GABA$_B$ receptors are heterodimeric G protein-coupled receptors, which control neuronal excitability by mediating prolonged inhibition. The magnitude of GABA$_B$ receptor-mediated inhibition essentially depends on the amount of receptors in the plasma membrane. However, the factors regulating cell surface expression of GABA$_B$ receptors are poorly characterized. Cell surface GABA$_B$ receptors are constitutively internalized and either recycled to the plasma membrane or degraded in lysosomes. The signal that sorts GABA$_B$ receptors to lysosomes is currently unknown. Here we show that Mind bomb-2 (MIB2)-mediated Lys-63-linked ubiquitination of the GABA$_{B1}$ subunit at multiple sites is the lysosomal sorting signal for GABA$_B$ receptors. We found that inhibition of lysosomal activity in cultured rat cortical neurons increased the fraction of Lys-63-linked ubiquitinated GABA$_B$ receptors and enhanced the expression of total as well as cell surface GABA$_B$ receptors. Mutational inactivation of four putative ubiquitination sites in the GABA$_{B1}$ subunit significantly diminished Lys-63-linked ubiquitination of GABA$_B$ receptors and prevented their lysosomal degradation. We identified MIB2 as the E3 ligase triggering Lys-63-linked ubiquitination and lysosomal degradation of GABA$_B$ receptors. Finally, we show that sustained activation of glutamate receptors, a condition occurring in brain ischemia that down-regulates GABA$_B$ receptors, considerably increased the expression of MIB2 and Lys-63-linked ubiquitination of GABA$_B$ receptors. Interfering with Lys-63-linked ubiquitination by overexpressing ubiquitin mutants or GABA$_{B1}$ mutants deficient in Lys-63-linked ubiquitination prevented glutamate-induced down-regulation of the receptors. These findings indicate that Lys-63-linked ubiquitination of GABA$_{B1}$ at multiple sites by MIB2 controls sorting of GABA$_B$ receptors to lysosomes for degradation under physiological and pathological conditions.

The number of neurotransmitter receptors at the cell surface available for signaling in neurons needs to be precisely tuned to a given cellular state and consequently must be dynamically adjusted to altered conditions. One key player regulating their amount as well as their life span is protein degradation. Two major cellular protein degradation systems control the number of neurotransmitter receptors, lysosomes and proteasomes. Interestingly, both systems rely on ubiquitination as a signal that tags most membrane proteins for degradation. For proteasomal degradation, primarily Lys-48-linked polyubiquitination is required, and for lysosomal degradation, primarily Lys-63-linked polyubiquitination is required (1). Both degradation pathways are involved in the regulation of G protein-coupled GABA$_B$ receptors. GABA$_B$ receptors are heterodimers assembled from GABA$_{B1}$ and GABA$_{B2}$ subunits and are activated by γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, to regulate excitability of neurons. At presynaptic locations, GABA$_B$ receptors suppress neurotransmitter release mainly by inhibiting voltage-gated Ca$^{2+}$ channels, whereas at postsynaptic sites they induce slow inhibitory postsynaptic currents by activating Kir3-type K$^+$ channels (2). GABA$_B$ receptors are involved in the regulation of all main brain functions ranging from synaptic plasticity (3), neuronal network activity (4, 5), to neuronal development (6).

An important factor regulating GABA$_B$ receptor signaling is the dynamic control of their cell surface expression via protein degradation. So far, the following two mechanisms have been identified: 1) proteasomal degradation of the receptors in the endoplasmic reticulum (ER), and 2) lysosomal degradation of receptors internalized from the plasma membrane. The amount of GABA$_B$ receptors in the ER available for forward trafficking to the cell surface is determined by the rate of their proteasomal degradation via the ER-associated degradation (ERAD) machinery (7). Proteasomal degradation of ER-residing GABA$_B$ receptors is regulated by the activity state of the neuron via Lys-48-linked ubiquitination of GABA$_{B2}$ and requires interaction of the GABA$_{B2}$ C terminus with the proteasomal AAA-ATPase Rpt6 (7, 8). In contrast, GABA$_B$ receptors at the cell surface are constitutively endocytosed and either recycled to the plasma membrane or degraded in lysosomes (9–13). Lysosomal degradation of GABA$_B$ receptors is most likely mediated via the ESCRT (endosomal sorting complex required for transport) machinery (13), which sorts ubiquitinated membrane proteins to lysosomes (14). Interestingly, USP14 (ubiquitin-specific protease 14) has been implicated in

---

*This work was supported by Swiss National Science Foundation Grant 31003A_138382 (to D. B.). The authors declare that they have no conflicts of interest with the contents of this article.

†Author’s Choice—Final version free via Creative Commons CC-BY license.

‡To whom the correspondence should be addressed: Institute of Pharmacology and Toxicology, University of Zurich, the Neuroscience Center Zurich, University of Zurich and ETH Zurich, and the Drug Discovery Network Zurich (DDNZ), Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

§Author’s Choice

1 The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; Ub, ubiquitin; ANOVA, analysis of variance; PLA, proximity ligation assay; EGFP, enhanced GFP.

2 The abbreviations used are: ER, endoplasmic reticulum; ERAD, ER-associated degradation; Ub, ubiquitin; ANOVA, analysis of variance; PLA, proximity ligation assay; EGFP, enhanced GFP.
sorting ubiquitinated GABA<sub>B</sub> receptors to lysosomal degradation (15). Lysosomal degradation of GABA<sub>B</sub> receptors appears to be tightly regulated because excessive activity of glutamate receptors, a condition occurring in brain ischemia, rapidly down-regulates GABA<sub>B</sub> receptors by preferential sorting them to the lysosomal degradation pathway at the expense of receptor recycling (16–19). The specific signal(s) that sorts GABA<sub>B</sub> receptors to lysosomal degradation under normal as well as pathological conditions is currently unknown. Here we show that Lys-63-linked ubiquitination by the E3 ligase mindbomb-2 (MIB2) of GABA<sub>B1</sub> at multiple sites targets GABA<sub>B</sub> receptors to the lysosomal degradation pathway.

Results

Lysosomal Degradation Regulates Cell Surface Expression of GABA<sub>B</sub> Receptors—GABA<sub>B</sub> receptors undergo fast constitutive dynamin and clathrin-dependent endocytosis. Most of the receptors are recycled to the plasma membrane, although a minor fraction is sorted to the lysosomal degradation pathway (9–11, 20, 21). However, it is currently not known whether interfering with lysosomal degradation affects the expression of cell surface expression of GABA<sub>B</sub> receptors. Blocking lysosomal degradation in cultured cortical neurons with leupeptin for 12 h considerably increased total (GABA<sub>B1</sub>, 151 ± 6%; GABA<sub>B2</sub>, 160 ± 7% of control; Fig. 1A) as well as cell surface expression of GABA<sub>B</sub> receptors (GABA<sub>B1</sub>, 146 ± 9%; GABA<sub>B2</sub>, 147 ± 9% of control; Fig. 1B) to a similar extent. This suggests that constitutive lysosomal degradation is one factor determining the availability of GABA<sub>B</sub> receptors at the cell surface for signaling.

Lys-63-linked Ubiquitination Is Involved in Lysosomal Degradation of GABA<sub>B</sub> Receptors—The signal that sorts GABA<sub>B</sub> receptors to lysosomal degradation is unknown. Lys-48-linked ubiquitination tags proteins for degradation in proteasomes, whereas Lys-63-linked ubiquitination is involved in non-proteolytic functions and can serve as a sorting signal for lysosomal degradation (1). To test whether Lys-63-linked ubiquitination is involved in degrading GABA<sub>B</sub> receptors, we transfected neurons with a mutant of ubiquitin that is not able to form Lys-63-linked chains (Ub(K63R)) and analyzed them for cell surface expression of GABA<sub>B</sub> receptors. Inhibition of Lys-63-linked ubiquitination by overexpression of Ub(K63R) increased the expression level of cell surface GABA<sub>B</sub> receptors (GABA<sub>B1</sub>, 162 ± 12%; GABA<sub>B2</sub>, 136 ± 9% of control neurons transfected with wild-type ubiquitin; Fig. 2A), suggesting that GABA<sub>B</sub> receptor levels are regulated by Lys-63-linked ubiquitination.

Next we tested whether regulation of GABA<sub>B</sub> receptor levels by lysosomal degradation requires direct Lys-63-linked ubiquitination of the receptor by in situ PLA using antibodies directed against GABA<sub>B1</sub> and Lys-63-linked ubiquitin. Under basal conditions, GABA<sub>B</sub> receptors exhibited Lys-63-linked ubiquitination, which considerably increased upon inhibition of lysosomal activity with leupeptin (164 ± 8% of control, Fig. 2B). In contrast, Lys-48-linked ubiquitination (which targets the receptors to proteasomal degradation (7, 8)) remained unaffected by blocking lysosomal activity (Fig. 2C). This suggests that direct Lys-63-linked ubiquitination of GABA<sub>B</sub> receptors regulates lysosomal degradation of GABA<sub>B</sub> receptors.

Identification of Lys-63-linked Ubiquitination Sites in GABA<sub>B</sub>B Receptors—For identification of Lys-63-linked ubiquitination sites in GABA<sub>B</sub> receptors, we first determined whether GABA<sub>B1</sub> or GABA<sub>B2</sub> is the main target. HEK293 cells were either transfected with a GABA<sub>B1</sub> mutant (GABA<sub>B1</sub>(RSAR)) containing an inactivated ER retention signal, which permits ER exit and cell surface targeting of the subunit when expressed in the absence of GABA<sub>B2</sub> (22), or with a combination of GABA<sub>B1</sub> and GABA<sub>B2</sub> and tested for Lys-63-linked ubiquitination with in situ PLA using antibodies directed against GABA<sub>B1</sub> or GABA<sub>B2</sub> and Lys-63-linked ubiquitin. We detected no difference in Lys-63-linked ubiquitination between HEK cells expressing GABA<sub>B1</sub> alone and those expressing GABA<sub>B1</sub> plus GABA<sub>B2</sub>, suggesting that GABA<sub>B1</sub> is the main target for Lys-63-linked ubiquitination (Fig. 3A).

We then searched for potential lysine residues serving as ubiquitination sites in the GABA<sub>B1</sub> sequence by an in silico analysis. Four lysines with a high probability of being ubiquitinated were identified as follows: two in the cytoplasmic loop linking transmembrane domains three and four and two
Lysosomal Degradation of GABAB Receptors

A Cell surface GABA_B receptors

![Image of GABA_B receptors with and without ubiquitin modification]

B GABA_B1/K63-Ub

Control Leupeptin

![Image showing PLA signals]

C GABA_B2/K48-Ub

Control Leupeptin

![Image showing PLA signals]

FIGURE 2. Expression level of GABA_B receptors is regulated by Lys-63-linked ubiquitination. A, interference with Lys-63-linked ubiquitination increased the expression level of cell surface GABA_B receptors. Neurons were transfected with wild-type ubiquitin (Ub) or a ubiquitin mutant unable to form Lys-63-linked chains (Ub(K63R)) and analyzed for GABA_B receptor expression using GABA_B antibodies. Left, representative images of stained neuronal somata (scale bar, 5 μm). Right, quantification of fluorescence intensities. The fluorescence signal of neurons transfected with wild-type ubiquitin was set to 100%. The data represent the mean of 30–34 neurons from three (GABA_B1) and two (GABA_B2) independent experiments. **, p < 0.004; ***, p < 0.0001; two-tailed unpaired t test. B, inhibition of lysosomal activity enhanced Lys-63-linked ubiquitination of GABA_B receptors. Cortical neurons were incubated for 12 h with or without (control) 100 μM leupeptin (Leup) and analyzed for Lys-63-linked ubiquitination by in situ PLA using antibodies directed against GABA_B1 and Lys-63-linked ubiquitin (white dots in representative images; scale bar, 5 μm). Right, quantification of in situ PLA signals. The data represent the mean ± S.E. of 30–40 neurons from three independent experiments. ***, p < 0.00001; two-tailed unpaired t test. Ctrl, control. C, inhibition of lysosomal activity did not affect Lys-48-linked ubiquitination of GABA_B receptors. Cortical neurons were treated as in B and analyzed for Lys-48-linked ubiquitination by in situ PLA using antibodies directed against GABA_B2 and Lys-48-linked ubiquitin (white dots in representative images; scale bar, 5 μm). Right, quantification of in situ PLA signals. The data represent the mean ± S.E. of 27–37 neurons from three independent experiments; n.s., p > 0.05; two-tailed unpaired t test.

in the C-terminal domain (Fig. 3B). Inactivation of these sites by mutation to arginine (Lys → Arg) yielded the three mutants GABA_B1a(K697R/K698R), GABA_B1a(K892R), and GABA_B1a(K960R). To test whether these sites are ubiquitinated, HEK293 cells were transfected with either wild-type GABA_B1a or one of the GABA_B1a(Lys → Arg) mutants along with GABA_B2 and analyzed for Lys-63-linked ubiquitination by in situ PLA. Numerous in situ PLA signals in cells transfected with wild-type GABA_B1a indicated that a fraction of GABA_B1a is Lys-63-linked ubiquitinated under basal conditions. In contrast, all three mutant GABA_B1a displayed strongly reduced Lys-63-linked ubiquitination (GABA_B1a(K697R/K698R), 43 ± 3%; GABA_B1a(K892R), 38 ± 3%; GABA_B1a(K960R), 37 ± 3%, of wild-type GABA_B1a; Fig. 3A). This result indicates that lysines 697 and/or 698 and lysine 960 in GABA_B1a can be Lys-63-linked ubiquitinated under basal conditions.

Ubiquitination of GABA_B2 Regulates Cell Surface Expression of GABA_B Receptors—To analyze the effect of Lys-63-linked ubiquitination on cell surface expression of GABA_B receptors, we transfected neurons with wild-type GABA_B1a or GABA_B1a(Lys → Arg) mutants along with GABA_B2 and immunostained for their total and cell surface expression levels. Total as well as cell surface expression of all three GABA_B1a mutants was considerably increased as compared with transfected wild-type GABA_B1a (total, GABA_B1a(K697R/K698R), 457 ± 26%; GABA_B1a(K892R), 511 ± 30%; and GABA_B1a(K960R), 551 ± 22%, of wild-type GABA_B1a; cell surface, GABA_B1a(K697R/K698R), 508 ± 52%; GABA_B1a(K892R), 504 ± 48%; and GABA_B1a(K960R), 482.2 ± 42% of wild-type GABA_B1a; Fig. 4A and B). Likewise, the cell surface expression of GABA_B2 in neurons transfected with GABA_B1a(Lys → Arg) mutants was significantly increased (GABA_B2 in GABA_B1a(K697R/K698R)-transfected neurons, 158 ± 14%; GABA_B2 in GABA_B1a(K892R)-transfected neurons, 187 ± 17%; GABA_B2 in GABA_B1a(K960R)-transfected neurons, 178 ± 16% of control; Fig. 4B). The considerably lower increase in GABA_B2 cell surface expression as compared with mutant GABA_B1a was due to the fact that in the case of GABA_B2, only transfected subunits were assayed (HAV-tagged), but in the case of GABA_B1a transfected as well as endogenously expressed subunits were detected. The results demonstrate that inactivation of any of the ubiquitination sites in GABA_B1a (Lys-697/698, Lys-892, and Lys-960) decreased or prevented degradation of GABA_B receptors and therefore increased their cell surface expression.

Lysosomal Targeting of GABA_B Receptors Is Regulated by Ubiquitination of GABA_B—The increased total and cell surface expression levels of GABA_B1a(Lys → Arg) mutants and their reduced Lys-63-linked ubiquitination suggest that ubiquitination of these lysine residues serves as signals for sorting the receptors to lysosomes for degradation. If this is the case, GABA_B1a(Lys → Arg) mutants should be resistant to lysosomal degradation, and their expression levels should not increase upon blocking lysosomal degradation. Indeed, in contrast to the expression level of wild-type GABA_B1a, those of all three GABA_B1a(Lys → Arg) mutants remained unaffected by inhibition of lysosomal degradation with leupeptin (wild-type GABA_B1a, 249 ± 30%; GABA_B1a(K697R/K698R), 111 ± 5%; GABA_B1a(K892R), 109 ± 5%; and GABA_B1a(K960R), 108 ± 4% of control; Fig. 5A).

To confirm this finding, we prevented lysosomal degradation by overexpressing a functionally inactive mutant of the small GTPase Rab7 (Rab7(DN)). Rab7 mediates trafficking from early endosomes via late endosomes to lysosomes (23), and therefore overexpression of Rab7(DN) disrupts this pathway. In line with the pharmacological data, overexpression of Rab7(DN) considerably enhanced total expression of wild-type GABA_B1a but did
not significantly affect the expression levels of GABA_{1a}(Lys → Arg) mutants (wild-type GABA_{1a}, 174 ± 11%; GABA_{1a}(K697R/K698R), 105 ± 8%; GABA_{1a}(K892R), 117 ± 8%; and GABA_{1a}(K960R), 126 ± 9% of control; Fig. 5B). This indicates that preventing ubiquitination of specific sites in GABA_{1a} excluded the mutant receptors from entering the endosomal pathway that directs proteins to the lysosome. Therefore, our observations suggest that ubiquitination of multiple lysine residues in GABA_{1a} receptors regulates lysosomal degradation of GABA_{1a} receptors.

E3 Ligase Mindbomb-2 (MIB2) Mediates Lys-63-linked Ubiquitination of GABA_{1a}—In the next step, we aimed at identifying the E3 ligase mediating Lys-63-linked ubiquitination of GABA_{1a} receptors. A recent comprehensive proteomic study determined proteins that robustly interact with GABA_{1a} receptors and most likely build basic GABA_{1a} receptor signaling complexes (24). A few E3 ligases, which did not pass their stringent criteria for a robustly associated protein and thus were not regarded as a permanent member of a basic GABA_{1a} receptor signaling complex, emerged in their screens (MIB2, TRIM9, and MYCBP2; additional tested E3 ligases were RNF112, RNF144, RNF167, and RNF152). Upon overexpression of those E3 ligases in neurons, we found that MIB2 significantly reduced cell surface (59 ± 3% of control, Fig. 6A) as well as total (78 ± 3% of control, Fig. 6B) GABA_{1a} receptor expression. MIB2 extensively colocalized with GABA_{1a} receptors in neurons (Fig. 6C) and interacted with GABA_{1a} receptors as tested by in situ PLA (Fig. 6D).

Next we analyzed whether MIB2 mediates Lys-63-linked ubiquitination of GABA_{1a} receptors. To demonstrate directly Lys-63-linked ubiquitination of GABA_{1a} receptors by MIB2, we overexpressed MIB2 in neurons and tested for increased Lys-63-linked ubiquitination using in situ PLA. In fact, overexpression of MIB2 in neurons increased Lys-63-linked ubiquitination of GABA_{1a} receptors to 156 ± 16% of controls (Fig. 7A). This result was corroborated by the observation that overexpression of mutant ubiquitin, which cannot form Lys-63 linkages (Ub(K63R)), inhibited the MIB2 effect on GABA_{1a} receptors (Fig. 7B). In contrast overexpression of MIB2 in neurons with either wild-type ubiquitin (WT Ub (59 ± 3% of control), mutant ubiquitin that can only form Lys-63 linkages (Ub(Lys-63), 64 ± 6% of control), or mutant ubiquitin that is deficient in Lysosomal Degradation of GABA_{1a} Receptors

FIGURE 3. Identification of Lys-63-linked ubiquitination sites in GABA_{1a}. A, GABA_{1a} is the main target for Lys-63-linked ubiquitination. HEK293 cells were either transfected with a GABA_{1a} mutant containing an inactivated ER retention signal (GABA_{1a}(RSAR)), which permits ER exit and cell surface targeting of the subunit when expressed alone, or with GABA_{1a} and GABA_{2a} and tested for Lys-63-linked ubiquitination by in situ PLA using GABA_{1a} antibodies in combination with an antibody detecting Lys-63-linked ubiquitin (white dots in representative images, scale bar, 7 μm). The data represent the mean ± S.E. of 47–49 neurons from three independent experiments. ns, p > 0.05; two-tailed unpaired t test. B, decreased Lys-63-linked ubiquitination of GABA_{1a}(Lys → Arg) mutants. Cortical neurons were transfected with HA-tagged wild-type GABA_{1a}, HA-tagged GABA_{1a}(K697R/K698R), HA-tagged GABA_{1a}(K892R), or HA-tagged GABA_{1a}(K960R) together with wild-type GABA_{1a} and analyzed for Lys-63-linked ubiquitination by in situ PLA using antibodies directed against the HA tag and Lys-63-linked ubiquitin (white dots in representative images, scale bar, 7 μm). B’, quantification of in situ PLA signals. B”, schematic depicting the location of Lys → Arg mutations in GABA_{1a}. The data represent the mean ± S.E. of 26–35 neurons from three independent experiments. ns, p > 0.05; ***, p < 0.0001; one-way ANOVA, Bonferroni’s Multiple Comparison test.
To further substantiate that Lys-63-linked ubiquitination is mediated via MIB2, we analyzed the effect of overexpression of MIB2 on the three GABAB1a (Lys → Arg) mutants, which are partially resistant to Lys-63-linked ubiquitination. In this set of experiments, overexpression of MIB2 reduced cell surface expression of wild-type GABAB1 to 33 ± 4% of controls (Fig. 8). However, cell surface expression of all three mutants remained unaffected by overexpression of MIB2 (Fig. 8).

**Sustained Activation of Glutamate Receptors Increases Lys-63-Linked Ubiquitination of GABAB Receptors via MIB2—Prolonged activation of glutamate receptors (AMPA as well as NMDA receptors) leads to down-regulation of GABAB receptors via lysosomal degradation (16–18). To investigate whether Lys-63-linked ubiquitination of GABAB receptors serves as a lysosomal sorting signal in this process, we first tested whether the three GABAB1a (Lys → Arg) mutants, which are partially resistant to Lys-63-linked ubiquitination, are resistant to glutamate-induced down-regulation. In contrast to the cell surface expression of wild-type GABAB1a (56 ± 8% of control, Fig. 9), the levels of all three GABAB1a (Lys → Arg) mutants remained unaffected by glutamate (GABAB1a (K697R/K698R), 90 ± 12%; GABAB1a (K892R), 115 ± 11%; GABAB1a (K960R), 108 ± 5% of control; Fig. 9). This suggests that Lys-63-linked ubiquitination of GABAB1a is the signal for down-regulating the receptors.

To directly test for ubiquitination of the receptors in this mechanism, we exposed cortical neurons for 30 min to glutamate and determined Lys-63-linked ubiquitination of the receptors via in situ PLA. As expected, sustained activation of glutamate receptors strongly increased Lys-63-linked ubiquitination of GABAB receptors (203 ± 34% of control; Fig. 10A).

Next we tested whether preventing Lys-63-linked ubiquitination inhibits the down-regulation of GABAB receptors after treating neurons with glutamate. For this, cortical neurons were transfected either with wild-type Ub, a mutant of ubiquitin in which all lysines were mutated to arginines thereby preventing chain elongation and thus any kind of polyubiquitination (Ub(KO)), or with a mutant in which all lysines were mutated to arginines except for Lys-63 (Ub(Lys-63), able to form only Lys-63-linked ubiquitination) and stained for cell surface GABAB receptors after sustained glutamate application. Glutamate induced down-regulation of GABAB receptors from the plasma membrane in neurons expressing wild-type ubiquitin (Ub(WT), 53 ± 5% of control; Fig. 10B) or the mutant that only permits Lys-63-linked ubiquitination (Ub(Lys-63), 61 ± 6% of control, Fig. 10B) but not in neurons expressing the mutant unable to build polyubiquitin chains (Ub(KO), 95 ± 11% of control, Fig. 10B).

Finally, we analyzed whether MIB2 is involved in glutamate-induced down-regulation of the receptors. Interestingly, treatment of neurons with glutamate significantly increased MIB2 expression in neurons (15 min of glutamate, 150 ± 9% of control; 30 min of glutamate, 179 ± 8% of control; Fig. 11A) and strongly increased the interaction of MIB2 with GABAB receptors as tested with in situ PLA (15 min of glutamate, 155 ± 13% of control; 30 min of glutamate, 218 ± 25% of control; Fig. 11B).

These findings suggest that sustained activation of glutamate receptors induces MIB2-mediated Lys-63-linked ubiq-
ultination of GABA<sub>B</sub> receptors, promoting their lysosomal degradation.

**Discussion**

The signaling strength of G protein-coupled receptors largely depends on the number of receptors present in the plasma membrane. The mechanisms determining cell surface expression of the receptors include exocytosis, endocytosis, recycling, and degradation. GABA<sub>B</sub> receptors assemble into heterodimeric GABA<sub>B1,2</sub> complexes in the ER, which is a prerequisite for their ER exit and forward trafficking to the plasma membrane. After reaching the cell surface, GABA<sub>B</sub> receptors are constitutively internalized and either recycled to the plasma membrane or degraded in lysosomes (25). Both forward trafficking of GABA<sub>B</sub> receptors to the cell surface as well as their residence time at the cell surface are tightly regulated by controlled degradation of the receptors. The amount of GABA<sub>B</sub> receptors available for forward trafficking to the plasma membrane in the ER is adjusted by proteasomal degradation of the receptors via the ERAD machinery depending on the activity level of the neuron (7, 8). In contrast, the amount of receptors degraded in lysosomes after internalization from the cell surface depends on mechanisms sorting the endocytosed receptors to either lysosomes or recycling endosomes. Interfering with recycling rapidly depletes the receptors from the cell surface by redirecting them to the lysosomal degradation pathway (10). Rapid down-regulation of cell surface GABA<sub>B</sub> receptors by rerouting the receptors to lysosomes appears to be associated with pathological conditions as it is induced by sustained activation of glutamate receptors, which is a characteristic of brain ischemia (16–19). The factors triggering lysosomal degradation of GABA<sub>B</sub> receptors were unknown, however. The results of this study provide evidence that MIB2-mediated Lys-63-linked ubiquitination of GABA<sub>B1</sub> sorts GABA<sub>B</sub> receptors to lysosomes for degradation under physiological and pathological conditions.

We found that pharmacological inhibition of lysosomal activity increased not only total GABA<sub>B</sub> receptor levels, which was expected due to the intracellular accumulation of the receptors (9), but also considerably enhanced cell surface expression of the receptors. This finding implies that regulating lysosomal degradation of GABA<sub>B</sub> receptors directly affects their cell surface expression, which in turn determines the strength of GABA<sub>B</sub> receptor signaling (7). Here we provide evidence that Lys-63-linked ubiquitination of GABA<sub>B1</sub> is required for lysosomal degradation of GABA<sub>B</sub> receptors. First, blocking global Lys-63-linked ubiquitination by overexpressing a ubiquitin mutant (K63R) that is unable to form Lys-63-linked chains significantly increased cell surface expression of GABA<sub>B</sub> receptors. Second, blocking lysosomal activity considerably increased the level of Lys-63-linked ubiquitination of GABA<sub>B1</sub> receptors while leaving the level of Lys-48-linked ubiquitination, which tags the receptors for proteasomal degradation (7), unaffected. Mutational inactivation of potential ubiquitination sites in GABA<sub>B1</sub> (Lys-697/Lys-698, Lys-892, and Lys-960)
strongly decreased Lys-63-linked ubiquitination of GABAB receptors containing the respective GABAB1 mutant and prevented their lysosomal degradation as indicated by their dramatically increased expression level and insensitivity to the effect of blocking lysosomal degradation (either by inhibiting lysosomal proteases by leupeptin or by overexpression of a functionally inactive mutant of Rab7, which inhibits transport of cargo from late endosomes to the lysosome and blocks lysosome biogenesis). Any of the three GABAB1 mutants (K697R/K698R, K892R, and K960R) appeared to completely prevent lysosomal degradation of the receptors, suggesting that ubiquitination of Lys-697/Lys-698, Lys-892, and Lys-960 in GABAB1 is mandatory for lysosomal degradation of GABAB receptors. A similar situation was reported for targeting EGF receptors to lysosomal degradation. Multiple Lys-63-linked ubiquitination sites were identified, and mutation of each site prevented degradation of the receptors (26). It is currently unclear at which stage of intracellular sorting Lys-697/Lys-698, Lys-892, and Lys-960 in GABAB1 need to be ubiquitinated. They may be ubiquitinated simultaneously at a certain sorting step, or alternatively, they may be sequentially ubiquitinated at distinct sorting checkpoints. Addressing this issue in relation to the ESCRT pathway for sorting the receptors to lysosomes is an important question that requires further investigation.

Lysosomal degradation of G protein-coupled receptors is predominantly mediated via the ESCRT machinery (27), which guides mono- and Lys-63-linked ubiquitinated membrane pro-
Therefore, our observation that Lys-63-linked ubiquitination tags GABAB receptors for lysosomal degradation indicates that the ESCRT machinery also sorts GABAB receptors to lysosomes. This view is supported by the finding that the ESCRT I complex component TGS101 (29) is required for lysosomal degradation of GABAB receptors (13). In addition, the deubiquitination enzyme USP14 has been implicated in lysosomal degradation of GABAB receptors (15). Deubiquitination of proteins is an integral part of ESCRT-mediated degradation. Deubiquitinases associated with the ESCRT-0 complex are thought to rescue proteins from degradation by deubiquitination at an early step of lysosomal targeting, whereas deubiquitinases recruited to ESCRT-III recycle ubiquitin before the cargo protein is being degraded in the lysosome (14). However, USP14 appears not to be involved in these classical functions. Instead, USP14 interacts with GABAB receptors and contributes to their lysosomal targeting independent of its deubiquitinating activity, in an as yet undefined way (15).

So far, information on the E3 ubiquitin ligases mediating ubiquitination of GABAB receptors is almost entirely lacking. We previously found that the prototypical ERAD E3 ligase Hrd1 interacts with GABAB receptors residing in the ER and is most likely responsible for Lys-48-linked ubiquitination of GABAB2, which tags the receptors for proteasomal degradation (7). Here we identified MIB2 as the E3 ubiquitin ligase mediating Lys-63-linked ubiquitination of GABAB1, tagging the receptors for lysosomal degradation. MIB2 was detected in a proteomic screen, but it did not fulfill the rigorous criteria of the authors for a robustly GABAB receptor-associated protein (24). However, we found that MIB2 in fact colocalized with GABAB receptors in neurons and interacted with GABAB receptor complexes as tested by in situ PLA.

MIB2 belongs to the class of RING (really interesting new gene) domain E3 ligases composed of two separate substrate recognition domains in its N-terminal portion and two RING domains with the ubiquitin ligase activity in the C-terminal portion (30). The best described function of MIB2 is the ubiquitination and internalization of Notch ligands (31). Because Notch signaling in the adult brain is involved in synaptic plasticity, memory, and learning, MIB2-deficient mice displayed impaired hippocampal long-term potentiation and spatial memory as well as contextual fear memory (32). Apart from regulating Notch signaling, MIB2 has been shown to control diverse systems. For instance, it mediates Lys-63-linked ubiquitination of TANK-binding kinase 1 resulting in interferon regulatory factor 3/7 activation (33); it controls NF-κB activation (34), and it ubiquitinates the NR2B subunit of NMDA receptors to down-regulate their activity (35). Our experiments using mutant ubiquitin, GABAB receptor ubiquitination-deficient mutants, as well as in situ PLA indicate that MIB2 medi-
conceivable that sustained activation of glutamate receptors may increase the activity of an MIB2-associated deubiquitase, which prevents auto-ubiquitination and proteasomal degradation of MIB2. The enhanced expression of MIB2 was accompanied by an increased interaction of MIB2 with GABA_B receptors and an elevated Lys-63-linked ubiquitination. Interfering with Lys-63-linked ubiquitination by overexpressing ubiquitin mutants or our GABA_B_Lys → Arg) mutants prevented glutamate-induced down-regulation of the receptors. These results indicate that MIB2-mediated Lys-63-linked ubiquitination is indispensable for down-regulating the receptors via the lysosomal pathway and that the level of lysosomal degradation of the receptors is, at least in part, dependent on the expression level of MIB2.

In conclusion, our data suggest that MIB2-mediated Lys-63-linked ubiquitination of GABA_B sorts GABA_B receptors to lysosomes for degradation under physiological as well as pathological conditions.

### Experimental Procedures

**Antibodies**—The following antibodies were used: mouse anti-HA (1:1000 for immunofluorescence, 1:500 for in situ PLA, Sigma); rabbit GABA_B directed against the N terminus of GABA_B (affinity-purified, 1:200 for immunofluorescence, custom-made by GenScript) (37); rabbit GABA_B directed against the N terminus of GABA_B (affinity-purified, 1:500 for immunofluorescence; custom-made by GenScript) (38); guinea pig GABA_B (1:500 for immunofluorescence; Millipore catalog no. AB2255, lot no. 2484228); mouse GABA_B (1:100 for PLA; NeuroMab, clone N93A/49, catalog no. 7-183); rabbit ubiquitin Lys-48-specific (clone Aup2, 1:50 for in situ PLA; Millipore, catalog no. 05-1307, lot no. 2385989); rabbit ubiquitin Lys-63-specific (clone Aup3, 1:50 for in situ PLA; Millipore, catalog no. 05-1308, lot no. 2575910); and rabbit MIB2 (1:1000 for immunofluorescence, 1:250 for PLA; MyBiosource catalog no. MBS2014413, lot no. A20160407515). Secondary antibodies were purchased from Jackson ImmunoResearch labeled with either Alexa Fluor 488 (1:800), Cy-3 (1:500), or Cy-5 (1:1000).

**Drugs**—The following chemicals were used for this study: glutamate (50 μM, Sigma) and leupeptin (100 μM, Sigma).

**Plasmids**—The following DNAs were used: HA-tagged GABA_B1 (39); GABA_B1 (RSAR) (22); GABA_B2 (40); HA-tagged ubiquitin (Addgene plasmid 17608); HA-tagged ubiquitin (Addgene plasmid 17603); HA-tagged ubiquitin Lys-63 (Addgene plasmid 17606); and HA-tagged ubiquitin K48R (Addgene plasmid 17604) (41). HA-tagged ubiquitin K63R was kindly provided by L.-Y. Liu-Chen, Temple University, Philadelphia; wild-type EGFP-tagged Rab7 was from Addgene (plasmid 12605); the functionally inactive mutant EGFP-tagged Rab7 (DN) was from Addgene (plasmid 12606); and HA-tagged MIB2 was from Addgene (plasmid 35312) (34).

**Mutation of GABA_B**—Lysines 697, 698, 892, and 960 in GABA_B were mutated to arginines using the QuikChange II XL site-directed mutagenesis kit from Stratagene according to the manufacturer’s instructions.

**Culture and Transfection of Cortical Neurons**—Primary neuronal cultures of cerebral cortex were prepared from 18-day-old embryos of Wistar rats as described previously (10). Neur-
Lysosomal Degradation of GABA<sub>B</sub> Receptors

**FIGURE 11. Glutamate exposure increases the expression level of MIB2 and the MIB2-GABA<sub>B</sub> receptor interaction.** **A**, increased MIB2 expression after glutamate exposure. Neurons were treated either for 15 or 30 min with glutamate and analyzed for MIB2 expression. Left, representative images, scale bar, 10 µm. Right, quantification of fluorescence intensities. The data represent the mean ± S.E. of 30 neurons from two independent experiments. **B**, increased interaction of GABA<sub>B</sub> receptors with MIB2 after glutamate exposure. Neurons were treated either for 15 or 30 min with glutamate and analyzed for the interaction of MIB2 with GABA<sub>B</sub> receptors using in situ PLA. Left, representative images, scale bar, 5 µm. Right, quantification of the in situ PLA signals. The data represent the mean ± S.E. of 14 neurons from two independent experiments. ***, p < 0.0003; one-way ANOVA, Dunnett’s Multiple Comparison test.

**Author Contributions**—K. Z. conceived and conducted most of the experiments, analyzed the data, and contributed to writing the manuscript. C. T. conducted and analyzed the experiments shown in Figs. 3B, 4, and 5. D. B. conceived the project, analyzed the data, and contributed to writing the manuscript.

**Acknowledgments**—We are grateful to Dr. Jean-Marc Fritschy for continued support in microscopy and for providing E18 rat cortex and Thomas Grampp for excellent technical assistance.

**References**

1. Komander, D., and Rape, M. (2012) The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203–229
2. Benke, D., Zemoura, K., and Maier, P. J. (2012) Modulation of cell surface GABA<sub>B</sub> receptors by desensitization, trafficking and regulated degradation. *World J. Biol. Chem.* **3**, 61–72
3. Pinard, A., Seddik, R., and Bettler, B. (2010) GABA<sub>B</sub> receptors: physiological functions and mechanisms of diversity. *Adv. Pharmacol.* **58**, 231–255
4. Craig, M. T., and McBain, C. J. (2014) The emerging role of GABA<sub>B</sub> receptors as regulators of network dynamics: fast actions from a ‘slow’ receptor? *Curr. Opin. Neurobiol.* **26**, 15–21
Lysosomal Degradation of GABA<sub>B</sub> Receptors

5. Kohl, M. M., and Paulsen, O. (2010) The roles of GABA<sub>B</sub> receptors in cortical network activity. *Adv. Pharmacol.* 58, 205–229

6. Gaiarsa, J. L., and Porcher, C. (2013) Emerging neurotrophic role of GABA<sub>B</sub> receptors in neuronal circuit development. *Front. Cell. Neurosci.* 7, 206

7. Zemoura, K., Skenkel, M., Acuña, M. A., Yévenes, G. E., Zeullenhofer, H. U., and Benke, D. (2013) Endoplasmic reticulum-associated degradation (ERAD) controls cell surface expression of γ-aminobutyric acid, type B receptors. *J. Biol. Chem.* 288, 34897–34905

8. Zemoura, K., and Benke, D. (2014) Proteosomal degradation of γ-aminobutyric acid receptors is mediated by the interaction of the GABA<sub>B</sub><sub>2</sub>C terminus with the proteosomal ATPase Rpt6 and regulated by neuronal activity. *J. Biol. Chem.* 289, 7738–7746

9. Grampp, T., Sauter, K., Markovic, B., and Benke, D. (2007) γ-Aminobutyric acid type B receptors are constitutively internalized via the clathrin-dependent pathway and targeted to lysosomes for degradation. *J. Biol. Chem.* 282, 24157–24165

10. Grampp, T., Notz, V., Broll, I., Fischer, N., and Benke, D. (2008) Constitutive, agonist-accelerated, recycling and lysosomal degradation of GABA<sub>B</sub> receptors in cortical neurons. *Mol. Cell. Neurosci.* 39, 628–637

11. Vargas, K. J., Terunuma, M., Tello, J. A., Pangalos, M. N., Moss, S. J., and Couve, A. (2008) The availability of surface GABA<sub>B</sub> receptors is independent of γ-aminobutyric acid but controlled by glutamate in central neurons. *J. Biol. Chem.* 283, 24641–24648

12. Hannan, S., Wilkins, M. E., Dehghani-Tafti, E., Thomas, P., Baddeley, S. M., and Smart, T. G. (2011) GABA<sub>B</sub> receptor internalisation is regulated by the R2 subunit. *J. Biol. Chem.* 286, 24324–24335

13. Kantamneni, S., Holman, D., Wilkinson, K. A., Correia, S. A., Feligioni, M., Ogden, S., Fraser, W., Nishimune, A., and Henley, J. M. (2008) GISP binding to TSG101 increases GABA<sub>B</sub> receptor stability by down-regulating ESCRT-mediated lysosomal degradation. *J. Neurochem.* 107, 86–95

14. Raiborg, C., and Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* 456, 445–452

15. Lahaie, N., Krallkova, M., Prézeau, L., Blahos, J., and Bouvier, M. (2016) Post-endocytic deubiquitination and degradation of the metabotropic γ-aminobutyric acid receptor by the ubiquitin-specific protease 14. *J. Biol. Chem.* 291, 7156–7170

16. Guert, N., Abdel Aziz, S., Holbro, N., Turecek, R., Rose, T., Sieddik, R., Gassmann, M., Moes, S., Jenoe, P., Oertner, T. G., Casanova, E., and Betler, B. (2010) NMDA receptor-dependent GABA<sub>B</sub> receptor internalization via CaMKII phosphorylation of serine 867 in GABA<sub>B</sub>1. *J. Biol. Chem.* 285, 37147–37157

17. Maier, P. J., Marin, L., Grampp, T., Sommer, A., and Benke, D. (2010) Sustained glutamate receptor activation down-regulates GABA<sub>B</sub> receptors by shifting the balance from recycling to lysosomal degradation. *J. Biol. Chem.* 285, 35606–35614

18. Terunuma, M., Vargas, K. J., Wilkins, M. E., Ramirez, O. A., Jaureguiberry-Bravo, M., Pangalos, M. N., Smart, T. G., Moss, S. J., and Couve, A. (2010) Prolonged activation of NMDA receptors promotes dephosphorylation of the regional and subcellular distribution. *J. Biol. Chem.* 285, 37147–37157

19. Jurd, R., Thornton, C., Wang, J., Luong, K., Kharazia, V., Gibson, S. L., and Ron, D. (2008) Mind-2 is an E3 ligase that ubiquitinates the N-methyl-D-aspartate receptor NR2B subunit in a phosphorylation-dependent manner. *J. Biol. Chem.* 283, 301–310

20. Tseng, L. C., Zhang, C., Cheng, C. M., Xu, H., Hsu, C. H., and Ji, Y. (2014) Lysine 63-linked TANK-binding kinase 1 ubiquitination by mindbomb E3 ubiquitin protein ligase 2 is mediated by the mitochondrial antiviral signaling protein. *J. Virol.* 88, 12765–12776

21. Stempin, C. C., Shi, L., Giraldo-Vela, J. P., High, A. A., Häcker, H., and Redecke, V. (2011) The E3 ubiquitin ligase mind bomb-2 (MIB2) protein controls B-cell CLL/lymphoma 10 (BCL10)-dependent NF-κB activation. *J. Biol. Chem.* 286, 37147–37157

22. Lim, K. L., Chew, K. C., Tan, J. M., Wang, C., Chung, K. K., Zhang, Y., et al. (2002) C-terminal interaction is essential for the formation and function of the Mind bomb E3 ligase in the context of Notch signal transduction. *Curr. Opin. Biol.* 14, 38–45

23. Benke, D. (2010) Mechanisms of GABA<sub>B</sub> receptor exocytosis, endocytosis, and degradation. *Adv. Pharmacol.* 58, 93–111

24. Schweng, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) Differential regulation of EGF receptor internalization and degradation by multiple ubiquitination within the kinase domain. *Mol. Cell.* 21, 737–748

25. Dores, M. R., and Trejo, I. (2014) Atypical regulation of G protein-coupled receptor intracellular trafficking by ubiquitination. *Curr. Opin. Cell Biol.* 27, 44–50

26. Lauwers, E., Jacob, C., and André, B. (2009) Lys-63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway. *J. Cell Biol.* 185, 493–507

27. Hurley, J. H., and Emr, S. D. (2006) The ESCRT complexes: structure and mechanism of a membrane-trafficking network. *Annu. Rev. Biophys. Biomol. Struct.* 35, 277–298

28. Koo, B. Y., Koon, K. J., Yoo, K. W., Lim, H. S., Song, R., So, J. H., Kim, C. H., and Kang, Y. Y. (2005) Mind bomb-2 is an E3 ligase for Notch ligand. *J. Biol. Chem.* 280, 22335–22342

29. Kim, S., Kim, T., Lee, H. R., Kong, Y. Y., and Kang, K. B. (2015) Mind bomb-2 regulates hippocampus-dependent memory formation and synaptic plasticity. *Korean J. Physiol. Pharmacol.* 19, 515–522

30. Ye, J. S., Kim, N., Lee, K. J., Nam, Y. R., Lee, U., and Joo, C. H. (2014) Lysine 63-linked TANK-binding kinase 1 ubiquitination by mindbomb E3 ubiquitin protein ligase 2 is mediated by the mitochondrial antiviral signaling protein. *J. Virol.* 88, 12765–12776

31. Steppin, C. C., Shi, L., Giraldo-Vela, J. P., High, A. A., Häcker, H., and Redecke, V. (2011) The E3 ubiquitin ligase mind bomb-2 (MIB2) protein controls B-cell CLL/lymphoma 10 (BCL10)-dependent NF-κB activation. *J. Biol. Chem.* 286, 37147–37157

32. Kaupmann, K., Hugel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W., and Better, B. (1997) Expression cloning of GABA<sub>B</sub> receptors uncovers similarity to metabolotropic glutamate receptors. *Nature* 386, 239–246

33. Kaupmann, K., Malitschek, B., Schulert, V., Heid, J., Froestl, W., Beck, P., Mosbacher, I., Bischoff, S., Kulik, A., Shimemoto, R., Karschin, A., and Better, B. (1998) GABA<sub>B</sub>-receptor subtypes assemble into functional heteromeric complexes. *Nature* 396, 683–687

34. Lim, K. L., Chew, K. C., Tan, J. M., Wang, C., Chung, K. K., Zhang, Y., Tanaka, Y., Smith, W., Engelender, S., Ross, C. A., Dawson, V. L., and Dawson, T. M. (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. *J. Neurosci.* 25, 2002–2009

35. Choudhury, A., Dominguez, M., Puri, V., Sharma, D. K., Narita, K., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2002) Rab proteins mediate Golgi transport of caveola-internized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J. Clin. Invest.* 109, 1541–1550
43. Buerli, T., Pellegrino, C., Baer, K., Lardi-Studler, B., Chudotvorova, I., Fritschy, J. M., Medina, I., and Fuhrer, C. (2007) Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. *Nat. Protoc.* 2, 3090–3101

44. Maier, P. J., Zemoura, K., Acuña, M. A., Yévenes, G. E., Zeilhofer, H. U., and Benke, D. (2014) Ischemia-like oxygen and glucose deprivation mediates down-regulation of cell surface γ-aminobutyric acid, receptors via the endoplasmic reticulum (ER) stress-induced transcription factor CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP). *J. Biol. Chem.* 289, 12896–12907

45. Leuchowius, K. J., Jarvius, M., Wickström, M., Rickardson, L., Landegren, U., Larsson, R., Söderberg, O., Fryknäs, M., and Jarvius, J. (2010) High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol. Cell. Proteomics* 9, 178–183

46. Söderberg, O., Gullberg, M., Jarvius, M., Ridderstråle, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods* 3, 995–1000