Cellular/Molecular

$G\alpha_{o2}$ Regulates Vesicular Glutamate Transporter Activity by Changing Its Chloride Dependence

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Classical neurotransmitters, including monoamines, acetylcholine, glutamate, GABA, and glycine, are loaded into synaptic vesicles by means of specific transporters. Vesicular monoamine transporters are under negative regulation by α subunits of trimeric G-proteins, including $G\alpha_{o2}$ and $G\alpha_q$. Furthermore, glutamate uptake, mediated by vesicular glutamate transporters (VGLUTs), is decreased by the nonhydrolysable GTP-analog guanylylimidodiphosphate. Using mutant mice lacking various $G\alpha$ subunits, including $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, and $G\alpha_{11}$, and a $G\alpha_{o2}$-specific monoclonal antibody, we now show that VGLUTs are exclusively regulated by $G\alpha_{o2}$. G-protein activation does not affect the electrochemical proton gradient serving as driving force for neurotransmitter uptake; rather, $G\alpha_{o2}$ exerts its action by specifically affecting the chloride dependence of VGLUTs. All VGLUTs show maximal activity at ~5 mM chloride. Activated $G\alpha_{o2}$ shifts this maximum to lower chloride concentrations. In contrast, glutamate uptake by vesicles isolated from $G\alpha_{o2}^{-/-}$ mice have completely lost chloride activation. Thus, $G\alpha_{o2}$ acts on a putative regulatory chloride binding domain that appears to modulate transport activity of vesicular glutamate transporters.

Key words: **$G\alpha_{o2}$; VGLUT; regulation; vesicular transmitter transporter; presynaptic; plasticity; chloride dependence**

Introduction

Neurons transmit signals by means of neurotransmitters. In presynaptic terminals, neurotransmitters are stored in synaptic vesicles that sequester them from the surrounding cytosol via specific transporters. After an action potential, synaptic vesicles fuse with the plasma membrane and release their transmitter content into the synaptic cleft, resulting in the activation of postsynaptic receptors.

The amount of neurotransmitter released by an action potential at a given synapse is subject to multiple regulatory mechanisms that together constitute the presynaptic contribution to synaptic plasticity. Whereas most of the attention has been focused on the molecular mechanisms underlying regulation of the vesicular release probability, relatively little attention has been given to presynaptic mechanisms involved in the regulation of quantal size. It is known that the transmitter content of individual synaptic vesicles may vary substantially (Van der Kloot et al., 2000, 2002) and that overexpression or deletion of vesicular amine as well as of vesicular glutamate transporters (VGLUTs) leads to an increase or decrease in intravesicular transmitter content (Song et al., 1997; Pothos et al., 2000; Travis et al., 2000; Fremeau et al., 2004; Wojcik et al., 2004). Thus, overall transport activity of the vesicular transporters directly influences synaptic performance.

Glutamate is the main excitatory transmitter in brain, and most studies addressing mechanisms of synaptic plasticity focus on glutamatergic synapses. Three homologous vesicular glutamate transporters have been identified, termed VGLUT1 (Bellocchio et al., 2000; Takamori et al., 2000), VGLUT2 (Bai et al., 2001; Fremeau et al., 2001; Hayashi et al., 2001; Takamori et al., 2001), and VGLUT3 (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002; Takamori et al., 2002). They are specific for glutamate (Takamori et al., 2000; Gras et al., 2002) and exhibit a relatively low substrate affinity (Bellocchio et al., 2000; Gras et al., 2002). VGLUT1 is the dominant transporter in cortex, hippocampus, and cerebellum (Fremeau et al., 2001; Fujiyama et al., 2001; Kaneko and Fujiyama, 2002), whereas VGLUT2 occurs preferentially in thalamic and hypothalamic regions (Hisano et al., 2000; Sakata-Haga et al., 2001; Fremeau et al., 2002; Fujiyama et al., 2001; Kaneko and Fujiyama, 2002). VGLUT3 is found as an additional transporter in serotonergic, cholinergic (Fremeau et al., 2002; Gras et al., 2002; Schäfer et al., 2002), and GABAergic (Fremeau et al., 2002) terminals.

In our own work, we showed previously that trimeric GTPases regulate vesicular neurotransmitter transporters, thus contributing to synaptic plasticity. We found that $G\alpha_{o2}$ is responsible for

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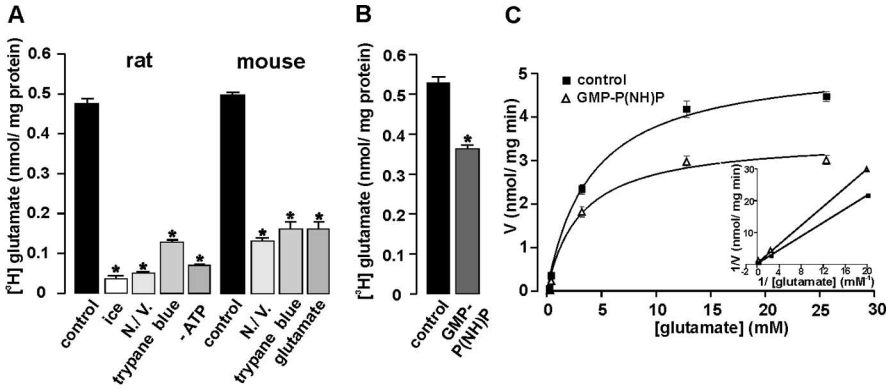


Figure 1. Inhibition of glutamate uptake into synaptic vesicles by GMP-P(NH)P. ***A***, Glutamate uptake was performed in KGC/ATP (2 mM ATP) for 10 min at 37°C using synaptic vesicles prepared from rat or mouse cortex. Specificity of uptake is shown by omitting ATP (−ATP), by incubating vesicles with KGC/ATP either on ice or in the presence of a mixture of 5 μM nigericine and 20 μM valinomycine (N./V.), or in the presence of 10 μM trypan blue or 10 mM glutamate. Values represent the mean ± SD of triplicates ($p < 0.00001$ according to Student's *t* test). ***B***, Glutamate uptake into rat brain vesicles was performed in KGC/ATP for 10 min at 37°C in the absence or presence of 50 μM GMP-P(NH)P. Nonspecific glutamate binding was subtracted. Values represent the mean ± SD of triplicates ($p < 0.001$ according to Student's *t* test). ***C***, Rat brain synaptic vesicles were incubated with increasing glutamate concentrations in either the absence (filled rectangles) or presence (open triangles) of 50 μM GMP-P(NH)P. Note that V_{max} (5.358) and K_m (4.387) are decreased by GMP-P(NH)P (V_{max} of 3.504 and K_m of 3.103). Values represent the mean of three determinations, and nonspecific binding was subtracted. The experiment was repeated with V_{max} of 5.287 and K_m of 4.035 in controls and V_{max} of 4.035 and K_m of 3.095 in the presence of GMP-P(NH)P.

inhibition of the vesicular monoamine transporters, VMAT1 and VMAT2, present on either large dense-core or small synaptic vesicles (Ahnert-Hilger et al., 1998; Höltje et al., 2000; Pahner et al., 2002). We identified different types of G-protein β subunits as well as $G\alpha_o$ subunits on glutamatergic vesicles characterized by either VGLUT1 or VGLUT2. Furthermore, glutamate uptake was reduced by guanylyl 5′-imidodiphosphate [GMP-P(NH)P], an activator of G-proteins (Pahner et al., 2003).

Here, we investigated which of the two $G\alpha_o$ subunits, $G\alpha_{o1}$ and/or $G\alpha_{o2}$, are involved in regulating glutamate uptake and by which mechanism this regulation is mediated.

Materials and Methods

Antibodies and chemicals. Mouse monoclonal antibodies against $G\alpha_o$-proteins were prepared according to standard procedures using recombinant $G\alpha_{o2}$ as antigen (clone 101.1, 101.4) (Jahn et al., 1985). A monoclonal antibody against synaptophysin (clone 7.2) (Jahn et al., 1985) and polyclonal antibodies against the vesicular glutamate transporters VGLUT1 (Takamori et al., 2000), VGLUT2 (Takamori et al., 2001), and VGLUT3 (Takamori et al., 2002) were obtained from Synaptic Systems (Göttingen, Germany). A monoclonal antibody for synaptosome-associated protein of 25 kDa (SNAP-25) was purchased from Sternberger Monoclonals (Baltimore, MD). Polyclonal antibodies against the chloride channel proteins ClC3 and ClC7 were obtained from either Chemicon (Hofheim, Germany) or Biotrend (Köln, Germany), respectively. Secondary antibodies for Western blot detection, horse anti-mouse and goat anti-rabbit conjugated with horseradish peroxidase, were purchased from Vector Laboratories (Burlingame, CA).

GMP-P(NH)P, nigericin, valinomycin, and trypan blue were purchased from Sigma (München, Germany).

G-protein deletion mutants. Wild-type and $G\alpha_q{}^{-/-}$ and $G\alpha_{11}{}^{-/-}$ mice were kindly supplied by S. Offermanns (Institut für Pharmakologie, Heidelberg, Germany) and bred as given (Offermanns et al., 1997, 1998). $G\alpha_{o1}$ and $G\alpha_{o2}$ splice variant-specific deletion mutants were generated and bred as given (Jiang et al., 1998; Dhingra et al., 2002). The respective wild-type animals (129/Sv × C57BL/6) were bred and analyzed in parallel. For littermate experiments, wild-type (129/Sv × C57BL/6) and $G\alpha_{o2}$ splice variant-specific deletion mutants were cross bred, and the resulting heterozygous offspring were used to generate littermates with the same genetic background.

Uptake of glutamate into synaptic vesicles and acidification assay. A preparation enriched in synaptic vesicles (lysis pellet 2, LP2 fraction in the following referred to as synaptic vesicles) were prepared from either rat or mouse whole brain following the procedure described by Huttner et al. (1983). For glutamate uptake, the vesicles were suspended in KGC-buffer (in mM: 150 K^+-gluconate, 20 PIPES, 4 EGTA, 2.5 $MgCl_2$, pH 7.0, KOH), and 25 μl of this suspension (corresponding to 30–60 μg of protein) was added to individual tubes containing 25 μl of KGC supplemented with 4 mM ATP (2 mM final ATP concentration in KGC/ATP buffer) and with 99 μM glutamate and 1 μM [^{3}H]glutamate (L-[G-^{3}H]glutamic acid; specific activity, 1220 Bq/mmol; Amersham Biosciences Braunschweig, Germany) and the additives indicated. Uptake was performed for 10 min at 36°C and stopped by adding 500 μl of ice-cold KGC/ATP buffer. Samples were spun down (10 min, 460.000 × *g*) and washed once using KGC/ATP buffer. The pellets were lysed with 0.4% Triton X-100 to determine radioactivity by scintillation counting and protein content by the BCA method (Höltje et al., 2000). In experiments analyzing the chloride dependence, $MgCl_2$ was exchanged for $MgSO_4$, and K^+-gluconate was replaced by corresponding amounts of KCl. Nonspecific binding was performed in the presence of 5 μM nigericin and 20 μM valinomycin, in the absence of ATP (KGC buffer), or in the presence of 10 μM trypan blue and reached values between 0.05 and 0.12 nmol/mg protein. Values were expressed as nanomoles per milligram of protein and corrected for nonspecific binding.

Acidification from LP2 preparations from wild-type and $G\alpha_{o2}{}^{-/-}$ mice was performed using the pH-sensitive dye acridine orange as described previously (Hartinger and Jahn 1993; Takamori et al., 2000, 2001). Acidification was measured as a decrease in the absorbance at 492 nm with 530 nm as a reference using an SLM-Aminco (Rochester, NY) DW double beam spectrophotometer.

Immunoblot analysis. Synaptic vesicles (LP2) were prepared from mouse brains and subjected to SDS-PAGE and immunoblot analysis using the antisera given (Becher et al., 1999).

Results

Glutamate uptake by VGLUTs into synaptic vesicles depends on an electrochemical gradient driven by the vacuolar proton ATPase (V-ATPase). It thus requires ATP and is sensitive to low temperatures, to ionophores such as a combination of nigericin and valinomycin, which dissipate the proton and the electrical gradient, respectively (Hell et al., 1990; Takamori et al., 2000, 2001, 2002), and to trypan blue, which directly inhibits the transporters (Fonnum et al., 1998). Furthermore, [^{3}H]glutamate uptake is competed for by excess unlabeled glutamate as expected for a saturable transporter (Fig. 1*A*). As reported previously, glutamate uptake was inhibited by the G-protein activator GMP-P(NH)P (Pahner et al., 2003) (Fig. 1*B*). Addition of GMP-P(NH)P decreased V_{max} and K_m of glutamate uptake (Fig. 1*C*).

Glutamatergic vesicles, defined by the presence of either VGLUT1 or VGLUT2, contain both $G\alpha_o$ and $G\alpha_{q/11}$ subunits (Pahner et al., 2003). To identify the G-protein responsible for downregulating glutamate uptake, we took advantage of mouse deletion mutants lacking $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, or $G\alpha_{11}$, thus allowing for studying the contribution of each of these G-proteins in a clean genetic background. As reported previously, $G\alpha_o{}^{-/-}$, $G\alpha_{o1}{}^{-/-}$, and $G\alpha_q{}^{-/-}$ mice are viable but have severe CNS defects (Offermanns et al., 1997; Jiang et al., 1998; Dhingra et al.,

2002). For $G\alpha_{11}{}^{-/-}$, a distinct alteration in motor control has been observed (Hartmann et al., 2004). In contrast $G\alpha_{o2}{}^{-/-}$ mice develop normally with no obvious change in their phenotype (Dhingra et al., 2002) (L. Birnbaumer and M. Jiang, unpublished observations). We prepared synaptic vesicles from these mice and examined whether glutamate uptake is reduced during incubation with GMP-P(NH)P. As shown in Figure 2*A*, GMP-P(NH)P inhibited glutamate uptake into all vesicles, except those from $G\alpha_{o2}{}^{-/-}$ mice, which showed reduced glutamate uptake with no additional reduction during GMP-P(NH)P addition. In contrast, the GMP-P(NH)P sensitivity of glutamate uptake by synaptic vesicles obtained from mice lacking $G\alpha_{o1}$, $G\alpha_q$, and $G\alpha_{11}$, respectively, was indistinguishable from that of wild-type vesicles (Fig. 2*C–E*). These data indicate that $G\alpha_{o2}$ is solely responsible for VGLUT regulation.

To confirm the involvement of $G\alpha_{o2}$ by an independent approach, we asked whether antibodies generated against $G\alpha_{o2}$ are able to prevent GMP-P(NH)P-mediated inhibition of uptake in otherwise fully equipped vesicles. Monoclonal antibodies were raised using recombinant $G\alpha_{o2}$ as antigen, resulting in four clones, two of which (clone 101.1 and clone 101.4) were further characterized. Both antibodies recognize recombinant $G\alpha_{o2}$ (data not shown). As revealed by the analysis of brain extracts obtained from $G\alpha_{o1}$ and $G\alpha_{o2}$ knock-out mice, clone 101.1 cross-reacts with $G\alpha_{o1}$ with a higher avidity for this splice form than for $G\alpha_{o2}$, whereas clone 101.4 only interacts with $G\alpha_{o2}$ (Fig. 3*A*). When added to vesicles prepared from rats or wild-type mice, both monoclonal antibodies prevented the GMP-P(NH)P-mediated inhibition (Fig. 3*B*,*C*). As a negative control, we used a monoclonal antibody directed against an unrelated protein [the SNARE (SNAP receptor) SNAP-25]. The SNAP-25 monoclonal antibody had no effect on GMP-P(NH)P-mediated inhibition, as observed in either set of experiments (Fig. 3*B*,*C*).

Synaptic vesicles prepared from whole brain contain both VGLUT1 and VGLUT2. Therefore, it is not possible to assess whether $G\alpha_{o2}$ regulates both isoforms or whether regulation is confined to only one of the VGLUTs. However, it has been shown recently that, in cerebellum between postnatal day 1 (P1) and P10, VGLUT2 is the only expressed isoform, with expression switching to VGLUT1 later during postnatal development (Miyazaki et al., 2003; Boulland et al., 2004), providing the opportunity of isolating synaptic vesicles that contain exclusively VGLUT2. As can be seen in Figure 4, glutamate uptake by synaptic vesicles isolated at postnatal day 9 (P9) from both the cerebellum and whole brain was inhibited by GMP-P(NH)P. However, inhibition was less pronounced than in vesicles prepared from adult brains. Immunoblot analysis using synaptosomal membranes performed in parallel confirmed that both P9 preparations contained only VGLUT2 (Fig. 4*B*).

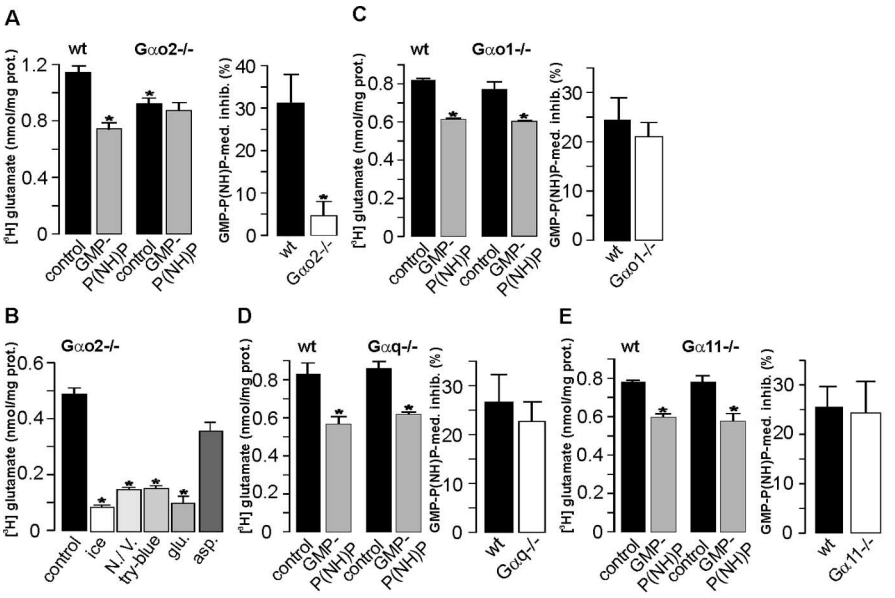


Figure 2. VGLUT activity and its inhibition by GMP-P(NH)P in deletion mutants of $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, and $G\alpha_{11}$. Synaptic vesicles were prepared from brains of $G\alpha_{o2}{}^{-/-}$ (***A***, ***B***), $G\alpha_{o1}{}^{-/-}$ (***C***), $G\alpha_q{}^{-/-}$ (***D***), and $G\alpha_{11}{}^{-/-}$ (***E***) mice and the corresponding wild types (wt). Glutamate uptake was performed in either the absence or presence of 50 μM GMP-P(NH)P. Values represent the mean of three determinations corrected for nonspecific binding. For each deletion mutant, experiments were repeated three times, and the respective GMP-P(NH)P-mediated inhibition of VGLUT activity is given in the smaller graphs in ***A–E***. Note that inhibition in all wild-type experiments was between 25 and 30%, as it was in the $G\alpha_{o1}{}^{-/-}$, $G\alpha_q{}^{-/-}$, and $G\alpha_{11}{}^{-/-}$ mutants. In $G\alpha_{o2}{}^{-/-}$ mice, GMP-P(NH)P did not downregulate VGLUT activity, which was also reduced in untreated controls compared with the wild-type vesicles (***A***). In $G\alpha_{o2}{}^{-/-}$ mice, nonspecific [^{3}H]glutamate accumulation was comparable with the one seen in wild-type mice and rats (compare Fig. 1*A*), whereas aspartate (10 mM) was almost not transported. $^*p < 0.04$ denotes significance according to Student's *t* test.

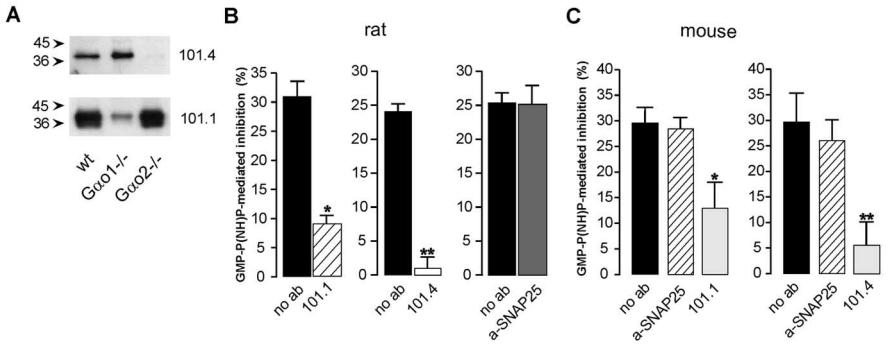


Figure 3. Abolition of GMP-P(NH)P-mediated inhibition of VGLUT activity by $G\alpha_{o2}$-specific antibodies. ***A***, Monoclonal antibodies clone 101.1 and clone 101.4 generated against recombinant $G\alpha_{o2}$ were tested for specificity by Western blotting using synaptic vesicle preparations from wild-type (wt), $G\alpha_{o1}{}^{-/-}$, or $G\alpha_{o2}{}^{-/-}$ mice. Whereas clone 101.1 recognizes both splice variants, clone 101.4 is $G\alpha_{o2}$ specific. ***B***, ***C***, Synaptic vesicles from rat (***B***) or mouse brain (***C***) were incubated with or without (no ab) the indicated dialyzed antibodies for 30 min on ice before they were subjected to glutamate uptake in the absence or presence of 50 μM GMP-P(NH)P. Values are presented as GMP-P(NH)P-mediated inhibition. Each experiment was performed in triplicate, and nonspecific glutamate binding was subtracted. The graphs in ***B*** represent the mean of three (clone 101.1) or two (clone 101.4; SNAP-25) independent experiments, and the graphs in ***C*** represent the mean of three (clone 101.1) or two (clone 101.4) independent experiments ($p < 0.005$ according to Student's *t* test). The final IgG concentrations used in the experiments were 862 μg/ml for SNAP-25 for rat and mouse vesicles and 646 μg/ml for clone 101.1 and 431 μg/ml for clone 101.4 in the experiments with either rat or mouse vesicles, respectively. Note that both $G\alpha_o$-antibodies overcome the GMP-P(NH)P-mediated downregulation of VGLUT activity with the $G\alpha_{o2}$-specific clone 101.4 being more efficient, whereas the antibody against SNAP-25 has no effect.

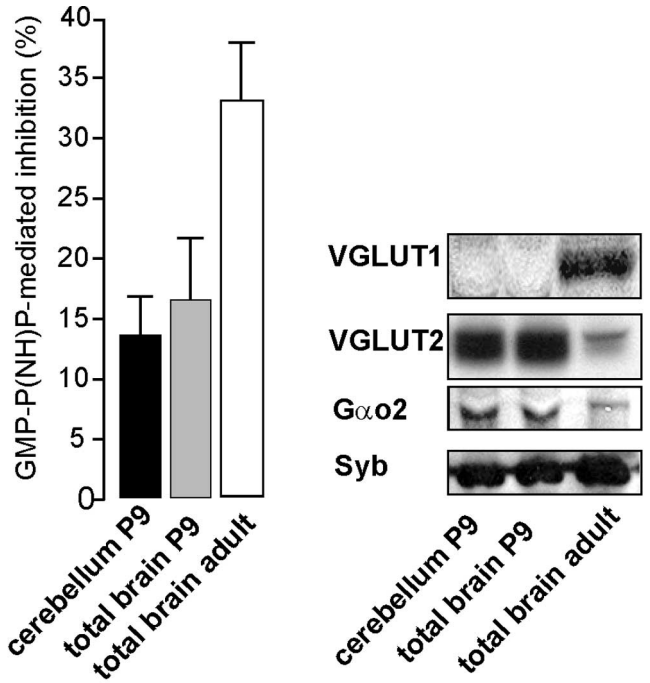


Figure 4. GMP-P(NH)P-mediated inhibition of VGLUT2. Synaptic vesicle-enriched membranes (LP2) were prepared from mouse cerebellum or whole brain either at P9 or from adult brain. Glutamate uptake was performed in either the absence or presence of 50 μM GMP-P(NH)P. Values represent the mean of three determinations corrected for nonspecific binding obtained in the presence of trypan blue and are presented as GMP-P(NH)P-mediated inhibition. Synaptosomal membranes (P2) from the same preparations were subjected to SDS-PAGE and Western blotting using the antibodies indicated. Synaptobrevin (Syb) and $G\alpha_{o2}$ were detected with the monoclonal antibody 69.1 or 101.4, respectively. Note that vesicle-enriched membranes from P9 contain only VGLUT2 and no VGLUT1, which is, however, clearly present in adult vesicular membranes. As expected, the amount of synaptobrevin analyzed for comparison was increased in adult synaptosomal membranes, whereas $G\alpha_{o2}$ appeared to be downregulated in adulthood.

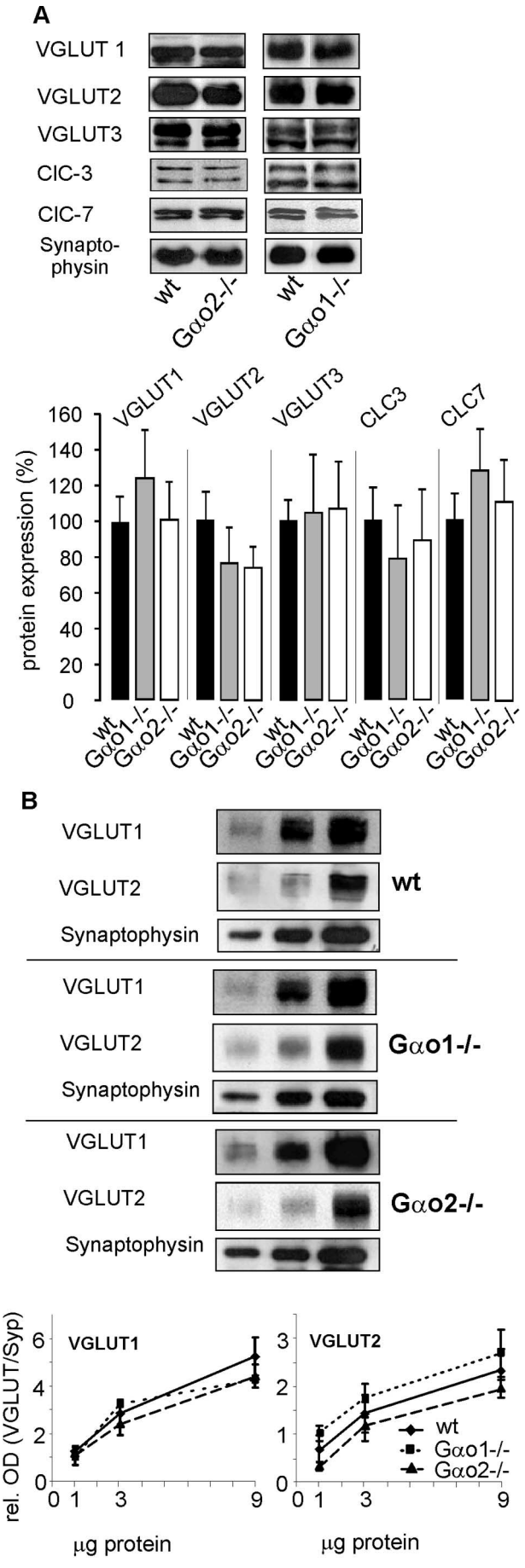
To rule out that the reduced uptake and the lack of regulation by G-protein activation in $G\alpha_{o2}^{-/-}$ mice is mediated by a diminished expression of vesicular proteins, especially VGLUTs and chloride channels, we compared the protein pattern of synaptic vesicle-enriched membranes prepared from wild-type mice with that prepared from $G\alpha_{o2}^{-/-}$ and $G\alpha_{o1}^{-/-}$ mice. The chloride channel ClC3 has been shown recently to be present on endosomes and synaptic vesicles, in which it appears to affect glutamate storage (Stobrawa et al., 2001), whereas ClC7 is a broadly expressed chloride channel localized to V-ATPases bearing endosomes and vesicles (Kornak et al., 2001). As can be seen the amounts of the chloride channels, CCl3 and CCl7 and of the three VGLUT isoforms were not changed in any of the knock-outs when synaptic vesicle-enriched membranes were used, with the exception of a slight but statistically not significant reduction in the amount of VGLUT2 (Fig. 5*A*) in $G\alpha_{o2}^{-/-}$ mice. A detailed analysis of synaptosomal membranes further confirmed that there was no change in the amounts of either synaptophysin or VGLUTs in both $G\alpha_o$ deletion mutants compared with wild-type mice (Fig. 5*B*).

Acidification of synaptic vesicles is mediated by the vacuolar H^+-ATPase, which needs a parallel chloride conductance for efficient pumping. The resulting electrochemical gradient $\Delta\mu H^+$ is the driving force for vesicular neurotransmitter uptake, with glutamate uptake being accompanied by increased proton influx under low chloride conditions. It is thus conceivable that G-protein regulation affects proton pumping rather than directly controlling VGLUT activity. To test for this possibility, ATP-stimulated acidification of synaptic vesicles from either wild-type or $G\alpha_{o2}^{-/-}$ mice was induced by 10 mM glutamate, followed by an addition of 30 mM chloride, which further increased acidification. There was no difference in the acidification by these subsequent additions. Similarly, no difference was observed when acidification was directly induced by either 30 or 150 mM chloride (Fig. 6).

The experiments described so far show that activation of $G\alpha_{o2}$ reduces glutamate uptake activity. These results were obtained under standard assay conditions, i.e., at a chloride concentration of 5 mM. Generally, VGLUTs are driven by an electrochemical proton gradient composed of a membrane potential ($\Delta\psi$, inside positive) and a pH gradient (ΔpH, inside acidic). Unlike positively charged monoamines that are directly exchanged for protons pumped in by the V-ATPase, glutamate is negatively charged, that excludes a similar mechanism unless charge compensation is mediated by other ions. At low chloride, glutamate uptake is driven by $\Delta\psi$ (Hell et al., 1990; Maycox et al., 1990), and charge neutrality is maintained by proton cotransport. This results in a glutamate-dependent acidification of the vesicle interior. However, it is unlikely that glutamate is taken up and stored as free glutamic acid under physiological conditions. Rather, charge balance may be maintained by chloride efflux through vesicular chloride channels (Maycox et al., 1990). Because recycling vesicles are likely to contain a high intravesicular chloride concentration, glutamate uptake would thus involve a net glutamate–chloride exchange. At high chloride, however, i.e., when ΔpH is high, glutamate uptake is coupled directly to proton exchange (Tabb et al., 1992; Wolosker et al., 1996). Under these conditions, transport is driven by ΔpH. Low amounts of chloride between 4 and 6 mM increase transport activity in a manner that is independent of the chloride effects on the balance between $\Delta\psi$ and ΔpH (which constitute the driving $\Delta\mu H^+$) but rather involves a direct activation of the transport system (Naito and Ueda, 1985; Hartinger and Jahn, 1993; Wolosker et al., 1996).

To gain insight into the mechanism of G-protein-mediated inhibition, we tested the chloride dependence of glutamate uptake in vesicles obtained from $G\alpha_{o2}^{-/-}$ mice compared with the other Gα deletion mutants. Surprisingly, chloride activation was completely abolished in vesicles obtained from $G\alpha_{o2}^{-/-}$ mice (Fig. 7*A*,*B*) and reduced in $G\alpha_{o2}^{+/-}$ mice (Fig. 7*B*), whereas it was unchanged in vesicles obtained from $G\alpha_{o1}^{-/-}$ (Fig. 7*C*), $G\alpha_{q}^{-/-}$ (Fig. 7*D*), and $G\alpha_{11}^{-/-}$ (Fig. 7*E*) mice. To gain more insight into the relationship between chloride activation and G-protein regulation of glutamate uptake, we tested regulation of uptake at different chloride concentrations. G-protein activation inhibited the increase in transport activity observed in the presence of 5 mM chloride (Fig. 8*A*), whereas inhibition was much less pronounced either in the absence of chloride or at high chloride concentrations. Thus, it appears that both G-protein activation and deletion of $G\alpha_{o2}$ shut down chloride-dependent regulation of VGLUT.

To find an explanation for these puzzling data, we analyzed VGLUT activity in the presence of GMP-P(NH)P using less favorable chloride concentrations below 5 mM. In the presence of GMP-P(NH)P, VGLUT was activated at 1 mM chloride, and this effect decreased with increasing chloride concentrations, whereas maximal activation in control vesicles was only observed at 5 mM (Fig. 8*B*). Chloride sensitivity of VGLUT activation was even more pronounced when ΔpH was clamped with nigericin (Fig. 8*C*). GMP-P(NH)P-dependent activation of transport activity at



1 mM chloride was also observed in the presence of nigericin (data not shown), suggesting that chloride has an allosteric effect on the transporter, as suggested previously (Hartinger and Jahn, 1993).

As expected, activation even at lower chloride concentrations was absent in vesicles from Gα$_{o2}^{-/-}$ mice (Fig. 8*D*), whereas transport was still enhanced in the presence of nigericin (data not shown). However, preincubation of vesicles with the Gα$_{o2}$ antibody, to abolish putative effects of Gα$_{o2}$, did not prevent the activation by 5 mM chloride (Fig. 8*E*). Together, our results show that activation of G-proteins shifts both the stimulatory and the inhibitory effects of chloride on VGLUT to lower chloride concentrations, such that, at ~5 mM, chloride is already inhibitory, whereas genetic deletion of Gα$_{o2}$ causes a loss of the effects of chloride on VGLUT activity, resulting in reduced activity at those concentrations of chloride at which it would have shown a stimulatory effects, 4–5 mM in the experiments of this report.

Discussion

In the present study, we have shown that the activity of VGLUT is selectively controlled by the α subunit of the trimeric G-protein G$_{o2}$. Our data provide the first change in phenotype for Gα$_{o2}^{-/-}$ mice. Furthermore, our data show that the functional target of G$_{o2}$ is the chloride regulation of the transporter.

Alterations of transport activity of VGLUTs by chloride

Changing the chloride concentration has two effects on the transport rate that need to be distinguished. First, increasing chloride shifts the balance between ΔpH and Δψ by providing charge compensation for the electrogenic V-ATPase attributable to the presence of vesicular chloride channels. Thus, at high chloride, i.e., when ΔpH is high, glutamate uptake is coupled directly to proton exchange (Tabb et al., 1992; Wolosker et al., 1996). Second, chloride stimulates uptake at concentrations in the low millimolar range (Naito and Ueda, 1985), which is probably attributable to a direct or indirect action on the transporter itself (Hartinger and Jahn, 1993). In fact, it has been suggested that chloride binding is responsible for shifting the transporter from the "symport" into the "antiport" mode (Schuldiner et al., 1995). Under standard assay conditions, i.e., when Gα$_{o2}$ is present in its inactive form, VGLUT activity shows a clear maximum at ~5 mM chloride, in accordance with previous reports (Naito and Ueda, 1985; Wolosker et al., 1996). Activation of Gα$_{o2}$ by GTP-analogs shifts the activity maximum and the decline in transport activity

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Figure 5. Comparison of the amounts of VGLUTs in synaptic vesicle-enriched membranes from wild-type, Gα$_{o1}^{-/-}$, and Gα$_{o2}^{-/-}$ mice. ***A***, Synaptic vesicle-enriched membranes were prepared from whole brains of respective wild-type (wt) and either Gα$_{o2}^{-/-}$ or Gα$_{o1}^{-/-}$ mice and subjected to SDS-PAGE and Western blotting using the antibodies indicated. Quantification was performed by calculating the ratio between the amount of synaptophysin used as internal standard and the respective transporters or chloride channel proteins. Synaptophysin-based ratios obtained from wild-type animals were set as 100%. The graphs represent the mean ratios of vesicle preparations obtained from three animals ± SD. There was a slight reduction of VGLUT2 in the Gα$_{o2}^{-/-}$ mice and no changes in the amounts of VGLUT1, VGLUT3, or the chloride channel proteins ClC3 and ClC7. ***B***, Synaptosomal membranes (P2) were prepared from whole brains of respective wild-type and either Gα$_{o2}^{-/-}$ or Gα$_{o1}^{-/-}$ mice and extracted using Triton X-114 partitioning. One, 3, or 9 μg of protein were subjected to SDS-PAGE and Western blotting using the antibodies indicated. Quantification was performed by calculating the ratio between the amount of synaptophysin used as internal standard and the respective transporters. The relative optical densities (OD) were given for wild-type and either Gα$_{o1}^{-/-}$- or Gα$_{o2}^{-/-}$-derived membranes. Values represent the mean ratios of synaptosomal preparations obtained from three animals ± SD. There was no difference between the amounts of VGLUT1 or VGLUT2 in the deletion mutants compared with wild type.

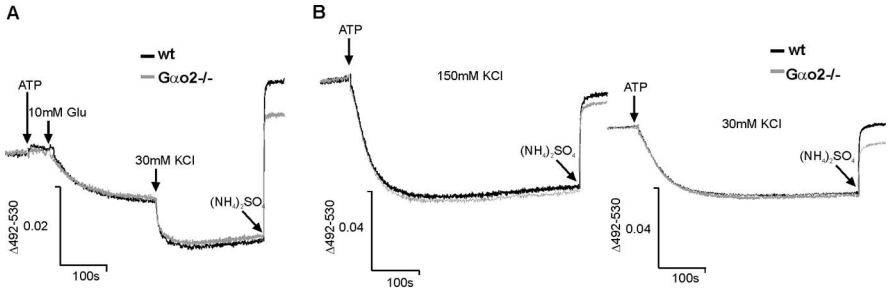

Figure 6. Acidification of synaptic vesicles from wild-type (wt) and $G\alpha_{o2}{}^{-/-}$ mice. Synaptic vesicles form brains of either genotype were subjected to ATP-induced acidification using either glutamate or chloride. ***A***, Addition of 10 mM glutamate followed by an addition of 30 mM chloride resulted in an identical acidification (indicated by a downward reflection) in both wild-type (black line) and $G\alpha_{o2}{}^{-/-}$ (gray line) vesicle preparations. ***B***, Similar data were obtained when 150 or 30 mM chloride was added directly. Addition of 10 mM $(NH_4)_2SO_4$ reverted the signal caused by neutralization of the vesicular pH.

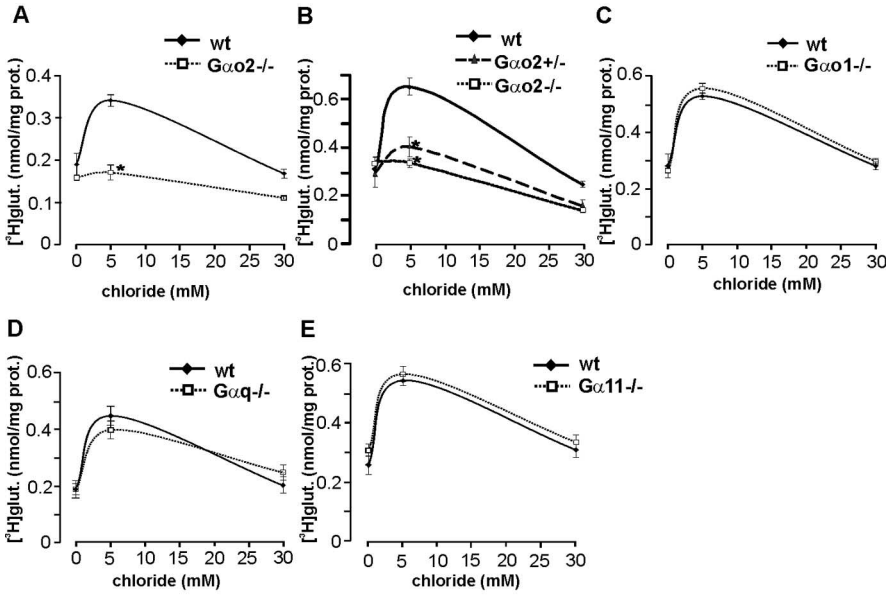

Figure 7. Chloride dependence of VGLUT activity in deletion mutants of $G\alpha_{o1}$, $G\alpha_{o2}$, $G\alpha_q$, and $G\alpha_{11}$. Synaptic vesicles were prepared from brains of $G\alpha_{o2}{}^{-/-}$ (***A***), $G\alpha_{o1}{}^{-/-}$ (***C***), $G\alpha_q{}^{-/-}$ (***D***), and $G\alpha_{11}{}^{-/-}$ (***E***) mice and the corresponding wild types (wt) or from wild-type, heterozygous, or knock-out $G\alpha_{o2}$ littermates (***B***). Glutamate uptake was performed in KGC/ATP buffer using the indicated chloride concentrations. Values represent the mean of three determinations, and nonspecific binding was subtracted. Experiments were performed at least two or three times. Note that 5 mM chloride increases glutamate uptake approximately twofold in wild-type as well as in $G\alpha_{o1}{}^{-/-}$ (***C***), $G\alpha_q{}^{-/-}$ (***D***), and $G\alpha_{11}{}^{-/-}$ (***E***) mice. To exclude the influence of a different genetic background and to analyze the effects of gene doses, the chloride dependency was repeated using littermates generated from heterozygous $G\alpha_{o2}{}^{+/-}$ mice (***B***). There is no chloride dependence in $G\alpha_{o2}{}^{-/-}$ mice and a reduced chloride dependence in $G\alpha_{o2}{}^{+/-}$ heterozygous ones ($p < 0.01$).

to lower chloride concentrations, whereas deletion of $G\alpha_{o2}$ completely abolishes chloride dependence.

$G\alpha_{o2}$ may directly interact with the transporter, thereby tonically modulating its chloride sensitivity and transport activity. Persistent activation by GMP-P(NH)P may cause a release of the G-protein from the vesicular membrane, causing a decrease in VGLUT activity. In fact, an increased release of $G\alpha_o$ subunits out of streptolysin O-permeabilized PC12 cells has been shown after stimulation with GTPγS or GMP-P(NH)P (Ahnert-Hilger et al., 1992). However, at low chloride concentration, G-protein activation increases VGLUT activity, rendering it unlikely that the dual chloride-dependent role of $G\alpha_{o2}$ is caused by its removal from vesicle membranes. In addition, no direct interaction between G-protein and transmitter transporters could be demonstrated so far, suggesting an indirect mechanism for the control of VGLUT activity by $G\alpha_{o2}$.

The data may be explained by a so far hypothetical presence of a separate regulatory protein that binds chloride and that is controlled by $G\alpha_{o2}$. In the absence of chloride, the regulator protein may be inactive, and VGLUT activity is exclusively driven by $\Delta\psi$. At low chloride, the regulator protein is activated and in turn binds and thus activates VGLUT. Furthermore, under these conditions, ΔpH may add to the driving force (Tabb et al., 1992; Schuldiner et al., 1995; Wolosker et al., 1996). Higher concentrations of chloride lead to a dissociation of the regulator protein, with the transporter gradually shifting from being preferentially driven by $\Delta\psi$ to a stage in which transport activity depends on both $\Delta\psi$ and ΔpH. Activation of $G\alpha_{o2}$ increases the chloride affinity of the assumed regulator protein, shifting the peak of binding and VGLUT activation to lower chloride concentrations. In $G\alpha_{o2}{}^{-/-}$ mice, the assumed regulator protein is lost. Probably, $G\alpha_{o2}$ is required for its stable expression or its protection from degradation. The >50% reduction of chloride sensitivity in the heterozygous mouse suggests that the relationships between regulator protein, $G\alpha_{o2}$, and VGLUT are not linear.

$G\alpha_{o2}$ as a modulator of vesicular transmitter transporter activity

In addition to VMATs (Höltje et al., 2000), VGLUTs constitute the second group of vesicular transporters to be regulated by $G\alpha_{o2}$. G_o-proteins are heterotrimers of composition α_o, β, and γ that are activated by heptahelical receptors of the plasma membrane but occur also on various types of secretory vesicles, including VGLUT1 and VGLUT2 types (Pahner et al., 2003). Generally, the $G\beta\gamma$ dimers and the GTP-α_o complexes are presumed to regulate specific downstream effectors. $G\alpha_o$ subunits are highly expressed in neuronal cells in which they may reach 1–2% of membrane protein (Sternweis and Robishaw, 1984). However, effects mediated by the α_o subunits are among the least understood of the G-protein α subunits. This is complicated still further by the fact that there are two molecularly distinct $G\alpha_o$-proteins. $G\alpha_{o1}$ and $G\alpha_{o2}$ are the result of an alternative transcript splicing process that, after translation, produces α subunits that differ in 25 of their 116 C-terminal amino acids. The C-terminal region of G-protein α subunits is known to be engaged in receptor interaction and recognition in the context of the trimeric state (cf. Grishina and Berlot, 2000). Differences in receptor recognition, leading to the selective activation of either G_{o1} or G_{o2} regulating the same downstream effector system, were documented (Kleuss et al., 1991; Chen and Clarke, 1996). The selective loss of retinal bipolar ON cell activation by glutamic acid from retinal photoreceptor cells in mice lacking $G\alpha_{o1}$ (Dhingra et al., 2000), but not in mice lacking $G\alpha_{o2}$ (Dhingra et al., 2002), has

provided additional proof for the nonequivalence of the two G_o-proteins under *in vivo* conditions.

G-protein activation may be a mechanism by which transmitter transporters are regulated, allowing the presynaptic terminal to modulate vesicular transmitter content. In brain, $G\alpha_{o2}$ is the only $G\alpha$-subunit involved in the regulation of vesicular transmitter transporter activity so far. This activity is not shared by the closely related splice variant of $G\alpha_{o1}$, underscoring the functional divergence of these two G_o-proteins in neurons.

Putative signal transduction and consequences of G-protein-mediated regulation of VGLUT activity

Which factors control the activity status of $G\alpha_{o2}$ on glutamatergic vesicles, and what are the physiological consequences? Using Tph1$^{-/-}$ mice, which have serotonin-depleted platelets (Walther et al., 2003), we have shown recently that, in monoaminergic vesicles, it is the intravesicular monoamine concentration itself that triggers G-protein activation (Höltje et al., 2003), thus providing an inhibitory feedback loop that downregulates the transporters when the vesicle is full. However, it is unknown by which mechanism the intravesicular transmitter concentration is sensed and how this signal is transferred to $G\alpha_{o2}$ on the cytoplasmic side of the vesicle. For glutamatergic vesicles, the situation is more complex because the efficacy of G-protein regulation is linked to the cytoplasmic chloride concentration. If there is signaling from the vesicle lumen, the trigger could be either the vesicular glutamate or the vesicular proton concentration that was suggested previously to exert a regulatory influence on the transporter (Tabb et al., 1992). In this respect, it is remarkable that proton-sensing G-protein-coupled receptors have been identified recently that work best at pH 6.8 and are inhibited by pH over 7.8 or beneath 5.8 (Ludwig et al., 2003). Furthermore, it cannot be excluded that $G\alpha_{o2}$ is regulated by cytosolic factors, thus linking it to intracellular signaling pathways as a means to regulate synaptic efficacy.

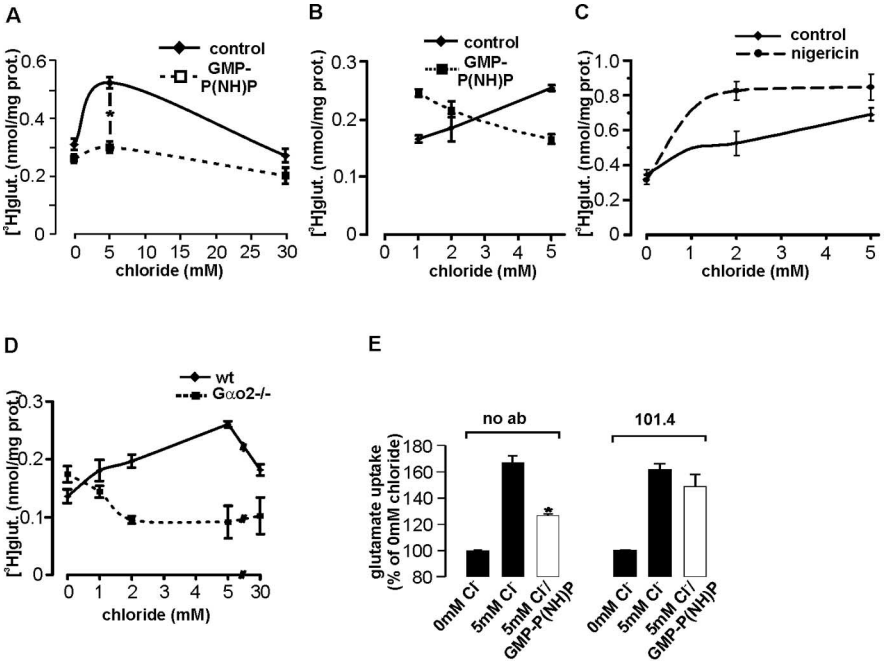

Figure 8. Abolition of the chloride dependence of VGLUT activity by GMP-P(NH)P. ***A***, Synaptic vesicles from rat brain were subjected to glutamate uptake using KGC/ATP buffer containing no chloride, 5 mM chloride, or 30 mM chloride as given by the abscissa. Uptake for each chloride concentration was performed in either the absence (filled rectangles) or presence (open squares) of 50 μM GMP-P(NH)P. Values represent the mean of three determinations, and nonspecific uptake in the absence of ATP was subtracted. Note that chloride dependence is abolished in the presence of GMP-P(NH)P ($p < 0.006$). ***B***, Synaptic vesicles from rat brain were subjected to glutamate uptake using KGC/ATP buffer containing 1, 2, or 5 mM chloride as given by the abscissa in the absence (filled rectangles) or presence (filled squares) of 50 μM GMP-P(NH)P. Experiment was performed in triplicate, and nonspecific glutamate binding was subtracted. G-protein activation stimulates glutamate uptake at 1 mM, but it inhibits it at 5 mM chloride. ***C***, Synaptic vesicles from rat brain were incubated with increasing chloride concentrations (abscissa) in the absence or presence of 0.5 μM nigericin. Values represent the mean of three determinations corrected for nonspecific uptake obtained in the presence of trypan blue. Note that clamping ΔpH by nigericine increases chloride sensitivity of VGLUT. ***D***, Synaptic vesicles from either wild-type (filled rectangles) or $G\alpha_{o2}^{-/-}$ brains were subjected to glutamate uptake in the presence of the chloride concentrations given at the abscissa. Experiment was performed in triplicate, and nonspecific glutamate binding was subtracted. There is no chloride activation in $G\alpha_{o2}^{-/-}$ mice but a decrease in the VGLUT activity with increasing chloride concentrations. ***E***, Synaptic vesicles from rat brain were incubated without or with dialyzed $G\alpha_{o2}$ antibody 101.4 for 30 min on ice before they were subjected to glutamate uptake using KGC/ATP buffer containing no chloride or 5 mM chloride either in the absence or presence of 50 μM GMP-P(NH)P. Values are expressed as percentage of glutamate uptake obtained in the nominal absence of chloride (set as 100%) and represent the mean of triplicates. Nonspecific glutamate binding was subtracted. Note that the $G\alpha_{o2}$-specific antibody prevented GMP-P(NH)P-mediated inhibition at 5 mM chloride ($p < 0.05$) without changing the activation of VGLUT activity by chloride. Generally one of two or three experiments is shown.

The effectors of $G\alpha_{o2}$ involved in the regulation of VGLUT appear to be different from those linked to VMAT regulation. The latter probably involves a cAMP-dependent pathway (Nakanishi et al., 1995) so far not seen in the regulation of VGLUT (Pahner et al., 2003). Uptakes of glutamate and GABA have been reported to be modulated by a 103 kDa inhibitory protein factor present in synaptosomal cytosol (Özkan et al., 1997). Whether this factor represents one of the missing links in the $G\alpha_{o2}$-mediated regulation of VGLUTs remains to be established.

Under physiological conditions, the cytosolic chloride concentration in neurons varies considerably between 8 and 20 mM in an activity-dependent manner, whereas hyperexcitability is increased by an increase in the intracellular chloride concentration (Zhu et al., 2005). At the higher chloride concentrations, VGLUT activity is not in the optimal range, leading to moderately filled glutamatergic vesicles, thereby slowing the effects of hyperexcitability. It may, therefore, be speculated that the $G\alpha_{o2}$ regulation of the chloride dependence of VGLUT activity represents a protection mechanism to prevent overexcitability under physiological conditions by keeping the transporter in a less efficient state. G_{o2} may work to increase chloride sensitivity and thereby transport activity when chloride is reduced and/or reduce activity when the chloride-activating concentration is shifted to higher chloride by metabolic changes (Reis et al., 2000). The concept of VGLUT regulation presented here might help to understand some aspects of seizure development and lead to new therapeutic concepts in their management. Future experiments will show whether defects may show up earlier or later in $G\alpha_{o2}^{-/-}$ mice when metabolism or other parameters run out of balance.

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