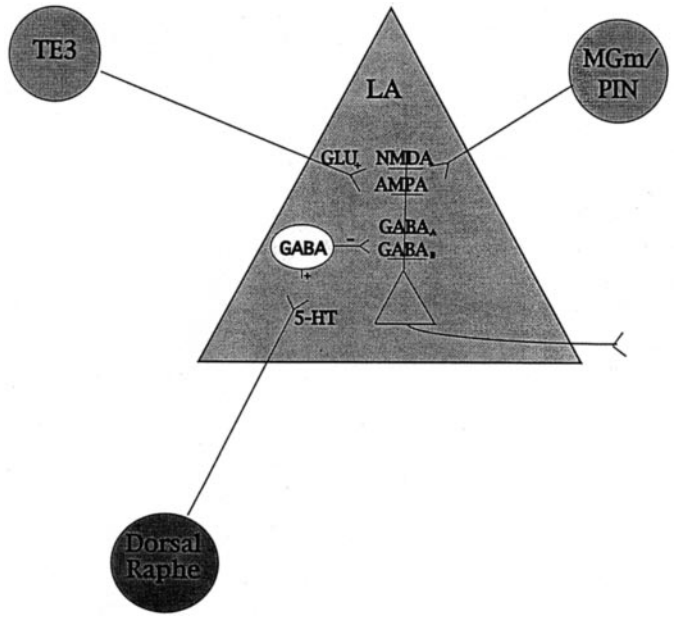


*Figure 4.* Schematic illustrating possible modulatory mechanisms of sensory input to the LA. Glutamatergic auditory afferents impinge on LA projections neurons containing both glutamate and GABA receptors and GABAergic interneurons. Interneurons containing the 5-$HT_3$ or 5-$HT_2$ receptor receive a serotonergic input from the dorsal raphe nucleus. Activation of these excitatory receptors increases GABA release onto projection neurons, inhibiting activation from sensory afferents. The net effect is a reduction in sensory transmission to the central nucleus.

to be inhibitory and may therefore serve as a modulator of affective sensory processing.

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