Extracellular Signal-Regulated Kinases Mediate an Autoregulation of GABA_B-Receptor-Activated Whole-Cell Current in Locus Coeruleus Neurons

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The norepinephrine-releasing neurons in the locus coeruleus (LC) are well known to regulate wakefulness/arousal. They display active firing during wakefulness and a decreased discharge rate during sleep. We have previously reported that LC neurons express large numbers of GABA_B receptors (GABA_BRs) located at peri-/extrasynaptic sites and are subject to tonic inhibition due to the continuous activation of GABA_BRs by ambient GABA, which is significantly higher during sleep than during wakefulness. In this study, we further showed using western blot analysis that the activation of GABA_BRs with baclofen could increase the level of phosphorylated extracellular signal-regulated kinase 1 (ERK1) in LC tissue. Recordings from LC neurons in brain slices showed that the inhibition of ERK1/2 with U0126 and FR180204 accelerated the decay of whole-cell membrane current induced by prolonged baclofen application. In addition, the inhibition of ERK1/2 also increased spontaneous firing and reduced tonic inhibition of LC neurons after prolonged exposure to baclofen. These results suggest a new role of GABA_BRs in mediating ERK1-dependent autoregulation of the stability of GABA_B-activated whole-cell current, in addition to its well-known effect on gated potassium channels, to cause a tonic current in LC neurons.

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the forebrain. By acting at ionotropic GABA_A receptors (GABA_ARs) located within the synaptic acting zone, GABA can rapidly increase the membrane permeability to Cl− in target neurons and produce fast phasic inhibitory transmission. This type of signaling is referred to as conventional synaptic transmission and features a specific method of communication between neurons with high temporal and spatial precision that enables the presynaptic neuron to shape the spiking pattern of the postsynaptic neuron. In addition to those located in the synaptic active zone, GABA_ARs containing specific subunits can also mediate a tonic form of inhibition that is not time-locked to presynaptic action potentials (APs) and is shown to profoundly modulate the input–output relationships of individual neurons. GABA_A-mediated tonic inhibition has been identified as an important player in both physiological and pathophysiological processes1,2.

In addition to GABA_ARs, GABA also acts on metabotropic GABA_B receptors (GABA_BRs) to produce a much slower but very long-lasting inhibition at both presynaptic and postsynaptic sites3–5 compared with the fast phasic transmission mediated by GABA_ARs. At the presynaptic site, the activation of GABA_BRs reduces the release probability of synaptic vesicles through inhibiting N-type or P/Q-type voltage-dependent Ca2+ channels; at the postsynaptic site, the activation of GABA_BRs produces hyperpolarization by increasing the potassium conductance of G protein-coupled inwardly rectifying K+ (GIRK) or inwardly rectifying K+3 (Kir3) channels8–10. GABA_BRs were the first G protein-coupled receptor (GPCR) to be identified as an obligate heterodimer; a functional GABA_B

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receptor is formed from the heterodimerization of the GABAB1 and GABAB2 receptor subunits, with the
former constituting the GABA binding site and the latter being coupled to the Gproteins, comprising α5, β and γ
subunits11–13. The binding of GABA to the GABAB1 receptor activates the coupled G protein to gate the pre-
and postsynaptic ion channels described above via the β and γ subunits11–13. Despite the well-understood functional
roles of the β and γ subunits, much remains to be learned about the role of receptor-induced lowering of CAMP
levels by the α subunit.

Electrophysiological studies have revealed that the subcellular distribution of GABA receptors is mostly at peri-
extrasynaptic loci45–47, implying that, similar to GABA receptors, these extrasynaptic GABA receptors can mediate a tonic form
of signaling by detecting ambient GABA. Indeed, it has been shown that ambient GABA can tonically induce a
low level of presynaptic and postsynaptic GABA receptor activation to provide the control of transmitter release at the
hippocampus and calyx of Held synapses and the control of the excitability of pyramidal neurons in the medial
prefrontal cortex and noradrenergic (NAergic) neurons in the locus coeruleus (LC)7,9,14–16. The physiological roles
of GABA receptor-mediated tonic inhibition have begun to emerge. Recently, it has been shown that tonic inhibition
of LC NAergic neurons (hereafter referred to as LC neurons) could be an important player in the regulation of
brain function states7,17. LC neurons have global NAergic projections to the forebrain and play important roles
in the control of behaviors through the regulation of vigilance18,19. Furthermore, GABAergic transmission in the
LC has been implied to be a mechanism underlying the effect of some anesthetics on consciousness20–24. It has
previously been shown that LC neurons and NAergic A7 neurons in the pons express a large amount of GABA receptors and are
subject to GABA receptor-mediated tonic inhibition in brain slice preparations and in vivo25–27. Moreover, the suppres-
sion of the tonic inhibition of LC neurons could accelerate the regain of consciousness from isoflurane-induced
deep anesthesia27. Tonic inhibition would require the activity of a substantial number of GABA receptors on the mem-
brane for a long period. Nevertheless, this would appear to conflict with the features of most GPCRs, including
GABA receptors, such that the receptor will undergo rapid desensitization upon activation by the ligand28. In this study, we
report that, in LC neurons, the activation of GABA receptors also activates extracellular signal-regulated kinase 1
(ERK) signaling pathways, which is consistent with previous studies in the hippocampus and cerebellum26–28. We further show that the activation of ERK signaling pathways by GABA receptors could prevent a rapid decline in the
GABA-activated whole-cell membrane current and help stabilize tonic inhibition.

Results

Activation of GABA receptors increases pERK levels in the LC. We first examined whether the activation
of GABA receptors could also increase phosphorylated-ERK (pERK) levels in the LC, as previously reported in
hippocampal and cerebellar tissue26–28. We examined pERK levels in LC tissue punched from slices (Fig. 1A)
bathed in 50 µM baclofen, a GABA receptor agonist, and the vehicle, artificial cerebrospinal fluid (aCSF) containing
synaptic blockers (see Materials and Methods), using western blot analysis. As pERK levels were reported to
peak at 10 min and start to decline at 20 min of baclofen stimulation in cultured cerebellar granule cells27, 15 min
of baclofen stimulation was used in this study. In comparison to the tissues from the vehicle-bathed slices, the
pERK level increased by 29.5 ± 8.2% in the LC tissues from the baclofen-bathed slices (Fig. 1B1) (p = 0.006; n = 9, Student's paired t-test). There was no increase in the pERK level (p = 0.183; n = 9, Student's paired t-test). We also compared pERK levels between LC tissues punched from slices bathed in baclofen and in baclofen plus
10 µM CGP54626, a GABA receptor antagonist. Compared to LC tissues from the baclofen-bathed slices, the inhibition
of GABA receptors with CGP54626 reduced the pERK level by 25.9 ± 4.1% (Fig. 1B2) (p = 0.003, n = 6, Student's paired t-test), showing that the increase in the pERK level by baclofen stimulation was specific to GABA receptor activation. Interestingly, compared to LC tissues from the baclofen-bathed slices, the pERK level also significantly decreased by 31.3 ± 6.1% in CGP54626-bathed slices (p = 0.018, n = 6, Student's paired t-test). As the ambient GABA in the
pontine area can continuously activate GABA receptors to exert tonic inhibition of LC neurons7,9,17, it could be that there
was a basal pERK level produced by continuous GABA receptor activation. Accordingly, tonic GABA receptor activation left
less room for a further increase in the pERK level by baclofen stimulation, and the inhibition of GABA receptors could result in a significant reduction.

Characterization of I_{GABABR} in LC neurons. To confirm that the increase in pERK levels upon GABA receptor activation occurred in LC neurons and to explore the possible physiological role of the elevated ERK activity, we performed whole-cell patch recording from LC neurons and tested the effects of ERK blockers on the whole-cell current induced by GABA receptor activation. All recordings described hereafter were performed with the addition of synaptic blockers to the bath medium to avoid secondary effects via fast synaptic transmissions. We adapted previously described criteria for identifying NAergic neurons in the dorsal pontine area7,29,30 to validate that the recorded neurons were LC neurons. The criteria were as follows: (1) the recorded neuron should be immunore-
active to anti-tyrosine hydroxylase (TH) antibody (Fig. 2A); (2) the recorded neuron should be able to spontane-
ously fire APs followed by prominent afterhyperpolarization; and (3) the recorded neuron should display a delay
in AP generation upon the injection of depolarizing current pulses, with V_m held at ~ -70 mV (Fig. 2B). A very interesting
observation from the whole-cell recordings of LC neurons was the appearance of spontaneous oscil-
lations at ~ 0.2 Hz of the membrane voltage in current-clamp recordings (Fig. 2C) or of the membrane current in
voltage-clamp recordings (Fig. 2D). The oscillating events displayed some similar features to those of spontaneous
APs, such as being biphasic and generated at a rate similar to spontaneous APs. Since LC neurons are electrically
coupled to gap junctions31,32, these events could be due to flow through the gap junctions of currents underlying
the APs generated from other LC neurons in the proximity and electrically coupled to the recorded neuron'.
This argument is further supported by the results showing that these events were blocked by CBX, a gap junction
blocker (Fig. 2D). We refer to the events recorded using the voltage clamp as I_{ Osc}.
Bath application of 100 μM baclofen induced an outward current that was blocked by subsequent application of 10 μM CGP54626 (Fig. 3A), showing that the current was mediated by GABA BRs. This observation is consistent with our previous reports7,9, further demonstrating that the current was generated by the opening of GABA BRs.

**Figure 1.** The activation of GABABRs increases pERK1 levels in LC tissue. (A) The images show two sagittal brainstem slices from an animal. The LC in the left slice was punched (A1) for western blot analysis, and the right slice was used for comparison (A2). IHC with anti-TH antibody was performed for the two slices, as shown in the insets showing merged fluorescence images of anti-TH (red) and DAPI (blue) staining of the dashed rectangular areas at high magnification. A comparison of the two slices shows that the punched area contained mostly TH-ir tissue. (B) Images show representative western blot analysis results for pERK1/2 in LC tissue punched from slices bathed in vehicle or baclofen (B1) and from slices bathed in baclofen or baclofen plus CGP54626 (B2). The plot in the right panels summarizes the results. Each paired circle and line indicates the result of a single experiment; bars and capped lines denote the mean and SEM, respectively. The asterisks denote significant differences compared to the control at p < 0.05 (*) and p < 0.01 (**); ns denotes no significance compared to the control.
GIRK channels downstream of the activation of GABABRs by baclofen. Hereafter, we refer to the current as the GABABR-mediated current (IGABABR). Interestingly, the induction of IGABABR was associated with the suppression of IOsc activity, and the activity reappeared upon subsequent application of CGP54626 to counteract the effect of baclofen (Fig. 3A–C).

The IGABABR underwent partial decline upon prolonged (15 minutes) exposure to baclofen in the control condition. As seen in Fig. 3A, upon the application of baclofen, IGABABR was quickly induced and peaked with a mean amplitude of 98.1 ± 20.4 pA (n = 11; Fig. 3D), followed by a gradual decline to approximately half the peak amplitude. We quantified the decline in IGABABR by measuring τ50, defined as the time required for the IGABABR to decline from its peak amplitude to half that value, and it was 356 ± 49 s in the control condition (Fig. 3E). The subsequent coapplication of CGP54626 15 min after baclofen application suppressed IGABABR to a level below baseline (the membrane current before baclofen application; see green dotted lines and asterisk in Fig. 3A), showing a basal tone of GABABR activation in LC neurons. The membrane current underlying the basal tone of GABABR activation is referred to as the tonic current (ITonic), measured as the difference between the membrane currents baseline and after CBX application.

Figure 2. Recordings from LC neurons (A) Images showing the identification of LC neurons with post hoc IHC using the anti-TH antibody. A1 and A3 show online phase contrast images of a sagittal brainstem slice at low (A1) and high magnification (A3). A2 and A4 show fluorescence images of anti-TH staining of the same field and magnification as shown in A1 and A3, respectively. A5 shows a fluorescence image of the same field and magnification as in A4 showing a recorded neuron filled with biocytin. This neuron also displayed TH-ir, as indicated by the asterisk. Abbreviations: Me5, mesencephalic trigeminal nucleus; scp, superior cerebellar peduncle. (B) Representative current-clamp recording from the TH-ir (LC) neuron shown in A, showing Vm responses (top traces) to current injection (bottom traces). Note the delay in the onset of AP (see arrow) elicited from Vm held at −70 mV. (C) A representative V-clamp (left bottom half) and I-clamp (right upper half) recording from an LC neuron. The arrow indicates switching of the recording from V-clamp to I-clamp mode. Note the biphasic IOsc in the V-clamp recording and the spontaneous APs and voltage oscillation in the I-clamp recording. The inserted green trace shows activity marked by the dashed rectangle on a faster and larger scale. Asterisks mark the voltage oscillations. The vertical bar to the left of the trace shows the amplitude scale for V-clamp (50 pA) or I-clamp (50 mV) recordings; the one to the right of the inserted trace shows the amplitude scale for the inserted trace; the bottom horizontal bar shows the time scale for the whole trace (120 s) and the inserted trace (20 s). (D) A representative experiment with V-clamp recordings from an LC neuron showing that IOsc are blocked by the application of 100 μM CBX, a gap junction blocker; top and bottom traces show recordings before and after CBX application, respectively.
Figure 3. The inhibition of ERK1/2 decreases the $\tau_{50}$ of $I_{\text{GABABR}}$ in LC neurons. (A–C) Representative recording of $I_{\text{GABABR}}$ from LC neurons in the control slice (A), U0126-treated slice (B), and FR180204-treated slice (C). The red double-headed line marks $\tau_{50}$, and the long and short green dashed lines mark the means of the membrane current recorded at baseline (before baclofen) and upon CGP54626 application. Note that the difference is measured as $I_{\text{Osc}}$, as indicated by the asterisk. The activity marked with the green square bracket is enlarged and shown at the bottom (traces a–c). Note the increased frequency of $I_{\text{Osc}}$, with CGP54626 application compared with baseline. (D–G) Plots show summarized results of the amplitude (D) and $\tau_{50}$ (E) of $I_{\text{GABABR}}$, $I_{\text{Tonic}}$ (F) and the rate of $I_{\text{Osc}}$ (G). Each circle (D–E) or dashed line (F–G) shows the result of an individual experiment; bars (D–E) or circles (F–G) denote the mean, and capped lines denote the SEM. The asterisks indicate a significant difference in $\tau_{50}$ (E) or in the increment of $I_{\text{Osc}}$ frequency (G) compared to the control at $p < 0.01$ (***) or at $p < 0.005$ (**__). $p$ denotes a significant increase in $I_{\text{Osc}}$ frequency after CGP54626 application (G); ns denotes no significant difference compared to the control.
at baseline and upon CGP54626 application (see asterisks in Fig. 3A). The $I_{\text{ tonic}}$ in the control was $-9.4 \pm 2.4$ pA ($n=11$) (Fig. 3F). Consistent with the observation of the basal tone of GABA$_B$R activation, we also observed a significantly higher frequency of IOsc during CGP54626 application than at baseline. The frequency of IOsc at baseline and during the CGP54626 application was $0.15 \pm 0.05$ Hz and $0.38 \pm 0.03$ Hz, respectively ($n=11; p=0.001$, post hoc Bonferroni test after two-way repeated-measures ANOVA) (Fig. 3Aa, Fig. 3G).

Inhibition of ERK$_{1/2}$ accelerates the decline of $I_{\text{GABA}_{B}}$ in LC neurons. We next tested the effects of two selective ERK blockers, U0126 and FR180204, on $I_{\text{GABA}_{B}}$ in LC neurons. We first examined whether there were possible effects of the two ERK$_{1/2}$ inhibitors on LC neurons by recording the basal spontaneous firing rate (SFR). The cell-attached configuration of the patch recording was used to avoid interference in the ion composition of the cytoplasm by the pipette solution. We found that the application of 20 μM U0126 for 30 min significantly decreased the SFR from $0.51 \pm 0.13$ to $0.33 \pm 0.09$ Hz (Fig. 4A,B) ($n=6$ cells, $p=0.028$, paired Wilcoxon-sign rank test). In contrast, the application of 20 μM FR180204 for 30 min significantly increased the SFR from $0.47 \pm 0.09$ to $0.73 \pm 0.08$ Hz (Fig. 4A,C) ($n=6$ cells, $p=0.006$, Student's paired t-test), and the application of the vehicle (0.1% DMSO) did not have an effect on the SFR (Fig. 4A,D) (baseline: $0.54 \pm 0.14$ Hz, DMSO: $0.55 \pm 0.14$ Hz, $n=8$ cells, $p=0.8$, Student's paired t-test). The differential effects of the two drugs on the SFR might be ascribed to the fact that FR180204 directly targets ERK$_{1/2}$, while U0126 targets the mitogen-activated protein kinase that acts upstream of ERK$_{1/2}$. The results also suggest that LC neurons might have basal ERK$_{1/2}$ activity, which could regulate various types of ion channels involved in the regulation of the membrane potential of LC neurons. To minimize the nonspecific effects, we pretreated the slices for 2 hrs and continuously perfused them throughout the recording with U0126 or FR180204 so that a stable baseline could be obtained before
the application of baclofen and CGP54626. In slices pretreated and perfused with U0126 or FR180204, the peak amplitude of $I_{\text{GABA}B}$ showed no difference in U0126- (n = 9 cells) and FR180204- (n = 10 cells) treated slices compared to the peak amplitude of $I_{\text{GABA}B}$ measured in control slices (Kruskal-Wallis test, p = 0.644 among the comparisons between control and ERK blocker groups) (Fig. 3A-D). In contrast, both drugs significantly accelerated the decline in $I_{\text{GABA}B}$ compared with the control condition. The $\tau_{\text{DLG}}$ measured from $I_{\text{GABA}B}$ recorded in the U0126-treated and FR180204-treated slices was 163 ± 21 s and 150 ± 21 s, respectively; both were significantly shorter than the measurements obtained using the control slice (Kruskal-Wallis test, p = 0.001 among the comparison between control and ERK blocker groups; p = 0.009 for control vs. U0126 and p = 0.002 for control vs. FR180204 using post hoc Dunn’s multiple comparisons test) (Fig. 3A-C,E). The $I_{\text{tonic}}$ revealed by the subsequent application of CGP54626 in slices pretreated with U0126 was $\sim 7.6 \pm 4.0$ pA; it was $0.9 \pm 7.0$ pA in slices pretreated with FR180204; no difference was observed in either case compared with the control (two-way repeated-measures ANOVA, sphericity assumed, F(1, 27) = 3.672, p = 0.066 for CGP effects; sphericity assumed, F(2, 27) = 1.337, p = 0.279 for the comparison among ERK blockers) (Fig. 3A-C,F).

Compared with baseline, the application of CGP54626 also increased the frequency of $I_{\text{osc}}$ in U0126- and FR180204-treated slices, with the extent of the increase being significantly less in FR180204-treated slices than that in the control slices (two-way repeated-measures ANOVA, sphericity assumed, F(1, 26) = 55.106, p = 0.000 for CGP effects; sphericity assumed, F(2, 26) = 6.255, p = 0.006 for the comparison among ERK blockers). Upon CGP54626 application, the frequency of $I_{\text{osc}}$ significantly increased from 0.24 ± 0.02 Hz to 0.33 ± 0.03 Hz in U0126-treated slices (n = 9 cells, p = 0.025, post hoc Bonferroni test); however, the extent of the increase showed no difference compared to the control (p = 1, post hoc Bonferroni test). In FR180204-treated slices, the frequency of $I_{\text{osc}}$ significantly increased from 0.48 ± 0.06 Hz to 0.57 ± 0.08 Hz (n = 9 cells, p = 0.032, post hoc Bonferroni test) and the extent of the increase was significantly less than that in the control (p = 0.001, post hoc Bonferroni test) (Fig. 3G). Again, the significant reduction in the extent of the increase in SFR upon CGP54626 application observed in FR180204-treated slices but not in U0126-treated slices might be ascribed to the fact that FR180204 specifically targets ERK1/2, while U0126 targets the mitogen-activated protein kinase that acts upstream of ERK1/2.

**ERK1/2 activated by GABA\textsubscript{B}R is essential to sustain tonic inhibition of LC neurons.** An interpretation of the above observations could be that the activation of GABA\textsubscript{B}R in LC neurons not only opened GIRK channels but also triggered ERK-dependent autoregulation of receptors to prevent the quick desensitization of GABA\textsubscript{B}R upon prolonged exposure to the agonist. To test this possibility, we examined the effects on tonic inhibition of LC neurons with prolonged exposure to the antagonist. If the above interpretation is correct, the inhibition of ERK-dependent autoregulation would result in a reduced functionality of GABA\textsubscript{B}R, as indexed by a reduction in the tonic inhibition of LC neurons after prolonged agonist exposure. We found that a precise assessment of tonic inhibition by directly measuring GABA\textsubscript{B}R-mediated $I_{\text{tonic}}$ was difficult due to the high noise level imposed by the high $I_{\text{osc}}$ activity. This phenomenon might account for the lack of a significant difference in $I_{\text{tonic}}$ in the U0126- or FR180204-treated slices compared to the control (Fig. 3F). Accordingly, we examined the SFR of LC neurons to assess GABA\textsubscript{B}R-mediated tonic inhibition.

To examine the effects on tonic inhibition of LC neurons with prolonged exposure of GABA\textsubscript{B}Rs to baclofen with the inhibition of ERK activity, we perfused the slices with 100 μM baclofen for an additional 15 min, followed by the co-administration of 20 μM baclofen and CGP54626 (Fig. 5A). Based on the dose-dependent relationship of $I_{\text{GABA}B}$ induced by baclofen, we estimated that 20 μM baclofen would produce 70% of the maximum GABA\textsubscript{B}R activation in LC neurons. Therefore, bathing the slices in 20 μM ambient baclofen could largely amplify tonic inhibition for easier observation. As seen (Fig. 5A,B), after a 15-min period of pre-exposure to the agonist at a saturating concentration, a significant increase in the SFR upon CGP54626 application was observed in LC neurons bathed in 20 μM baclofen in the control slices (n = 10 cells, p = 0.000, Student’s paired t-test), the U0126-treated slices (n = 7 cells, p = 0.012, Student’s paired t-test) and in the FR180204-treated slices (n = 7 cells, p = 0.001, Student’s paired t-test). The GABA\textsubscript{B}R-mediated tonic inhibition under the condition was defined as:

$$\left(\frac{\text{SFR}_{\text{Bac}} + \text{CGP54626} - \text{SFR}_{\text{Bac}}}{\text{SFR}_{\text{Bac}}} \times 100\%\right)$$

where SFR\textsubscript{Bac} and SFR\textsubscript{Bac+CGP54626} are the SFRs recorded in 20 μM baclofen and 20 μM baclofen plus CGP54626, respectively. The calculated tonic inhibition was $147.3 \pm 39.4$% in the control (n = 10), which was significantly stronger than that in U0126-treated slices of $34.8 \pm 8.9$% (n = 7) and in FR180204-treated slices of 16.6 ± 3.2% (n = 7) (One-way ANOVA, p = 0.008 among the comparison between control and ERK blocker groups; p = 0.045 for control vs. U0126 and p = 0.013 for control vs. FR180204 using post hoc Bonferroni test) (Fig. 5C).

**ERK1/2 activated by GABA\textsubscript{B}R does not have an effect on pGABA\textsubscript{B}R.** Finally, we examined the possible regulatory site of ERK1/2 regulation of GABA\textsubscript{B}R functionality upon receptor activation. To this end, we compared the level of phosphorylated GABA\textsubscript{B}R receptor subunit at the serine 783 (S783) site between LC tissue treated with baclofen and that treated with baclofen plus FR180204 using western blot analysis, as this regulatory site is shown to enhance GABA\textsubscript{B}R activation of GIRK[8, 9]. If ERK1/2 regulated GABA\textsubscript{B}R functionality by phosphorylating the S783 site of the GABA\textsubscript{B}R receptor subunit, the pGABA\textsubscript{B}R receptor level should be significantly higher in baclofen-treated LC tissue than in the tissue treated with baclofen plus FR180204. However, we did not find a difference between the two groups in the level of phosphorylation of the GABA\textsubscript{B}R receptor at the S783 site. Compared to the control (LC tissues from the baclofen-bathed slices), the pGABA\textsubscript{B}R level was 96.3 ± 6.9% of the
control in LC tissues from the slice bathed in baclofen plus FR180204 (Fig. 6) (n = 6, p = 0.651, Student’s paired t-test).

Discussion
In this study, we provide biochemical and electrophysiological evidence showing that GABABRs can mediate the autoregulation of GABA BR-activated whole-cell current through the activation of ERK 1 in LC neurons. Since ambient GABA in the LC is significantly higher during rapid eye movement (REM)/non-REM sleep than during wakefulness20,22, ERK1-dependent autoregulation of GABABR functionality could be a mechanism enabling the receptors to be continuously activated by the ambient GABA without undergoing severe desensitization, thereby providing a stable, tonic inhibition of LC neurons.

In many brain regions, including the LC, electron microscopic studies have shown that GABA<sub>3</sub>Rs are located predominantly close to neurotransmitter release sites at presynaptic terminals; in contrast, they are mainly located at peri-/extrasynaptic but not at synaptic active zones in postsynaptic neurons4–7. These locations of GABA<sub>3</sub>Rs imply that most of the receptors are not directly activated by synaptically released GABA in the synaptic cleft but by ambient GABA in peri-/extrasynaptic spaces. The concentration of ambient GABA critically depends on GABA spillover from the synaptic cleft, and the amount of GABA spillover is determined by the frequency and pattern of AP arrival at axonal terminals. It is also determined by the operation of GABA transporters located in the neuron and glia cell membrane34–39. GABA transporters not only serve in the reuptake of GABA in the synaptic cleft but also operate in a reverse mode that actually causes release but not reuptake of the neurotransmitter, a so-called nonvesicular release process, which would occur when high-frequency and repeated APs arrive at axonal terminals40–42. Accumulating reports have shown that ambient GABA generated by the abovementioned neuronal and glial activity could sufficiently and tonically activate peri-/extrasynaptic GABA<sub>3</sub>Rs at both presynaptic and postsynaptic sites, thereby exerting tonic inhibition for the control of transmitter release from
presynaptic terminals and the spiking activity in postsynaptic neurons\(^9,14-16,34-36,39\). The regulation of ambient GABA by AP-dependent vesicular and nonvesicular release could modulate tonic inhibition of neurons that express peri-/extrasynaptic GABA\(_A\)Rs and/or GABA\(_B\)Rs, and the processes could be linked to the long-term regulation of brain function. LC neurons are well known to be involved in the regulation of the wakefulness–sleep cycle; they fire APs in a brain state-dependent manner, displaying active firing during wakefulness, a decreased discharge rate during non-REM sleep and silence during REM sleep\(^35,43\). Obviously, a mechanism that provides prolonged and stable inhibition of LC neurons could promote REM/non-REM sleep.

In addition to ambient GABA, the operation of GABA\(_B\)R-mediated tonic inhibition would require a substantial and steady amount of GABA\(_B\)Rs on the membrane. Nevertheless, many GPCRs undergo desensitization, a process in which a receptor reduces its response after prolonged exposure to the agonist, which helps to attenuate or terminate signal transduction to protect the target cells from overstimulation\(^35\). It is now well known that desensitization involves the phosphorylation of GPCRs by G protein-coupled receptor kinases (GRKs), which lead to the uncoupling of the receptors from G proteins. This process is followed by the internalization of GPCRs through the recruitment of arrestins that trigger clathrin-mediated endocytosis of the phosphorylated receptor\(^44\). Interestingly, a growing amount of evidence suggests that GABA\(_B\)Rs, unlike many other GPCRs, are not the substrate for GRKs other than GRK4/5 and do not conform to the above-described agonist-induced internalization\(^25\). Nevertheless, GABA\(_B\)Rs exhibit significant rates of constitutive endocytosis via clathrin-dependent and dynamin-dependent mechanisms under basal conditions, followed by the recycling of large numbers of GABA\(_B\)Rs back to the plasma membrane to maintain steady-state cell surface numbers\(^45-48\). These processes perhaps reflect the long cell surface half-lives of GABA\(_B\)Rs and make them suitable for mediating a stable and persistent effect such as tonic inhibition. For example, unlike neurokinin receptors, which undergo almost complete desensitization after a few seconds of exposure to substance-P in NAergic A7 neurons\(^29\), GABA\(_B\)R desensitization progresses at a much slower rate in NAergic A7 neurons\(^8\) as well as in LC neurons. The regulation of the surface stability of the GABA\(_B\)R number usually involves the phosphorylation of the receptor molecule per se. It has been shown that the phosphorylation of S892 of the GABA\(_B\)A receptor by protein kinase-A promotes cell surface stability of the GABA\(_B\)A receptor number\(^46\); in cultured hippocampal neurons, S867 in GABA\(_B\)1 receptors is phosphorylated by Ca\(^{2+}\)/calmodulin-dependent protein kinases downstream of NMDA receptor activation, which induces the internalization of GABA\(_B\)A Rs. The phosphorylation of the receptor molecule also regulates GABA\(_B\)A signaling by altering the efficacy of receptor–effector coupling. The phosphorylation of S783 of the GABA\(_B\)B receptor by 5′AMP-dependent protein kinase (AMPK) has been shown to enhance GABA\(_B\)A activation of GIRKs\(^52\). In addition to the promotion of cell surface stability of the receptor number, the phosphorylation of S892 of the GABA\(_B\)A receptor by protein kinase-A facilitates receptor–effector coupling\(^53\) and slows KCTD12-induced desensitization of the GIRK current\(^50\). In addition to the receptor molecules, the phosphorylation of effectors, such as N-type Ca\(^{2+}\) channels, by tyrosine kinase can interact with regulators of G protein signaling (RGS) to form a regulatory complex that provides additional mechanism for regulation in the GABA\(_B\)A-mediated currents\(^21\). Another mechanism reported to be involved in the regulation of GABA\(_B\)A desensitization includes the agonist-induced association of the receptors with GRK4/5, which results in GABA\(_B\)A desensitization in a phosphorylation-independent manner. The interaction of GABA\(_B\)1 and GABA\(_B\)2 receptor subunits with NEM-sensitive fusion protein (NSF) primes the receptors for phosphorylation by protein kinase C (PKC) to uncouple them from G proteins. The association of GABA\(_B\)A Rs with various types of auxiliary proteins, such as potassium channel tetramerization domain-containing proteins (KCTDs), via the C-terminus of GABA\(_B\)2 receptors can render the receptor complex competent for desensitization.

Our western blot analysis showed that baclofen could increase pERK, levels in LC tissue, and this effect was not observed in LC tissue treated with CGP54626 to block GABA\(_B\)A Rs. These observations show that the
baclofen-induced increase in pERK, levels in the LC was GABA_{B}R-dependent, consistent with the results of previous reports in the hippocampus and cerebellum. In addition to the well-known effect on gating GIRKs, the results of these previous and present studies demonstrate an additional role of GABA_{B}R activation in activating ERK in hippocampal CA1 pyramidal neurons, cerebellar granule neurons and LC neurons. By performing whole-cell recordings directly from LC neurons, we further observed that the inhibition of ERK_{1/2} with two selective blockers produced consistent effects on the activity induced by baclofen application in LC neurons. These results confirmed that the elevation of pERK, levels by GABA_{B}R activation occurred in LC neurons, although we could not completely rule out the possibility that the effects on LC neuron activity were secondary. We did not validate the link between GABA_{B}R activation and the increased pERK, level, however, it has been reported that the increase in pERK_{1/2} levels by GABA_{B}R activation relies on a pertussis-toxin-sensitive G_{i/o} heterotrimeric protein-dependent pathway by releasing G_{i/o} in cultured cerebella granule neurons. Interestingly, this signaling pathway for ERK_{1/2} phosphorylation seems to be unique to GABA_{B}R, as the activation of G_{i/o}-coupled α_{7}-adrenergic receptors or G_{i/o}-coupled 5HT_{1A} receptors in the adult mouse CA1 region did not result in ERK_{1/2} phosphorylation. As shown in our electrophysiological experiments, the inhibition of ERK_{1/2} activity with two selective blockers accelerated the decay of I_{GABA_{B}R} and it is likely that the signaling mechanisms of GABA_{B}R traffic-ficking and/or desensitization may involve an ERK-dependent intermediate step that has not yet been identified. Therefore, the inhibition of the ERK-dependent intermediate step accelerated the decline of I_{GABA_{B}R}. Our western blot analysis showed that the activation of ERK_{i} downstream of GABA_{B}R activation did not have an effect on the level of phosphorylation of the GABA_{B}R receptor at the serine 783 site. Activated ERK_{i} could regulate GABA_{B}R functionality at other serine sites in GABA_{B}1 and/or GABA_{B}2 receptors, as previously described. Furthermore, it could be involved in the interaction between RGS and phosphorylated GIRKs by tyrosine kinase, as it has been recently suggested that the GABA_{B}R activates tyrosine kinase and downstream ERK_{i}. Apparently, the mechanisms underlying the regulation of GABA_{B}R desensitization by ERK_{i} remain to be clarified.

In conclusion, here, we report that the activation of GABA_{B}R with baclofen increases the phosphorylation of ERK_{i} in LC tissue and that the blockade of ERK_{1/2} activity with two selective blockers, U0126 and FR180204, accelerates the decay of I_{GABA_{B}R} induced by prolonged baclofen application in LC neurons. Furthermore, an increase in the spontaneous firing rate and a decrease in the tonic inhibition of LC neurons occurs after prolonged GABA_{B}R activation by baclofen at a saturating concentration. These results suggest a new functional role of G_{i/o} upon GABA_{B}R activation in mediating an ERK_{i/2}-dependent autoregulation of the stability of GABA_{B}R-activated whole-cell current in LC neurons, in addition to the well-known action of gating GIRKs with G_{i/o}, subunits. We argue that activated ERK_{i} signaling might help maintain a dynamic balance between the desensitization and recycling of receptors back to the membrane to sustain GABA_{B}R-mediated tonic inhibition of LC neurons. However, it should be noted that postnatal day 8–10 rats were used in this study, and there is a possible weakness that the same phenomena may not be fully present in adulthood.

Materials and Methods

Animals. The use of animals in this study was approved by the Institutional Animal Care and Use Committee for National Taiwan University (permission #NTU103-EL-00076) and the Institutional Animal Care and Use Committee for Chung Shan Medical University (permission #1423), the guidelines of which comply with the "Codes for Experimental Use of Animals" of the Council of Agriculture of Taiwan based on the Animal Protection Law of Taiwan. Every effort was made to minimize the number of animals used and their suffering. Sprague-Dawley rat pups of both sexes were used and sacrificed for slice preparation. Every effort was made to minimize the number of animals used and their suffering.

Preparation of brainstem slices and electrophysiology. The animals were anesthetized with 5% isoflurane in O_{2} and decapitated, followed by rapid exposure of the brain and chilling with ice-cold aCSF consisting of the following (in mM): 119 NaCl, 2.5 KCl, 1.3 MgSO_{4}, 26.2 NaHCO_{3}, 1 NaH_{2}PO_{4}, 2.5 CaCl_{2} and 11 glucose, oxygenated with 95% O_{2} and 5% CO_{2}, pH 7.4. Sagittal brainstem slices (500 μm) containing the LC were prepared using a vibratome (D.S.K. Super Microslicer Zero 1, Dosaka EM, Kyoto, Japan), maintained in a moist air-liquid (aCSF) interface chamber and allowed to recover for at least 90 minutes before being transferred to an immersion-type chamber mounted on an upright microscope (BX51WI, Olympus Optical Co., Ltd., Tokyo, Japan) for recording. Throughout the recording period, they were perfused at 2–3 ml/min with oxygenated aCSF containing synaptic blockers. The synaptic blockers contained 5 mM kynurenic acid, 100 μM picrotoxin and 1 μM strychnine to block glutamatergic, GABAergic and glycinegic synaptic transmission, respectively. The baseline described in the control slices refers to the recordings made under these conditions before administering baclofen and CGP54626. For recordings made in the U0126-treated slices and FR180204-treated slices, baseline refers to recording in the aCSF containing synaptic blockers, 0.1% DMSO and U0126 or FR180204.

Neurons were viewed using Nomarski optics. Patch pipettes were pulled from borosilicate glass tubing (1.5 mm outer diameter; 0.32 mm wall thickness; Warner Instruments Corp., Hamden, CT, USA) and had a resistance of approximately 3–5 MΩ. The pipette solution consisted of the following (in mM): 131 K-gluconate, 20 KCl, 10 HEPES, 3 NaCl, 2 ATP, and 0.3 GTP; pH adjusted to 7.2 with KOH. Recordings were performed at 29–31 °C in the whole-cell or cell-attached configuration with a patch amplifier (Multiclamp 700 B; Axon Instruments Inc., Union City, CA, USA). For current-clamp recordings of whole-cell configuration, the bridge was balanced, and the recordings were accepted only if the recorded neuron had a membrane potential (V_{m}) of at least −45 mV without applying a holding current and if the APs were able to overshoot 0 mV. For voltage-clamp recordings, the V_{m} was clamped to −70 mV unless specified. The serial resistance was monitored throughout the recording, and the data were discarded if the values varied by more than 20% of the original value, which was usually less than 20 MΩ. In a series of experiments, the cell-attached configuration was used. In the recording, the patch amplifier was set in...
voltage-clamp mode with the pipette voltage set to 0 mV (holding current = 0 pA) so that the recorded neurons were at their resting membrane potential. All signals were low-pass filtered at a corner frequency of 2 kHz and digitized at 10 kHz using the Micro 1401 interface running Signal software for episode-based capture and Spike2 software for continuous recording (Cambridge Electronic Design, Cambridge, UK). All data are presented as the mean±the standard error of the mean (SEM). For statistical comparisons, the normality of the data was first tested using the Shapiro-Wilk test. Student’s paired t-test and the nonparametric paired Wilcoxon-sign rank test were used for the comparison of data collected before and after drug application (Fig. 4, Fig. 5B). One-way ANOVA was used for the comparison of I\textsubscript{GABA\text{\textsubscript{A}}} parameters (Fig. 3D,E) and tonic inhibition (Fig. 5C) among the control, U0126 and FR180204 groups. Two-way repeated-measures ANOVA was used for the comparison of I\textsubscript{GABA\text{\textsubscript{B}}} and oscillation rates (Fig. 3F,G) between baseline and CGP54626 application among the control, U0126 and FR180204 groups. The criterion for significance was p < 0.05.

All chemicals used to prepare the aCSF and pipette solution were from Merck (Frankfurt, Germany); baclofen, biocytin, carbamoxolone (CBX), kynurenic acid, picROTOXin, and strychnine were from Sigma (St. Louis, USA); and CGP54626 hydrochloride (CGP), U0126, and FR180204 were from Tocris-Cookson (Bristol, UK).

### Biocytin histochemistry and immunohistochemistry

In some experiments, 6.7 mM biocytin was included in the internal solution to fill the recorded neurons. The detailed procedures for viewing the biocytin-filled neurons and post hoc immunohistochemistry (IHC) for cell type identification of the recorded neurons have been described previously. Briefly, after recording, the slices were fixed overnight at 4 °C in 4% paraformaldehyde (Merck) in 0.1 M phosphate buffer (PB), pH 7.4, and then subjected to biocytin histochemistry and IHC procedures without further sectioning. The slices were incubated for 1 hr at room temperature in phosphate-buffered saline (PBS) containing 0.03% Triton X-100 (PBST), 2% bovine serum albumin (BSA), and 10% normal goat serum (NGS), followed by incubation overnight at 4 °C in PBST containing a 1/2000 dilution of rabbit antibodies against TH (Merck Millipore, Darmstadt, Germany) and a 1/200 dilution of streptavidin (Vector Laboratories), followed by avidin-biotinylated horseradish peroxidase (HRP) complex (Vector Laboratories), followed by avidin-biotinylated horseradish peroxidase (HRP) complex (Vector Laboratories) in PBST. The bound antibodies were detected using an enhanced chemiluminescence (ECL) detection system (Fujifilm), and the intensities of the bands were quantified using the ImageGauge program (Fujifilm Laboratories) in TTBS. The bound antibodies were detected using an enhanced chemiluminescence (ECL) detection system (Fujifilm), and the intensities of the bands were quantified using the ImageGauge program (Fujifilm Laboratories). The supernatant was then collected, and the protein concentration was determined using a BCA Protein Assay Kit (Pierce) with bovine serum albumin (BSA) as the standard. The samples (30 μg of protein) were separated by 8–10% SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was incubated with 5% BSA and 5% nonfat milk in Tris-Tween-buffered saline (TTBS) buffer containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.1% Tween 20 for 1 hr at room temperature, followed by an overnight incubation at 4 °C with primary antibodies in TTBS buffer. In Fig. 1A, the primary antibodies were polyclonal antibodies against phospho-p44/42 MAPK (ERK\text{\textsubscript{1,2}}) (1/1000, Cell Signaling Technology) or rabbit monoclonal antibodies against phospho-p44/42 MAPK (ERK\text{\textsubscript{1,2}}) (1/750, Cell Signaling Technology); in Fig. 5, the primary antibodies were rabbit monoclonal antibodies against the GABA\text{\textsubscript{A}} receptor (1/1000, Cell Signaling Technology) and rabbit polyclonal antibodies against phospho-S783 of the GABAB\text{\textsubscript{2}} receptor (1/1000, Rockland Immunocchemicals). The membrane was sequentially incubated for 1 hr at room temperature with biotinylated goat anti-rabbit IgG antibodies (Vector Laboratories), followed by avidin-biotinylated horseradish peroxidase (HRP) complex (Vector Laboratories) in TTBS. The bound antibodies were detected using an enhanced chemiluminescence (ECL) detection system (Fujifilm), and the intensities of the bands were quantified using the ImageGauge program (Fujifilm Software). For statistical comparisons, Student’s paired t-test was used, as the data all passed the normality test using the Shapiro-Wilk test (Fig. 1 and 6).

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### References

1. Belelli, D. et al. Extrasynaptic GABA\text{\textsubscript{A}} receptors: form, pharmacology, and function. J. Neurosci. 29, 12757–12763 (2009).
2. Walker, M.C.&Kullmann, D.M. Tonic GABAA receptor-mediated signaling in epilepsy. In:Jasper's Basic Mechanisms of the Epilepsies [Internet]. J. Noebels, J. L., Avoli, M., Rogawski, M. A., Olsen, R. W. & Delgado-Escueta, A. V.) 4th edition. (Bethesda (MD): National Center for Biotechnology Information (US)) (2012).
3. Ulrich, D. & Bettler, B. GABA\text{\textsubscript{B}} receptors: synaptic functions and mechanisms of diversity. Curr. Opin. Neurobiol. 17, 298–303 (2007).
4. Kulik, A. et al. Subcellular localization of metabotropic GABA\text{\textsubscript{B}} receptor subunits GABA\text{\textsubscript{B1a}}, and GABA\text{\textsubscript{B2}} in the rat hippocampus. J. Neurosci. 23, 11026–11035 (2003).
5. Chen, L., Boyes, J., Yung, W. H. & Bolam, J. P. Subcellular localization of GABA\text{\textsubscript{B}} receptor subunits in rat globus pallidus. J. Comp. Neurol. 474, 340–352 (2004).
6. Luján, R., Shigemoto, R., Kulik, A. & Juuz, J. M. Localization of the GABAB receptor 1a/b subunit relative to glutamatergic synapses in the dorsal cochlear nucleus of the rat. J. Comp. Neurol. 475, 36–46 (2004).

7. Wang, H.-Y. et al. GABAB receptor-mediated tonic inhibition regulates the spontaneous firing of locus coeruleus neurons in developing rats and citalopram-treated rats. J. Physiol. (London). 593, 161–180 (2015).

8. Bettler, B., Kaupmann, K., Mosbacher, I. & Gassmann, M. Molecular structure and physiological functions of GABAB receptor. Physiol. Rev. 84, 835–867 (2004).

9. Wu, Y. et al. GABAB receptor-mediated tonic inhibition of noradrenergic A7 neurons in the rat. J. Neurophysiol. 105, 2715–2728 (2011).

10. Gassmann, M. & Bettler, B. Regulation of neuronal GABAB receptor functions by subunit composition. Nat. Rev. Neurosci. 13, 380–394 (2012).

11. Jones, K. A. et al. GABAB receptors function as a heteromeric assembly of the subunits GABABR1 and GABABR2. Nature 396, 674–679 (1998).

12. Kaupmann, K. et al. GABAB receptor subtypes assemble into functional heteromeric complexes. Nature 396, 683–687 (1998).

13. White, J. H. et al. Heterodimerization is required for the formation of a functional GABAB receptor. Nature 396, 679–682 (1998).

14. Vanhoose, A. M., Emery, M., Jimenez, L. & Winder, D. G. ERK activation by G-protein-coupled receptors in mouse brain is receptor identity-specific. Cell Signal. 28, 403–450 (2005).

15. Nelson, L. E. & Waterhouse, B. Locus coeruleus: From global projection system to adaptive regulation of behavior. Brain Res. 1645, 75–78 (2016).

16. Nitz, D. & Siegel, J. M. GABA release in the locus coeruleus as a function of sleep/wake state. Neuroscience 78, 795–801 (1997).

17. Moore, J. T. et al. Direct Activation of Sleep-Promoting VLPO Neurons by Volatile Anesthetics Contributes to Anesthetic Hypnosis. Curr. Biol. 22, 2008–2016 (2012).

18. Nelson, L. E. et al. The delta2 adrenoceptor agonist dexmedetomidine converges on an endogenous sleep-promoting pathway to exert its sedative effects. Anesthesiology 98, 428–436 (2003).

19. Vazey, E. M. & Aston-Jones, G. Designer receptors manipulations reveal a role of the locus coeruleus noradrenergic system in modulating activity in the rostral prefrontal cortex in vitro. Eur. J. Neurosci. 31, 1582–1594 (2010).

20. Wang, T. et al. Regulation of neuronal GABAB receptor functions by subunit composition. J. Physiol. 591, 4877–4894 (2013).

21. Hung, W.-C. et al. GABAB receptor-mediated tonic inhibition of locus coeruleus neurons plays a role in deep anesthesia induced by isoflurane. Neuroreport 31 (7), 557–564 (2020).

22. Aston-Jones, G. & Cohen, J. D. An Integrative Theory of Locus Coeruleus- Norepinephrine Function: Adaptive Gain and Optimal Performance. Annu. Rev. Neurosci. 28, 403–450 (2005).

23. Nelson, L. E. & Waterhouse, B. Locus coeruleus: From global projection system to adaptive regulation of behavior. Brain Res. 1645, 75–78 (2016).

24. Vazey, E. M. & Aston-Jones, G. Designer receptors manipulations reveal a role of the locus coeruleus noradrenergic system in modulating activity in the rostral prefrontal cortex in vitro. Eur. J. Neurosci. 31, 1582–1594 (2010).

25. Min, M.-Y. et al. Physiological and morphological properties of, and effect of substance P on, neurons in the A7 catecholamine cell group in rats. Neuroscience 153, 1020–1033 (2008).

26. Min, M.-Y. et al. Roles of A-type potassium currents in tuning spike frequency and integrating synaptic transmission in noradrenergic neurons of the A7 catecholamine cell group in rats. Neuroscience 166, 633–645 (2010).

27. Ishimatsu, M. & Williams, J. T. Synchronous activity in locus coeruleus results from dendritic interactions in pericoerulear regions. J. Neurosci. 16, 5196–5204 (1996).

28. Ballantyne., D., Andrzejewski, M., Muckenhoff, K. & Scheid, P. Rhythms, synchrony and electrical coupling in the Locus coeruleus. J. Neurosci. 673–681 (2000).

29. Scanziani, M. GABA spillover activates postsynaptic GABAB receptors to control rhythmic hippocampal activity. Neuron 53, 233–247 (2007).

30. Isaacson, J. S., Solis, J. M. & Nicoll, R. A. Local and diffuse synaptic actions of GABA in the hippocampus. Neuron 10, 165–175 (1993).

31. Zilberter, Y., Kaiser, K. M. & Sakemann, B. Dendritic GABA release depresses excitatory transmission between layer 2/3 pyramidal and bitufted neurons in rat neocortex. J. Physiol. 548, 97–110 (2003).

32. Scanziani, M. GABA spillover activates postsynaptic GABAB receptors to control rhythmic hippocampal activity. Neuron 25, 673–681 (2000).

33. Smith, T. C. & Jahr, C. E. Self-inhibition of olfactory bulb neurons. Nat. Neurosci. 5, 760–766 (2002).

34. Alle, H. & Geiger, J. R. GABAergic spillover transmission onto hippocampal mossy fiber boutons. J. Neurosci. 27, 942–960 (2007).

35. Rosi, D., Hamann, M. & Attwell, D. Multiple modes of GABAAergic inhibition of rat cerebellar granule cells. J. Physiol. 548, 97–110 (2003).

36. Wu, Y., Yang, W., Diez-Sampedro, A. & Richerson, G. B. Nonvesicular inhibitory transmission via reversal of the GABA transporter GAT-1. Neuron 56, 851–867 (2007).

37. Koch, U. & Magnusson, A. K. Unconventional GABA release: mechanisms and function. Curr. Opin. Neurobiol. 19, 305–310 (2009).

38. Berridge, C. W. & Foote, S. L. Effects of locus coeruleus activation on electroencephalographic activity in neocortex and hippocampus. J. Neurosci. 11, 3135–3145 (1991).

39. Krupnick, J. G. & Benovic, J. L. The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu. Rev. Pharmacom. Ther. 13, 289–319 (1998).

40. Terunuma, M., Pangalos, M. N. & Moss, S. J. Functional Modulation of GABAB receptors by Protein Kinases and Receptor Trafficking. Adv. Pharmacoc. 58, 113–122 (2010).

41. Brown, W. P. et al. Phosphorylation and chronic agonist treatment atypically modulate GABAB receptor cell surface stability. J. Biol. Chem. 279, 12565–12573 (2004).

42. Vagas, K. J. et al. The availability of surface GABAB receptors is independent of gamma-aminobutyric acid but controlled by glutamate in central neurons. J. Biol. Chem. 283, 24641–24648 (2008).

43. Benke, D., Zemoura, K. & Maier, P. J. Modulation of cell surface GABA receptors by desensitization, trafficking and regulated degradation. World. J. Biol. Chem. 3, 61–72 (2012).

44. Gouve, A. et al. Cyclic AMP-dependent protein kinase phosphorylation facilitates GABAB receptor-effector coupling. Nat. Neurosci. 5, 415–424 (2002).
50. Adelfinger, L. et al. GABA\(_\text{B}\) receptor phosphorylation regulates KDTC12-induced K\(^+\) current desensitization. Biochem. Pharmacol. 91, 369–379 (2014).
51. Schiff, M. L. et al. Tyrosine-kinase-dependent recruitment of RGS12 to the N-type calcium channel. Nature 408, 723–727 (2000).
52. Padgett, C. L. & Slesinger, P. A. GABA\(_\text{B}\) receptor coupling to G-proteins and ion channels. Adv. Pharmacol. 58, 123–147 (2010).
53. Jiang, X. et al. GABA\(_\text{B}\) receptor complex as a potential target for tumor therapy. J. Histochem. Cytochem. 60(4), 269–279 (2012).

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Author contributions

R.-N. Wu and C.-C. Kuo collected and analyzed the data. M.-Y. Min, R.-F. Chen and H.-W. Yang conceived and designed the experiments and wrote the first draft of the paper. All authors have contributed to the writing of this paper and have approved the final version.

Competing interests

The authors declare no competing interests.

Additional information

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