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Activation and modulation of concatemeric GABA-A receptors expressed in human  
embryonic kidney cells

Gustav Akk, Ping Li, John Bracamontes and Joe Henry Steinbach

Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO  
63110

Running title: single-channel properties of concatemeric GABA-A receptors

Correspondence to: Gustav Akk, Dept. of Anesthesiology, Campus Box 8054, 660 S. Euclid Ave, St. Louis, MO 63110. Tel: (314) 362-3877; Fax: (314) 362-8571; Email: akk@morpheus.wustl.edu

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## ABSTRACT

We have employed whole-cell and single-channel electrophysiology to examine the kinetic and pharmacological properties of GABA-A receptors consisting of  $\gamma 2L$ - $\beta 2$ - $\alpha 1$  and  $\beta 2$ - $\alpha 1$  subunit concatemeric constructs expressed in human embryonic kidney cells. Concatemeric receptors activated by GABA exhibited the same single-channel conductance, channel opening rate constant, and basic open and closed time properties as receptors containing free subunits. However, the whole-cell GABA dose-response and the single-channel effective opening rate curves were shifted to higher GABA concentrations, suggesting that the concatemeric receptors have a lower affinity to GABA. Pharmacological tests demonstrated that the concatemeric receptors were potentiated by pentobarbital, diazepam and the neurosteroid  $3\alpha 5\alpha P$ , and were insensitive to  $Zn^{++}$ . Introduction of the  $\alpha 1Q241L$  mutation, previously shown to abolish  $\alpha 1\beta 2\gamma 2L$  channel potentiation by neurosteroids, selectively into one of the two concatemeric constructs had a relatively small effect on receptor activation by GABA or macroscopic potentiation by the neurosteroid ( $3\alpha, 5\alpha$ )-3-hydroxypregnan-20-one. Single-channel measurements showed that the kinetic mechanism of action of the steroid is unchanged when the mutation is introduced to the  $\gamma 2L$ - $\beta 2$ - $\alpha 1$  concatemer. We infer that a single wild-type  $\alpha$  subunit is capable of mediating the full set of kinetic effects in the presence of steroids. Introduction of the  $\alpha 1Q241W$  mutation, previously shown to mimic the effect of the steroid on  $\alpha 1\beta 2\gamma 2L$  channels, selectively into either concatemeric construct altered the mode of activity elicited by P4S, but the presence of mutations in both  $\alpha$  subunits was required to affect open time distributions. The data indicate that the  $\alpha 1Q241W$  mutation acts as a partial steroid modulator.

## INTRODUCTION

The GABA-A receptor is a pentameric protein consisting of five homologous subunits. Functional receptors containing one or two types of subunits can be formed under certain conditions, but the majority of mammalian GABA-A receptors likely contain three kinds of subunits. The most common type of GABA-A receptors in the mammalian brain is one consisting of two  $\alpha$  subunits, two  $\beta$  subunits and one  $\gamma$  subunit (McKernan and Whiting, 1996).

Studies of GABA-A receptors expressed in heterologous expression systems allow the manipulation of subunit composition as well as easy introduction of mutations to the receptor, and are therefore widely used. However, in many instances heterologous expression results in multiple subunit populations, needlessly complicating the interpretation of results. For example, cells nominally expressing  $\alpha\beta\gamma$  receptors may contain a population of receptors that lack the  $\gamma$  subunit. These receptors are functional, and, as shown by heterologous expression of  $\alpha$  and  $\beta$  subunits, possess distinctive biophysical and pharmacological characteristics. Similarly, the analysis of the data from conventional mutational studies is hindered when the target subunit is present in two (or more) copies per receptor because of multiple changes being introduced to receptor structure. This raises issues of additivity and specificity of the effects of structural changes. In the case of neurosteroids, that have two potentiating sites per receptor, mutagenesis of the  $\alpha$  subunit results in structural changes in both binding sites, preventing study of a single modified binding site to determine whether the individual binding sites are functionally equivalent.

Previous work has shown that functional GABA-A receptors can be formed from concatemeric or tandem subunits where the carboxyterminus of one subunit is joined with a short linker region to the aminoterminal of the other subunit. The simplest tandem



subunit consists of linked  $\alpha$  and  $\beta$  subunits, which, when coexpressed with a single monomeric subunit, produces receptors that respond to GABA (Im et al., 1995; Baumann et al., 2001; Boileau et al., 2005). Further work has shown that combination of a trimeric concatemer (e.g.,  $\gamma\beta\alpha$ ) with a dimer ( $\beta\alpha$ ) produces receptors that are activated by GABA and potentiated by the benzodiazepine diazepam (Baumann et al., 2002). The presence of the  $\gamma$  subunit in the concatemeric construct, at least theoretically, ensures that the subunit gets included in the receptor-complex. The use of nonidentical concatemeric constructs, neither of which alone can produce functional receptors, allows an approach in which mutations are selectively introduced to one of the two  $\alpha$  or  $\beta$  subunits of the receptor.

In this study, we have examined the kinetic and pharmacological properties of GABA-A receptors formed of  $\gamma 2L$ - $\beta 2$ - $\alpha 1$  and  $\beta 2$ - $\alpha 1$  concatemeric constructs, using single-channel and whole-cell macroscopic recordings. We show that the linkage of subunits does not affect the single-channel conductance, the channel opening rate constant, or the basic pharmacological properties, including the mechanism of action of the neurosteroid  $3\alpha 5\alpha P$ . However, compared to receptors containing free subunits, the concatemeric receptors have lower affinity to GABA. Introduction of a mutation that confers insensitivity to the potentiating action of the neurosteroid  $3\alpha 5\alpha P$  to a single  $\alpha$  subunit did not affect the mechanism of action of the steroid indicating that steroid interaction with a single  $\alpha$  subunit is sufficient to produce the range of kinetic effects observed in the presence of steroids.

## **MATERIALS AND METHODS**

The experiments were conducted on human embryonic kidney cells (HEK 293 and tsA 201) transiently expressing rat GABA-A receptors. The cells were grown in

Dulbecco's modified Eagle's medium / F-12 with 10 % (v/v) fetal bovine serum (Hyclone, Logan, UT), penicillin (100 U ml<sup>-1</sup>) and streptomycin (100 µg ml<sup>-1</sup>) in a humidified atmosphere with 5 % CO<sub>2</sub> at 37°C, and passaged twice a week at 80-90 % confluency.

Concatemeric subunits were created using the rat α1, β2, and γ2L subunits as described earlier (Bracamontes and Steinbach 2009). In brief, we first generated the β2-α1 concatemer (referred to as the βα construct) that had a linker consisting of 23 amino acid residues: Q3(Q2A3PA)2AQ5. The subunit contained a FLAG tag on the N-terminus of the β2 subunit between residues 4 and 5 of the mature peptide. The subunits were joined together with the linker through site-directed mutagenesis by overlap extension (Ho et al., 1989) and subcloned into pcDNA3. The γ2L-β2-α1 concatemer (referred to as the γβα construct) was generated by joining the γ2L and β2 subunits using the 26 amino acid residue linker: Q5A3PAQ2(QA)2A2PA2Q5. The resulting PCR product was subcloned into the βα construct. Mutated concatemers containing Q241L and Q241W were made by DNA subcloning, digesting mutated single subunits and ligating them into fragments of digested concatemers.

The GABA-A receptor free subunit or concatemeric construct cDNAs were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA), and transiently transfected into HEK 293 or tsA 201 cells using a calcium phosphate precipitation-based technique. A total of 3 µg of cDNA in the ratio of 1:1 (γβα : βα) or 1:1:1 (α : β : γ) was mixed with 12.5 µl of 2.5 M CaCl<sub>2</sub>, and dH<sub>2</sub>O to a final volume of 125 µl. The solution was added slowly, without mixing, to an equal volume of 2x BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) buffered solution. The combined mixture was incubated at room temperature for 10 min followed by mixing the contents and an additional 15 min incubation. The precipitate was added to the cells in a 35 mm dish for overnight incubation at 37 °C, followed by replacement of medium in the dish. The experiments were conducted during the next two days after changing the medium.

Cells expressing GABA-A receptors were identified using a bead-binding technique. The aminotermini of the  $\beta\alpha$  concatemeric construct and the free  $\alpha 1$  subunit were tagged with the FLAG epitope (Ueno et al., 1996). Surface expression of the FLAG peptide was determined using a mouse monoclonal antibody to the FLAG epitope (M2, Sigma-Aldrich, St. Louis, MO), which had been adsorbed to immuno-beads with a covalently attached goat anti-mouse IgG antibody (Dynal, Great Neck, NY).

The experiments were carried out using whole-cell voltage clamp and single-channel patch clamp methods. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 D-glucose and 10 HEPES; pH 7.4. In whole-cell recordings, the pipette solution contained (in mM): 140 CsCl, 4 NaCl, 4 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 5 EGTA, 10 HEPES, pH 7.4. In single-channel recordings, the pipette solution contained (in mM): 120 NaCl, 5 KCl, 10 MgCl<sub>2</sub>, 0.1 CaCl<sub>2</sub>, 20 tetraethylammonium, 5 4-aminopyridine, 10 D-glucose, 10 HEPES; pH 7.4.

The agonist and modulator were applied through the bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT) in whole-cell experiments, or added to the pipette solution in single-channel recordings. The recording and analysis of whole-cell currents were carried out as described previously (Li et al., 2006). In most experiments, the cells were clamped at -60 mV. The currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Union City, CA), low-pass filtered at 2 kHz and digitized with a Digidata 1320 series interface (Molecular Devices) at 10 kHz. The analysis of whole-cell currents was carried out using the pClamp 9.0 software package.

The recording and analysis of single-channel currents have been described in detail previously (Akk et al., 2001; 2004). Most experiments were conducted in the cell-attached configuration. The pipette potential was held at +60 to +80 mV, which translates to an approximately -120 to -100 mV potential difference across the patch membrane. Channel activity was recorded using an Axopatch 200B amplifier, low-pass

filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software.

All experiments were conducted at room temperature. The statistical analysis was done using the Systat 7.0 (SPSS Inc, Chicago, IL) software package. The statistical tests used and the significance levels are given in the text. Curve fitting was carried out using the program NFIT (The University of Texas Medical Branch at Galveston).

## RESULTS

### ***Macroscopic activation properties of receptors formed of wild-type $\gamma\beta\alpha$ and $\beta\alpha$ concatemeric constructs***

Cells transfected with cDNA for the wild-type  $\gamma 2L$ - $\beta 2$ - $\alpha 1$  and  $\beta 2$ - $\alpha 1$  constructs ( $\gamma\beta\alpha$  -  $\beta\alpha$  receptors) responded to applications of GABA in a concentration-dependent manner. Sample macroscopic currents and the GABA dose-response relationship are shown in Figure 1. Compared to receptors formed of free  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 2L$  subunits ( $\alpha 1\beta 2\gamma 2L$  receptors), the GABA dose-response curve for the concatemeric receptors was shifted by ~10-fold to higher agonist concentrations. The  $EC_{50}$  for the concatemeric receptor was  $92 \pm 2 \mu M$ , and the Hill coefficient was  $1.7 \pm 0.1$  (best-fit  $\pm$  SD of the parameter estimate).

### ***Single-channel properties of wild-type concatemeric receptors activated by GABA***

We examined the properties of single-channel currents arising from concatemeric receptors. Sample currents elicited by 20, 100, 500 or 2000  $\mu M$  GABA are shown in Figure 2. The increase in transmitter concentration led to specific changes in receptor

kinetics. At 20  $\mu\text{M}$  GABA, the activity consisted of single openings and isolated bursts, but at higher concentrations channel activity was condensed into easily distinguishable clusters of activity.

At all GABA concentrations studied (20-5000  $\mu\text{M}$ ), the channel open time histograms were best-fitted to sums of three exponentials. The mean durations of the three components were 250-500  $\mu\text{s}$  (OT1), 3-5 ms (OT2), and 10-13 ms (OT3). The OT2 component was the most prevalent, with slightly more than one-half of all open events falling to this class of openings. The open time findings are summarized in Table 1. Compared to receptors containing free  $\alpha 1\beta 2\gamma 2\text{L}$  subunits, the concatemeric receptors demonstrate significant increases in the mean durations of OT2 ( $p < 0.01$ ; t-test) and OT3 ( $p < 0.01$ ) (Figure 3).

The intracluster closed time distributions were best-fitted to sums of three (at 50-200  $\mu\text{M}$  GABA) or four (at 500-5000  $\mu\text{M}$  GABA) exponentials. The summary of the closed time analysis is given in Table 2. The briefest closed time component, CT1, was observed at all agonist concentrations. It had a mean duration of  $0.14 \pm 0.02$  ms (mean  $\pm$  SD; averaged from data from 30 patches at 20-5000  $\mu\text{M}$  GABA), and it formed  $52 \pm 7$  % of all intracluster closed events. A closed time component with a mean duration of  $1.9 \pm 1.8$  ms and a prevalence of  $12 \pm 8$  % was designated CT2. In the presence of high (500-5000  $\mu\text{M}$ ) concentrations of GABA, we observed a long-lived closed state (CT4). This closed state had a mean duration of  $23 \pm 10$  ms and a relative frequency of  $1 \pm 1$  %. This closed interval likely originates from sojourns in a short-lived desensitized state (Steinbach and Akk, 2001). In addition, we observed a closed state whose duration is dependent on GABA concentration ( $\text{CT}_\beta$ ). The  $\text{CT}_\beta$  closed time component arises from receptor occupation of mono- and unliganded closed states. As the agonist concentration is increased, the time spent in the unliganded and monoliganded closed states is reduced, and the mean duration of  $\text{CT}_\beta$  decreases.

The relationship between the inverse of  $CT_{\beta}$ , defined as the effective opening rate, and GABA concentration is shown in Figure 4A. The curve was fitted with the Hill equation, yielding the maximal effective opening rate (i.e., the channel opening rate constant) of  $2950 \pm 686 \text{ s}^{-1}$  and an  $EC_{50}$  of  $1452 \pm 488 \text{ }\mu\text{M}$ . The data indicate that in concatemeric receptors the effective opening rate curve is shifted to higher GABA concentrations. The rightward shift likely results from reduced affinity to agonist. In addition, the channel opening rate constant, estimated from fitting the effective opening rate curve, is somewhat higher for concatemeric receptors compared to  $\alpha 1\beta 2\gamma 2L$  receptors. We note, however, that the estimates for the channel opening rate constant were poorly defined due to large error limits.

To more fully assess the gating properties of concatemeric receptors, we compared the intracluster closed time properties at 5 mM GABA for receptors formed of free vs. concatemeric subunits. The mean duration of the closed time component corresponding to the channel opening rate constant was  $0.45 \pm 0.05 \text{ ms}$  ( $n = 5$  patches) in receptors containing free subunits, and  $0.47 \pm 0.07 \text{ ms}$  ( $n = 5$  patches) in concatemeric receptors. The difference was not statistically significant ( $p > 0.75$ ; t-test). We conclude that the channel opening rate constant is unchanged in concatemeric receptors.

We have defined the rate of entry into the  $CT_{\beta}$  component as the channel closing rate constant. The averaged channel closing rate constant for the concatemeric receptors is  $104 \pm 35 \text{ s}^{-1}$  ( $n = 30$  patches at 20-5000  $\mu\text{M}$  GABA). For comparison, the closing rate constant for receptors containing free subunits is  $135 \pm 65 \text{ s}^{-1}$  (Steinbach and Akk, 2001).

Figure 4B shows the relationship between cluster open probability ( $P_o$ ) and GABA concentration. The curve was fitted to the Hill equation yielding a maximal  $P_o$  of  $0.88 \pm 0.03$ , an  $EC_{50}$  of  $70 \pm 9 \text{ }\mu\text{M}$ , and a Hill coefficient of  $1.4 \pm 0.2$ .

### ***Single-channel conductance of concatemeric receptors***

The single-channel conductance of  $\gamma\beta\alpha$  -  $\beta\alpha$  concatemeric receptors was estimated in inside-out patches, where the membrane is exposed to similar chloride concentrations on both sides. The receptors were exposed to 20  $\mu\text{M}$  GABA, and the membrane voltage was held at -100 mV. In 3 patches, the conductance was  $25.6 \pm 1.1$  pS. This is statistically indistinguishable from the single-channel conductance of receptors formed of free  $\alpha 1\beta 2\gamma 2\text{L}$  subunits, estimated in an identical experimental setting ( $27.3 \pm 1.2$  pS,  $n = 4$  patches).

### ***Modulation of wild-type concatemeric receptors by diazepam, pentobarbital, $\text{Zn}^{++}$ , and $3\alpha 5\alpha\text{P}$***

We next examined the basic pharmacological properties of wild-type concatemeric receptors by probing receptor modulation by the benzodiazepine diazepam, pentobarbital,  $\text{Zn}^{++}$ , and the neurosteroid  $3\alpha 5\alpha\text{P}$ .

Coapplication of 10  $\mu\text{M}$  diazepam with 30  $\mu\text{M}$  GABA (approximately  $\text{EC}_{20}$ ) potentiated the peak whole-cell current to  $320 \pm 125$  % of control ( $n = 5$  cells). When 100  $\mu\text{M}$  pentobarbital was coapplied with 30  $\mu\text{M}$  GABA, the peak response was increased to  $644 \pm 331$  % of control ( $n = 5$  cells). Exposure to 100  $\mu\text{M}$   $\text{ZnCl}_2$  was without effect ( $97 \pm 3$  % of control;  $n = 4$  cells) on the peak response elicited by 1 mM GABA. These findings are consistent with previously published data on  $\alpha 1\beta 2\gamma 2\text{L}$  receptors (Draguhn et al., 1990; Pistis et al., 1997; Walters et al., 2000; Li et al., 2006). We note that the extent of potentiation is sensitive to the fractional activation of the control response. Accordingly, the findings merely qualitatively confirm the concatemeric receptor modulation by

diazepam and pentobarbital. The lack of effect of  $Zn^{++}$  on the peak response elicited by a saturating concentration of GABA is indistinguishable from the findings on  $\alpha 1\beta 2\gamma 2L$  receptors (Li et al., 2006).

Coapplication of the neurosteroid  $3\alpha 5\alpha P$  with GABA resulted in potentiation of whole-cell responses. Sample currents and the potentiation dose-response curve are shown in Figure 5A-B. The effect of the steroid was concentration-dependent, with the  $EC_{50}$  at  $63 \pm 9$  nM.

We examined the kinetic mechanism of steroid potentiation of concatemeric receptors using single-channel patch clamp. The receptors were activated by  $50 \mu M$  GABA in the absence and presence of  $1 \mu M$   $3\alpha 5\alpha P$ . Sample currents under both conditions are shown in Figure 5C, and the summary of kinetic parameters is given in Table 3. The presence of steroid affected both open and closed time distributions. In accordance with data from receptors consisting of free subunits (Akk et al., 2005), concatemeric receptors exposed to  $3\alpha 5\alpha P$  demonstrated an increase in the mean duration of OT3, and a reduction in the prevalence of  $CT_{\beta}$ . However, in contrast to our previous findings from  $\alpha 1\beta 2\gamma 2L$  receptors, the increase in the prevalence of OT3 in  $\gamma\beta\alpha - \beta\alpha$  receptors was not statistically significant.

***The  $\alpha 1Q241L$  mutation in a single  $\alpha$  subunit does not disrupt channel potentiation by the neurosteroid  $3\alpha 5\alpha P$***

Introduction of the Q241L mutation to the  $\alpha$  subunit in the receptor containing free  $\alpha 1\beta 2\gamma 2L$  subunits affects channel activation by GABA. The macroscopic dose-response curve is shifted to higher agonist concentrations. In single-channel records, the major effect of the mutation is to reduce the mean open time (Akk et al., 2008). In addition, the  $\alpha 1Q241L$  mutation disrupts channel potentiation by the neurosteroid



3 $\alpha$ 5 $\alpha$ P, possibly by preventing steroid binding to the steroid binding site (Hosie et al., 2006; Akk et al., 2008). In this study, we examined activation by GABA, and potentiation by 3 $\alpha$ 5 $\alpha$ P, of concatemeric receptors containing the  $\alpha$ 1Q241L mutation in the  $\gamma\beta\alpha$ , the  $\beta\alpha$ , or both concatemeric constructs.

In whole-cell recordings, the GABA concentration-response relationships for receptors containing the mutation were shifted to slightly higher agonist concentrations (Figure 6A). The midpoint of the GABA activation curve was 140  $\mu$ M or 128  $\mu$ M when the  $\alpha$ 1Q241L mutation was in the  $\gamma\beta\alpha$  or  $\beta\alpha$  constructs, respectively. When both  $\alpha$  subunits contained the mutation, the GABA EC<sub>50</sub> was 152  $\mu$ M. For comparison, the EC<sub>50</sub> of the wild-type  $\gamma\beta\alpha$  -  $\beta\alpha$  receptor is 92  $\mu$ M GABA (Figure 1B).

The presence of a single  $\alpha$ 1Q241L mutation per receptor had a relatively small effect on channel potentiation by the neurosteroid 3 $\alpha$ 5 $\alpha$ P. When the  $\gamma\beta\alpha$  concatemer contained the  $\alpha$ 1Q241L mutation, the midpoint of the steroid potentiation curve was at 140 nM. When the mutation was in the  $\beta\alpha$  concatemer, the steroid EC<sub>50</sub> was at 106 nM (Figure 6B). For comparison, the steroid EC<sub>50</sub> of the wild-type  $\gamma\beta\alpha$  -  $\beta\alpha$  receptor is at 63 nM (Figure 5B). The maximal potentiation was slightly reduced when one  $\alpha$ 1 subunit contains the Q241L mutation, but the effect is not statistically significant (Figure 6B). A concatemeric receptor with the Q241L mutation in both  $\alpha$  subunits was not potentiated by the neurosteroid 3 $\alpha$ 5 $\alpha$ P (Figure 6B).

Previous studies employing single-channel patch clamp have suggested that the open and closed time effects that collectively underlie channel potentiation by neuroactive steroids are mediated by steroid interactions with distinct sites (Akk et al., 2004; Li et al., 2007). This raises a possibility that the two  $\alpha$  subunits mediate a different set of kinetic effects, e.g., steroid interactions with one  $\alpha$  subunit may underlie the changes in open times while steroid binding to the other  $\alpha$  subunit leads to the closed

time effect. To test this hypothesis, we examined the kinetic mechanism of potentiation of the concatemeric mutant receptors by 3 $\alpha$ 5 $\alpha$ P.

The single-channel currents from the  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$  receptor activated by 100  $\mu$ M GABA demonstrated the presence of three intracluster open and three closed time components. The kinetic parameters (mean durations and prevalence) of the open and closed times (Table 4) were indistinguishable from those observed for the wild-type concatemeric receptor (Tables 1 and 2). Coapplication of 1  $\mu$ M 3 $\alpha$ 5 $\alpha$ P with GABA enhanced the channel open probability by prolonging the mean open duration and reducing the mean intracluster closed time duration (Figure 7 and Table 4). The effect in the mean open duration was mediated by an increase in the mean duration (from 8.0 ms to 21.4 ms) and the prevalence (from 19 % to 30 %) of the longest-lived open time component. The effect on the mean closed time duration was predominantly mediated by a reduction in the prevalence (from 24 % to 11 %) of the longest intracluster closed time component. We conclude that steroid interactions with a single wild-type  $\alpha$ 1 subunit (in the  $\beta\alpha$  concatemer) can cause the full set of kinetic changes observed in the presence of 3 $\alpha$ 5 $\alpha$ P.

Due to low expression levels, we were unable to record single-channel currents from the  $\gamma\beta\alpha$  -  $\beta\alpha$ Q241L or  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$ Q241L receptors. Based on the macroscopic measurements (Figure 6B) as well as previous work with receptors formed from free subunits (Akk et al., 2008), we believe that 3 $\alpha$ 5 $\alpha$ P is ineffective at modulating the single-channel currents from the  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$ Q241L receptor. The similarities in the macroscopic potentiation curves for the  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$  and  $\gamma\beta\alpha$  -  $\beta\alpha$ Q241L receptors suggest that the single-channel currents may be modified in a similar fashion. If so, this indicates that 3 $\alpha$ 5 $\alpha$ P interactions with either  $\alpha$  subunit is functionally equivalent.

***The  $\alpha$ 1Q241W mutation in a single  $\alpha$  subunit affects closed but not open times***

The  $\alpha 1Q241W$  is a gain-of-function mutation that affects the  $\alpha 1\beta 2\gamma 2L$  channel activation by the low-efficacy agonist piperidine-4-sulfonic acid (P4S) by increasing the mean open time and decreasing the mean closed time durations (Akk et al., 2008). This leads to the emergence of high open probability single-channel clusters in contrast to the monotonous, low-frequency channel openings seen in the absence of the mutation.

We have investigated the effect of the Q241W mutation in a single  $\alpha$  subunit on concatemeric channel activation by P4S. Exposure of wild-type concatemeric channels to 1 mM P4S resulted in low  $P_o$  (~0.05) channel activity with no clear-cut clusters present (Figure 8A). Segments of channel activity containing no overlapping currents were selected for open and closed time analysis. The open time analysis of channel activity revealed two components with the mean durations of 1.2 ms (OT1) and 2.4 ms (OT2). The OT2 component was less frequent, constituting 28 % of all open events (Table 5). We observed two closed time components within the records. The briefer closed event (CT1) had a mean duration of 0.42 ms and a prevalence of 10 % (Table 6). The longer-lived closed event had a mean duration of 42 ms. We caution, however, that in records without clear-cut clusters, even in the absence of overlapping currents, the neighboring openings may originate from the activation of different receptor-complexes. So, little mechanistic meaning can be assigned to the durations of long closed events.

In receptors where the  $\alpha 1Q241W$  mutation was introduced to both the  $\gamma\beta\alpha$  and  $\beta\alpha$  constructs, the prevalence of OT2 was increased to 73 % (Table 5). The mean durations of the open time components were not significantly altered. Interestingly, the presence of the mutation in both  $\alpha$  subunits did not result in the emergence of the third, long-lived open state (Akk et al., 2008). Additionally, the mutations altered the modal behavior of channel activity. Concatemeric receptors containing the  $\alpha 1Q241W$  mutation

in both  $\alpha$  subunits demonstrated clear-cut clusters when activated by 1 mM P4S (Figure 8D). The cluster  $P_o$  was  $0.40 \pm 0.08$  ( $n = 6$  patches).

The presence of a single Q-to-W mutation per receptor was without effect on the open time distributions (Table 5). In particular, a single mutation was incapable of producing a statistically significant increase in the prevalence of OT2. The data indicate that the major effect of the  $\alpha 1Q241W$  mutation on channel open distributions is an increase in the prevalence of OT2, but the mutation must be present in both  $\alpha$  subunits to produce the effect.

Both receptor types containing a single  $\alpha 1Q241W$  mutation exhibited single-channel clusters when exposed to 1 mM P4S (Figure 8B-C). Similar to the  $\gamma\beta\alpha Q241W$  -  $\beta\alpha Q241W$  receptor, the intracluster closed time histograms contained three components. The mean durations and prevalence of the three closed states are given in Table 6.

The comparison of closed time distributions for the four types of receptors is complicated by the absence of clusters in the data from wild-type receptors. Among the receptors containing the Q241W mutation, the major difference in the intracluster closed time distributions lies in the duration of the longest-lived closed time component (CT3). The mean duration of CT3 was 9 ms for receptors containing the Q241W mutation in both  $\alpha$  subunits, and 15 or 29 ms for receptors containing the mutation in the  $\gamma\beta\alpha$  or  $\beta\alpha$  constructs, respectively (Table 6).

### ***Modulation of concatemeric $\alpha 1Q241W$ mutant receptors by $3\alpha 5\alpha P$***

The  $\alpha 1\beta 2\gamma 2L$  receptor containing the Q241W mutation is not potentiated by the neurosteroid  $3\alpha 5\alpha P$  (Hosie et al., 2006). In an earlier study, we proposed that the tryptophan side chain acts by mimicking the presence of steroid in the steroid binding

pockets (Akk et al., 2008). Here, we examined how the Q-to-W mutation modifies concatemeric channel potentiation by 3 $\alpha$ 5 $\alpha$ P.

Single-channel currents elicited by 1 mM P4S from the  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$ Q241W receptor were not modulated by 3 $\alpha$ 5 $\alpha$ P (Tables 5 and 6). In contrast, exposure of the wild-type concatemeric receptor to 1  $\mu$ M 3 $\alpha$ 5 $\alpha$ P had a strong effect on the open time distributions. The mean duration of OT2 was prolonged from 2.4 to 12.5 ms, and the prevalence of OT2 was increased from 28 to 52 % (Table 5).

Receptors with the  $\alpha$ 1Q241W mutation in one of the two concatemeric constructs showed positive modulation by 3 $\alpha$ 5 $\alpha$ P. In receptors containing the mutated  $\gamma\beta\alpha$  construct, the average open duration was increased from 1.9 to 4.8 ms in the presence of steroid. The change was due to an increase in the mean duration and prevalence of OT2 (Table 5). When the  $\beta\alpha$  tandem contained the  $\alpha$ 1Q241W mutation, the average open duration was increased from 2.5 to 8.2 ms. Similarly, the effect was due to a prolonged and more prevalent OT2 component (Table 5).

It is noteworthy that the mean duration of OT2 in wild-type concatemeric receptors or receptors containing the Q241W mutation in only one of the two  $\alpha$  subunits, exposed to P4S + 3 $\alpha$ 5 $\alpha$ P, is prolonged compared to that in the  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$ Q241W receptor activated by P4S. The mean duration of OT2 was 12.5 ms, 6.9 ms, or 11.8 ms for  $\gamma\beta\alpha$  -  $\beta\alpha$ ,  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$ , or  $\gamma\beta\alpha$  -  $\beta\alpha$ Q241W receptors, respectively, in the presence of P4S + 3 $\alpha$ 5 $\alpha$ P (Table 5). In contrast, the mean duration of OT2 was 3.4 ms for the  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$ Q241W receptor activated by P4S. In the model where the tryptophan mutation in the  $\alpha$ 241 position acts by mimicking the presence of a steroid molecule, this finding suggests that the presence of the steroid molecule in the binding pocket is more efficacious, in terms of prolonging OT2, than the presence of the tryptophan residue.

The application of 3 $\alpha$ 5 $\alpha$ P was largely without effect on intracluster closed times in receptors containing a single  $\alpha$ 1Q241W mutation. In  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$  receptors, the

application of 3 $\alpha$ 5 $\alpha$ P increased the prevalence of CT1, while in  $\gamma\beta\alpha$  -  $\beta\alpha$ Q241W receptors the presence of steroid resulted in an increase in the prevalence of CT1 and a decrease in the prevalence of CT3.

## DISCUSSION

Concatemers of subunits can generate multimeric receptors of defined subunit composition and order. They also provide the opportunity to selectively mutate one copy of a subunit which is present in multiple copies in the assembled receptor. Concatemers of GABA-A receptor subunits have been successfully used to exploit both these advantages (e.g., Baumann et al., 2003; Boulineau et al., 2005; Baur et al., 2006). However, previous studies have almost exclusively used *Xenopus* oocytes as expression systems (but see Boileau et al., 2005; Baur et al., 2006), and have only utilized macroscopic measures of receptor function. It would be extremely valuable to study properties of concatemers expressed in a somatic expression system whose properties may be more similar to other cells in the organism, and which is more amenable to precise experimental analysis. The present results demonstrate that concatemers of wild-type GABA-A receptor subunits produce functional receptors when expressed in the human embryonic kidney cell line. More importantly, the receptors have functional properties essentially identical to those of receptors formed from the same subunits expressed as free subunits. Pharmacological properties of the receptors formed from concatemeric constructs are indistinguishable from those of receptors containing free subunits. Finally, we demonstrate that concatemeric constructs can be used to determine the effects of selective mutation of one copy of a subunit present in two copies in the assembled receptor, when expressed in HEK cells. Overall these studies significantly extend the use of concatemeric subunits in studies of GABA-A receptors

and validate the interpretation of results obtained in studies of receptors expressed in oocytes. To the best of our knowledge, this is the first study presenting single-channel data from concatemeric GABA-A receptors.

In the present study, we have investigated the properties of receptors consisting of concatemeric  $\gamma 2L$ - $\beta 2$ - $\alpha 1$  and  $\beta 2$ - $\alpha 1$  subunits ( $\gamma\beta\alpha$  -  $\beta\alpha$  receptors). Our data indicate that the subunit linkage does not affect the single-channel conductance, the channel opening rate constant or the mechanism of action of the neurosteroid  $3\alpha 5\alpha P$ .

In single-channel currents, the major effect of linkage of subunits is the rightward shift in the effective opening rate curve. The midpoint of the effective opening rate curve was at  $\sim 1.5$  mM in receptors formed of concatemeric subunits, but only 0.4 mM when free subunits were used. This suggests that the concatemeric receptors have lower affinity to GABA than receptors formed of free subunits. In whole-cell recordings, the GABA dose-response curve for concatemeric receptors is shifted to higher agonist concentrations. Interestingly, we observed no changes in the intracuster  $P_o$  properties, as the prolongation in the mean closed time duration (due to the shift in the effective opening rate) is offset by an increase in the mean open time duration.

Previous studies in *Xenopus* oocytes (Baumann et al., 2001) have observed a higher GABA  $EC_{50}$  for macroscopic responses from cells expressing concatemeric receptors. It has been suggested that eggs injected with cRNA for free  $\alpha 1$ ,  $\beta 2$ , and  $\gamma 2$  subunits express a population of high-affinity  $\alpha\beta$  receptors, whose presence affects the overall GABA dose-response properties. The use of concatemeric constructs presumably prevents that by forcing the incorporation of the  $\gamma$  subunit in receptor-complexes, thereby revealing the true dose-response relationship for the  $\gamma$  containing receptor.

We agree that the use of free subunits can result in the expression of  $\alpha\beta$  receptors (e.g., Li et al., 2006), but believe that subunit linkage *per se* is the major

contributor to the observed shift in the agonist dose-response relationship. First, the rightward shift in the GABA dose-response curve is also observed in the case of the tandem  $\beta\alpha$  construct coexpressed with the  $\beta$  subunit (Baumann et al., 2001). Second, the degree of shift can be dependent on the linker length (Baumann et al., 2001), as well as the location of the linker in identically arranged receptors (Baumann et al., 2002). We also note that the  $\alpha 1\beta 2$  receptors have lower conductance and a lower maximal open probability than  $\alpha 1\beta 2\gamma 2L$  receptors, limiting their relative contribution to macroscopic responses from cells expressing mixed receptor populations. However, the strongest argument against a contribution of  $\alpha 1\beta 2$  receptors comes from the present single-channel data. The single-channel conductance indicates that  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits are present, while the effective opening rate data (Figure 4A) demonstrate that a change in affinity has occurred.

The concatemeric wild-type receptors are potentiated by the benzodiazepine diazepam, pentobarbital, and the neurosteroid  $3\alpha 5\alpha P$ , while coapplication of  $ZnCl_2$  with GABA was without effect on the macroscopic peak current. These findings are in agreement with previously published data on  $\alpha 1\beta 2\gamma 2L$  receptors. We caution, however, that the linkage of subunits may have minor, yet measurable effects on the kinetic mechanisms of action of these drugs. For example, while the application of  $3\alpha 5\alpha P$  on  $\alpha 1\beta 2\gamma 2L$  receptors leads to a decrease in the prevalence of  $CT_\beta$  and an increase in the mean duration and prevalence of OT3 (Akk et al., 2005), only the first two effects were consistently reproduced in concatemeric receptors (Table 3). The change in the prevalence of OT3 was not statistically significant.

Conventional mutagenesis studies of ligand-gated ion channels have shortcomings when the target subunit is present in multiple copies per receptor. Mutagenesis of the  $\alpha$  (or  $\beta$ ) subunit in the  $\alpha 1\beta 2\gamma 2$  GABA-A receptor inevitably results in the introduction of two mutations per receptor. This complicates the interpretation of



studies aimed at examining the effects of modifications to a single locus. The use of concatemeric receptors allows an approach where the mutations are selectively introduced to one of the two  $\alpha$  (or,  $\beta$ ) subunits of the receptor. The present study suggests that concatemeric GABA-A receptors expressed in HEK cells are an acceptable model system for these purposes.

We used the concatemeric GABA-A receptor subunit constructs to examine the effect of  $\alpha 1Q241L$  and  $\alpha 1Q241W$  mutations on channel activation and modulation by the neurosteroid  $3\alpha 5\alpha P$ . Our previous work with receptors consisting of free subunits had indicated that the  $\alpha 1Q241L$  mutation prevents the interaction of  $3\alpha 5\alpha P$  with its binding site (Akk et al., 2008). In this study, we probed the effect of a single  $\alpha 1Q241L$  mutation per receptor on neurosteroid-mediated potentiation. The overall goal was to test the hypothesis that the two  $\alpha$  subunits, i.e., two individual steroid binding sites, mediate different kinetic actions (e.g., only the open time effect vs. only the closed time effect) of the steroid. When the  $\alpha 1Q241L$  mutation was included solely in the  $\gamma\beta\alpha$  concatemer, the application of  $3\alpha 5\alpha P$  resulted in the prolongation and increase in the prevalence of the longest open time component and a decrease in the prevalence of the longest intracluster closed time component. The effects were analogous to those found in the  $\alpha 1\beta 2\gamma 2L$  receptor (Akk et al., 2005) or the wild-type  $\gamma\beta\alpha - \beta\alpha$  receptor (Table 3). We infer from the data that a single wild-type  $\alpha$  subunit (in the  $\beta\alpha$  construct) can mediate the full set of kinetic effects observed in the presence of neurosteroids. Low expression levels prevented an analogous study on the  $\gamma\beta\alpha - \beta\alpha Q241L$  receptor. However, the similarities in the macroscopic potentiation curves (Figure 6B) strongly suggest that the wild-type  $\gamma\beta\alpha$  construct is similarly capable of mediating the full range of kinetic effects of neurosteroids. In sum, the data indicate that steroid interaction with a single  $\alpha$  subunit produces multiple kinetic changes.

When expressed in free subunits, the  $\alpha 1Q241W$  mutation prolongs the mean open time duration and reduces the mean closed time duration. The same kinetic effects are observed in the presence of neuroactive steroids prompting us to propose that the presence of the bulky tryptophan residue in the  $\alpha 241$  position mimics the presence of steroid (Akk et al., 2008).

The presence of the  $\alpha 1Q241W$  mutation in both concatemeric constructs resulted in an increase in the prevalence of long openings. In contrast, having the mutation in just one  $\alpha$  subunit was without effect. When  $3\alpha 5\alpha P$  was coapplied with P4S, the prevalence of OT2 was increased, indicating that the unmutated site can interact with the steroid molecule, and that this interaction produces an effect not unlike the presence of the tryptophan residue in the  $\alpha 241$  position. Interestingly, the mean duration of OT2 in receptors containing a single Q241W mutation (as well as in wild-type concatemeric receptors), exposed to P4S +  $3\alpha 5\alpha P$ , was significantly greater than in  $\gamma\beta\alpha Q241W - \beta\alpha Q241W$  receptors activated by P4S. We interpret this finding to mean that the presence of a steroid molecule in the binding pocket is more efficacious, in terms of prolonging OT2, than the presence of the tryptophan residue in the  $\alpha 241$  position.

No changes in the kinetics of the  $\gamma\beta\alpha Q241W - \beta\alpha Q241W$  receptor were observed when  $3\alpha 5\alpha P$  was coapplied with the agonist. The mean duration of OT2 under these conditions was significantly below the value reached when the wild-type  $\gamma\beta\alpha - \beta\alpha$  receptor was exposed to P4S +  $3\alpha 5\alpha P$ . We interpret this finding as the Q-to-W mutation being able to exclude the steroid from its binding site to prevent any additional effect, in addition to partially mimicking the effect of the steroid.

Overall, our results are in agreement with a recent study from our laboratory describing the effects of the  $\alpha 1Q241L$  and  $\alpha 1Q241W$  mutations on macroscopic currents from concatemeric receptors expressed in *Xenopus* oocytes (Bracamontes and Steinbach, 2009). The major conclusion of that study was that a single wild-type  $\alpha$

subunit can support the actions of a steroid. The present study extends these findings by providing a full kinetic and pharmacologic description of the wild-type concatemeric receptor activity. We also demonstrate that neurosteroid interactions with a single  $\alpha$  subunit produces multiple kinetic effects, and show that the Q241W mutations in the individual  $\alpha$  subunits act in concert to produce the effects seen when the mutant  $\alpha$  subunit is expressed in the context of free subunits.

In sum, the use of concatemeric receptors is a practical approach to forcing receptor assembly allowing to combine different isoforms or mutated subunits within the receptor-complex. Our work indicates that the wild-type concatemeric  $\gamma 2L\beta 2\alpha 1 - \beta 2\alpha 1$  receptors expressed in human embryonic kidney cells behave qualitatively similarly to receptors consisting of free  $\alpha 1$ ,  $\beta 2$ ,  $\gamma 2L$  subunits. The major conclusion from the work with the mutant concatemers is that steroid interaction with a single wild-type  $\alpha$  subunit is capable of producing the full set of kinetic effects seen in the wild-type receptor.

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## FOOTNOTES

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## LEGENDS FOR FIGURES

**Figure 1. Receptors formed of wild-type concatemeric  $\gamma\beta\alpha$  -  $\beta\alpha$  subunits are activated by GABA. A.** Sample responses from a HEK cell transfected with  $\gamma\beta\alpha$  and  $\beta\alpha$  constructs exposed to 10, 100 or 1000  $\mu\text{M}$  GABA. **B.** GABA dose-response properties for the  $\gamma\beta\alpha$  -  $\beta\alpha$  concatemeric receptors and receptors consisting of free  $\alpha 1\beta 2\gamma 2\text{L}$  subunits. The data show mean  $\pm$  SEM from 15 (concatemeric receptors) or 6 cells ( $\alpha 1\beta 2\gamma 2\text{L}$  receptors). The curves were fitted to the Hill equation. For concatemeric receptors, the best-fit parameters are:  $\text{EC}_{50} = 92 \pm 2 \mu\text{M}$ ,  $n_{\text{H}} = 1.7 \pm 0.1$ . For  $\alpha 1\beta 2\gamma 2\text{L}$  receptors, the best-fit parameters are:  $\text{EC}_{50} = 7.2 \pm 0.3 \mu\text{M}$ ,  $n_{\text{H}} = 1.3 \pm 0.1$ .

**Figure 2. Sample single-channel currents from cells expressing wild-type concatemeric  $\gamma\beta\alpha$  -  $\beta\alpha$  GABA-A receptors.** The cells were exposed to 20, 100, 500 or 2000  $\mu\text{M}$  GABA. The increase in agonist concentration results in an increase in cluster open probability. At 20  $\mu\text{M}$  GABA, only isolated openings and short bursts were observed. Channel openings are shown as downward deflections. The putative clusters at 100 to 2000  $\mu\text{M}$  GABA are shown with lines above the current traces.

**Figure 3. Comparison of open time properties for receptors containing concatemeric vs. free subunits. A.** Mean open durations for the three open time components at 20-5000  $\mu\text{M}$  GABA. Each symbol corresponds to data from one patch. The lines correspond to mean  $\pm$  SD for the open time durations from  $\alpha 1\beta 2\gamma 2\text{L}$  receptors (Steinbach and Akk, 2001). The data indicate that the mean durations of OT2 and OT3 are increased in concatemeric receptors. **B.** The prevalence (fraction) of the three open time components at 20-5000  $\mu\text{M}$  GABA. Each symbol corresponds to data from one

patch. The lines correspond to mean  $\pm$  SD for the relative frequencies of OT1-3 from  $\alpha 1\beta 2\gamma 2L$  receptors (Steinbach and Akk, 2001).

**Figure 4. Single-channel activation properties of the wild-type concatemeric  $\gamma\beta\alpha$  -**

**$\beta\alpha$  receptor. A.** Relationship between the effective opening rate and GABA concentration. The effective opening rate is the inverse duration of the GABA concentration-dependent closed time component. Each symbol corresponds to data from one patch. The curve was fitted to the Hill equation. The best-fit parameters are:  $\beta$  (maximal effective opening rate) =  $2950 \pm 686 \text{ s}^{-1}$ ,  $EC_{50} = 1452 \pm 488 \text{ }\mu\text{M}$ ,  $n_H = 1.4 \pm 0.1$ . The dashed line is from previous data on  $\alpha 1\beta 2\gamma 2L$  receptors (Steinbach and Akk, 2001), with the following best-fit parameters:  $\beta = 1883 \pm 686 \text{ s}^{-1}$ ,  $EC_{50} = 359 \pm 162 \text{ }\mu\text{M}$ ,  $n_H = 1.7 \pm 0.2$ . **B.** Relationship between the intracluster open probability ( $P_o$ ) and GABA concentration. Each symbol corresponds to data from one patch. The curve was fitted to the Hill equation. The best-fit parameters are:  $P_{o,max} = 0.88 \pm 0.03$ ,  $EC_{50} = 83 \pm 10 \text{ }\mu\text{M}$ ,  $n_H = 1.4 \pm 0.2$ . The dashed line is from previous data on  $\alpha 1\beta 2\gamma 2L$  receptors (Steinbach and Akk, 2001), with the following best-fit parameters:  $P_{o,max} = 0.82 \pm 0.04$ ,  $EC_{50} = 70 \pm 9 \text{ }\mu\text{M}$ ,  $n_H = 1.2 \pm 0.2$ .

**Figure 5. The neurosteroid 3 $\alpha$ 5 $\alpha$ P potentiates wild-type concatemeric  $\gamma\beta\alpha$  -  $\beta\alpha$**

**GABA-A receptors. A.** Sample whole-cell responses to 40  $\mu\text{M}$  GABA in the absence and presence of 1  $\mu\text{M}$  3 $\alpha$ 5 $\alpha$ P. **B.** Potentiation dose-response properties. The data show mean  $\pm$  SEM from 7 cells. The test applications lasted 4 s and were separated from flanking control (GABA alone) applications by 30 s washouts. The curve was fitted to the Hill equation with an offset (fixed at 100 %). The best-fit parameters are: maximal potentiation =  $386 \pm 11 \%$ ,  $EC_{50} = 63 \pm 9 \text{ nM}$ , and  $n_H = 1.5 \pm 0.2$ . The dashed line applies



to  $\alpha 1\beta 2\gamma 2L$  receptors (Akk et al., 2008), with best-fit parameters: maximal potentiation =  $351 \pm 4 \%$ ,  $EC_{50} = 41 \pm 2$  nM, and  $n_H = 1.2 \pm 0.1$ . **C.** Sample single-channel clusters elicited by 50  $\mu$ M GABA in the absence and presence of 1  $\mu$ M 3 $\alpha$ 5 $\alpha$ P. The clusters are shown with lines above the traces. The intracluster open and closed time histograms from the respective patches are shown next to data traces. For GABA, the open times were 0.17 ms (24 %), 4.3 ms (58 %) and 17 ms (18 %), and the closed times were 0.13 ms (54 %), 1.5 ms (10 %) and 42 ms (36 %). For GABA + 3 $\alpha$ 5 $\alpha$ P, the open times were 0.24 ms (47 %), 1.4 ms (14 %) and 26 ms (39 %), and the closed times were 0.19 ms (72 %), 1.5 ms (22 %) and 39 ms (6 %).

**Figure 6. The effect of the  $\alpha 1Q241L$  mutation on the activation and modulation of concatemeric receptors.** **A.** GABA dose-response properties for the  $\gamma\beta\alpha Q241L - \beta\alpha$  (hollow circles),  $\gamma\beta\alpha - \beta\alpha Q241L$  (hollow squares), and  $\gamma\beta\alpha Q241L - \beta\alpha Q241L$  receptors (filled diamonds). The data show mean  $\pm$  SEM from 8-19 cells. The curves were fitted to the Hill equation. The best-fit parameters are:  $EC_{50} = 140 \pm 13$   $\mu$ M,  $n_H = 1.4 \pm 0.2$  ( $\gamma\beta\alpha Q241L - \beta\alpha$ );  $EC_{50} = 128 \pm 8$   $\mu$ M,  $n_H = 1.5 \pm 0.1$  ( $\gamma\beta\alpha - \beta\alpha Q241L$ );  $EC_{50} = 152 \pm 14$   $\mu$ M,  $n_H = 1.5 \pm 0.2$  ( $\gamma\beta\alpha Q241L - \beta\alpha Q241L$ ). The dashed curve applies to data from the wild-type  $\gamma\beta\alpha - \beta\alpha$  receptor (reproduced from Figure 1B). **B.** Potentiation dose-response properties for the  $\gamma\beta\alpha Q241L - \beta\alpha$  (hollow circles),  $\gamma\beta\alpha - \beta\alpha Q241L$  (hollow squares), and  $\gamma\beta\alpha Q241L - \beta\alpha Q241L$  receptors (filled diamonds). The data show mean  $\pm$  SEM from 4-6 cells. The test applications lasted 4 s and were separated from flanking control (30  $\mu$ M GABA alone;  $\sim EC_{20}$ ) applications by 30 s washouts. The curve was fitted to the Hill equation with an offset (fixed at 100 %). The best-fit parameters for the  $\gamma\beta\alpha Q241L - \beta\alpha$  are: maximal potentiation =  $313 \pm 27 \%$ ,  $EC_{50} = 142 \pm 65$  nM. The best-fit parameters for the  $\gamma\beta\alpha - \beta\alpha Q241L$  are: maximal potentiation =  $334 \pm 28 \%$ ,  $EC_{50} = 106 \pm 41$  nM. No

fitting was attempted for the data from the  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$ Q241L receptor. The dashed curve applies to data from the wild-type  $\gamma\beta\alpha$  -  $\beta\alpha$  receptor (reproduced from Figure 5B).

**Figure 7. The potentiation of single-channel currents from the concatemeric**

**$\gamma\beta\alpha$ Q241L -  $\beta\alpha$  receptor by the neurosteroid 3 $\alpha$ 5 $\alpha$ P. (A)** A sample single-channel cluster from the  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$  receptor activated by 10  $\mu$ M GABA, and the open and closed time histograms. The open times were 0.13 ms (15 %), 2.5 ms (73 %) and 7.1 ms (12 %), and the closed times were 0.15 ms (57 %), 1.0 ms (11 %) and 13.0 ms (31 %).  
**(A)** A sample single-channel cluster from the  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$  receptor activated by 10  $\mu$ M GABA in the presence of 1  $\mu$ M 3 $\alpha$ 5 $\alpha$ P, and the open and closed time histograms. The open times were 0.33 ms (44 %), 1.6 ms (22 %) and 16.9 ms (34 %), and the closed times were 0.16 ms (72 %), 1.0 ms (22 %) and 15.6 ms (6 %). Averaged data are shown in Table 5.

**Figure 8. The presence of the  $\alpha$ 1Q241W mutation affects channel activation by**

**P4S. A.** Sample single-channel currents from the  $\gamma\beta\alpha$  -  $\beta\alpha$  receptor exposed to 1 mM P4S. No clusters were evident. A portion of the current trace is shown at higher resolution in the bottom trace. **B.** Sample single-channel currents from the  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$  receptor exposed to 1 mM P4S. One cluster is shown. A portion of the cluster is shown at higher resolution in the bottom trace. **C.** Sample single-channel currents from the  $\gamma\beta\alpha$  -  $\beta\alpha$ Q241W receptor exposed to 1 mM P4S. One cluster is shown. A portion of the cluster is shown at higher resolution in the bottom trace. **D.** Sample single-channel currents from the  $\gamma\beta\alpha$ Q241W -  $\beta\alpha$ Q241W receptor exposed to 1 mM P4S. One cluster is shown. A portion of the cluster is shown at higher resolution in the bottom trace.

[GABA]	OT1 (ms)	Fraction OT1	OT2 (ms)	Fraction OT2	OT3 (ms)	Fraction OT3	n
20 $\mu$ M	0.45 $\pm$ 0.09	0.30 $\pm$ 0.03	4.9 $\pm$ 0.5	0.57 $\pm$ 0.09	11.8 $\pm$ 1.6	0.14 $\pm$ 0.12	3
50 $\mu$ M	0.27 $\pm$ 0.13	0.24 $\pm$ 0.11	3.9 $\pm$ 0.6	0.58 $\pm$ 0.15	12.6 $\pm$ 4.0	0.18 $\pm$ 0.11	4
100 $\mu$ M	0.34 $\pm$ 0.20	0.20 $\pm$ 0.08	3.9 $\pm$ 1.0	0.62 $\pm$ 0.24	11.0 $\pm$ 2.2	0.17 $\pm$ 0.16	4
200 $\mu$ M	0.23 $\pm$ 0.16	0.16 $\pm$ 0.13	4.1 $\pm$ 1.4	0.53 $\pm$ 0.29	9.6 $\pm$ 3.7	0.30 $\pm$ 0.16	3
500 $\mu$ M	0.27 $\pm$ 0.10	0.10 $\pm$ 0.01	4.6 $\pm$ 0.8	0.64 $\pm$ 0.16	11.1 $\pm$ 4.2	0.26 $\pm$ 0.15	3
1000 $\mu$ M	0.40 $\pm$ 0.14	0.16 $\pm$ 0.06	3.8 $\pm$ 0.6	0.73 $\pm$ 0.10	10.2 $\pm$ 2.4	0.11 $\pm$ 0.10	5
2000 $\mu$ M	0.38 $\pm$ 0.07	0.17 $\pm$ 0.03	3.5 $\pm$ 0.4	0.67 $\pm$ 0.17	10.4 $\pm$ 2.4	0.16 $\pm$ 0.15	3
5000 $\mu$ M	0.42 $\pm$ 0.17	0.16 $\pm$ 0.13	2.8 $\pm$ 0.7	0.67 $\pm$ 0.18	6.5 $\pm$ 1.9	0.17 $\pm$ 0.11	5

**Table 1. Open times for wild-type concatemeric  $\gamma\beta\alpha$  -  $\beta\alpha$  receptors.** Receptors were activated by 20-5000  $\mu$ M GABA. The table gives the mean durations (OT1-3) and prevalence (fraction OT1-3) for the three open time components, and the number of patches for each condition. The data show mean  $\pm$  SD.

[GABA]	CT1 (ms)	Fraction CT1	CT2 (ms)	Fraction CT2	CT <sub>β</sub> (ms)	Fraction CT <sub>β</sub>	CT4 (ms)	Fraction CT4
20 μM	0.13 ± 0.02	0.57 ± 0.12	1.0 ± 0.2	0.12 ± 0.08	94 ± 52	0.31 ± 0.05	-	-
50 μM	0.13 ± 0.01	0.55 ± 0.05	3.6 ± 4.3	0.14 ± 0.09	34 ± 8	0.31 ± 0.08	-	-
100 μM	0.15 ± 0.02	0.53 ± 0.05	0.9 ± 0.2	0.14 ± 0.03	15 ± 8	0.33 ± 0.05	-	-
200 μM	0.14 ± 0.01	0.48 ± 0.11	1.6 ± 0.6	0.22 ± 0.06	9.4 ± 1.5	0.30 ± 0.10	-	-
500 μM	0.12 ± 0.00	0.52 ± 0.03	0.7 ± 0.1	0.20 ± 0.05	2.4 ± 0.6	0.27 ± 0.05	9.1 ± 1.1	0.02 ± 0.01
1000 μM	0.14 ± 0.02	0.49 ± 0.03	2.9 ± 1.6	0.06 ± 0.04	0.6 ± 0.1	0.44 ± 0.03	28 ± 9	0.003 ± 0.001
2000 μM	0.14 ± 0.03	0.53 ± 0.10	2.3 ± 0.4	0.09 ± 0.10	0.5 ± 0.1	0.37 ± 0.03	19 ± 8	0.01 ± 0.01
5000 μM	0.16 ± 0.01	0.50 ± 0.10	1.8 ± 0.3	0.09 ± 0.10	0.5 ± 0.1	0.43 ± 0.09	27 ± 6	0.003 ± 0.001

**Table 2. Closed times for wild-type concatemeric  $\gamma\beta\alpha$  -  $\beta\alpha$  receptors.** Receptors were activated by 20-5000 μM GABA. The intracluster closed time histograms were fitted to sums of three (50-200 μM GABA) or four exponentials. At 20 μM GABA, no clusters were evident. For analysis sections of data containing no overlapping currents were selected. The table gives the mean durations (CT1-4) and prevalence (fraction CT1-4) for the closed time components. CT<sub>β</sub> signifies the GABA concentration-dependent closed time component that arises from dwells in the diliganded, monoliganded and unliganded closed states. The data show mean ± SD. The number of patches for each condition is given in Table 1.

[GABA]	[3 $\alpha$ 5 $\alpha$ P]	OT1 (ms)	Fraction OT1	OT2 (ms)	Fraction OT2	OT3 (ms)	Fraction OT3
50 $\mu$ M	-	0.27 $\pm$ 0.13	0.24 $\pm$ 0.11	3.9 $\pm$ 0.6	0.58 $\pm$ 0.15	12.6 $\pm$ 4.0	0.18 $\pm$ 0.11
50 $\mu$ M	1 $\mu$ M	0.27 $\pm$ 0.05 <sup>†</sup>	0.45 $\pm$ 0.10*	2.1 $\pm$ 1.8 <sup>†</sup>	0.24 $\pm$ 0.06**	22.7 $\pm$ 5.5*	0.31 $\pm$ 0.09 <sup>†</sup>
[GABA]	[3 $\alpha$ 5 $\alpha$ P]	CT1 (ms)	Fraction CT1	CT2 (ms)	Fraction CT2	CT $\beta$ (ms)	Fraction CT $\beta$
50 $\mu$ M	-	0.13 $\pm$ 0.01	0.55 $\pm$ 0.05	3.6 $\pm$ 4.3	0.14 $\pm$ 0.09	34 $\pm$ 8	0.31 $\pm$ 0.08
50 $\mu$ M	1 $\mu$ M	0.16 $\pm$ 0.05 <sup>†</sup>	0.68 $\pm$ 0.07*	1.2 $\pm$ 0.3 <sup>†</sup>	0.27 $\pm$ 0.05*	23.4 $\pm$ 9.7 <sup>†</sup>	0.05 $\pm$ 0.03***

**Table 3. Neurosteroid 3 $\alpha$ 5 $\alpha$ P potentiates currents from the wild-type concatemeric  $\gamma\beta\alpha$  -  $\beta\alpha$  receptor.** The effect of 1  $\mu$ M 3 $\alpha$ 5 $\alpha$ P on currents elicited by 50  $\mu$ M GABA. The table gives mean durations and prevalence for the three open and closed time components. The data show mean  $\pm$  SD from 4 (GABA) or 5 patches (GABA + 3 $\alpha$ 5 $\alpha$ P). The significance levels apply to comparison with the data in the absence of steroid. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; <sup>†</sup>, not significant.

[GABA]	[3 $\alpha$ 5 $\alpha$ P]	OT1 (ms)	Fraction OT1	OT2 (ms)	Fraction OT2	OT3 (ms)	Fraction OT3
100 $\mu$ M	-	0.27 $\pm$ 0.17	0.16 $\pm$ 0.03	3.1 $\pm$ 0.6	0.65 $\pm$ 0.10	8.0 $\pm$ 2.1	0.19 $\pm$ 0.10
100 $\mu$ M	1 $\mu$ M	0.32 $\pm$ 0.08 <sup>†</sup>	0.36 $\pm$ 0.08 <sup>***</sup>	2.5 $\pm$ 1.1 <sup>†</sup>	0.39 $\pm$ 0.08 <sup>***</sup>	21.4 $\pm$ 5.9 <sup>**</sup>	0.39 $\pm$ 0.08 <sup>*</sup>
[GABA]	[3 $\alpha$ 5 $\alpha$ P]	CT1 (ms)	Fraction CT1	CT2 (ms)	Fraction CT2	CT $_{\beta}$ (ms)	Fraction CT $_{\beta}$
100 $\mu$ M	-	0.16 $\pm$ 0.02	0.64 $\pm$ 0.05	1.6 $\pm$ 0.7	0.12 $\pm$ 0.03	13 $\pm$ 2	0.24 $\pm$ 0.05
100 $\mu$ M	1 $\mu$ M	0.16 $\pm$ 0.04 <sup>†</sup>	0.64 $\pm$ 0.06 <sup>†</sup>	1.3 $\pm$ 0.3 <sup>*</sup>	0.25 $\pm$ 0.06 <sup>**</sup>	12 $\pm$ 5 <sup>†</sup>	0.11 $\pm$ 0.04 <sup>**</sup>

**Table 4. The neurosteroid 3 $\alpha$ 5 $\alpha$ P potentiates single-channel currents from the concatemeric mutant  $\gamma\beta\alpha$ Q241L -  $\beta\alpha$  receptor.** Effect of 1  $\mu$ M 3 $\alpha$ 5 $\alpha$ P on currents elicited by 100  $\mu$ M GABA. The table gives mean durations and prevalence for the three open and closed time components. The data show mean  $\pm$  SD from 5 (GABA) or 4 patches (GABA + 3 $\alpha$ 5 $\alpha$ P). The significance levels apply to comparison with the data in the absence of steroid. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; <sup>†</sup>, not significant.

Receptor	P4S	3α5αP	OT1 (ms)	Fraction OT1	OT2 (ms)	Fraction OT2	n
γβ <sub>α</sub> - β <sub>α</sub>	1 mM	-	1.2±0.2 <sup>†,†,†</sup>	0.72±0.16 <sup>†,†,**</sup>	2.4±0.6 <sup>†,†,†</sup>	0.28±0.16 <sup>†,†,**</sup>	4
γβ <sub>α</sub> - β <sub>α</sub>	1 mM	1 μM	0.6±0.2 <sup>**</sup>	0.48±0.03 <sup>*</sup>	12.5±0.5 <sup>***</sup>	0.52±0.03 <sup>*</sup>	4
γβ <sub>α</sub> Q241W - β <sub>α</sub>	1 mM	-	1.0±0.2 <sup>†,†,†</sup>	0.58±0.20 <sup>†,†,*</sup>	3.2±1.1 <sup>†,†,†</sup>	0.42±0.20 <sup>†,†,*</sup>	8
γβ <sub>α</sub> Q241W - β <sub>α</sub>	1 mM	1 μM	0.8±0.4 <sup>†</sup>	0.37±0.06 <sup>*</sup>	6.9±1.9 <sup>***</sup>	0.63±0.06 <sup>*</sup>	5
γβ <sub>α</sub> - β <sub>α</sub> Q241W	1 mM	-	1.4±0.7 <sup>†,†,†</sup>	0.61±0.28 <sup>†,†,*</sup>	4.0±1.2 <sup>†,†,†</sup>	0.39±0.28 <sup>†,†,*</sup>	6
γβ <sub>α</sub> - β <sub>α</sub> Q241W	1 mM	1 μM	1.1±0.6 <sup>†</sup>	0.33±0.08 <sup>*</sup>	11.8±4.8 <sup>**</sup>	0.67±0.08 <sup>*</sup>	7
γβ <sub>α</sub> Q241W - β <sub>α</sub> Q241W	1 mM	-	0.8±0.2 <sup>†,†,†</sup>	0.27±0.03 <sup>***,**</sup>	3.4±0.8 <sup>†,†,†</sup>	0.73±0.03 <sup>***,**</sup>	6
γβ <sub>α</sub> Q241W - β <sub>α</sub> Q241W	1 mM	1 μM	0.8±0.4 <sup>†</sup>	0.27±0.13 <sup>†</sup>	3.7±1.4 <sup>†</sup>	0.73±0.13 <sup>†</sup>	6

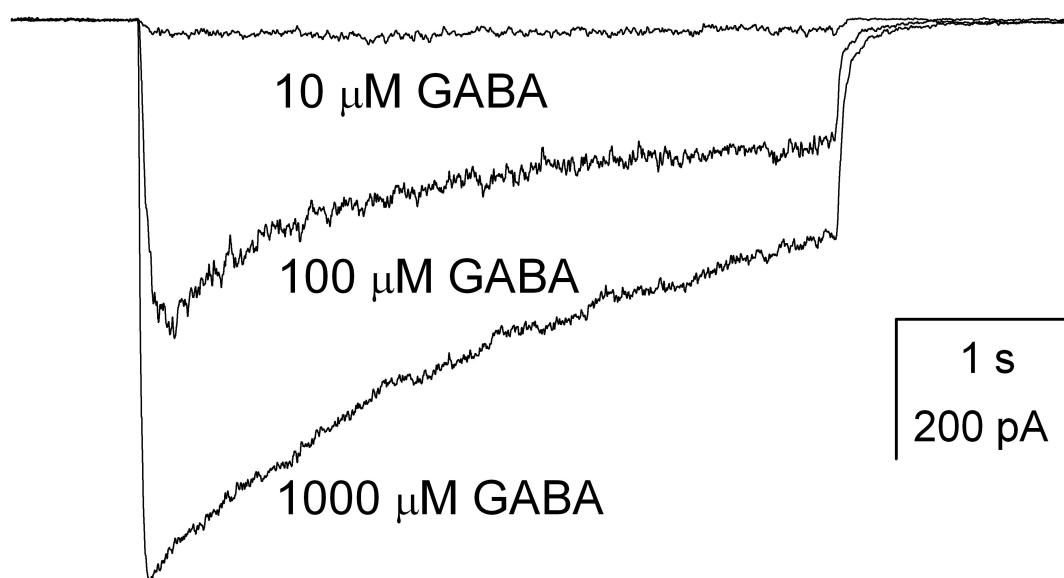
**Table 5. The effect of the  $\alpha$ Q241W mutation on channel activation by P4S in the absence and presence of 3 $\alpha$ 5 $\alpha$ P: open times.** The open time histograms were fitted to sums of two exponentials. The table gives the mean durations (OT1-2) and relative contributions (fraction OT1-2) for the open time components. For data obtained in the absence of 3 $\alpha$ 5 $\alpha$ P, the significance levels (ANOVA with Bonferroni correction) apply to comparison with the remaining three receptor types. For data obtained in the presence of P4S + 3 $\alpha$ 5 $\alpha$ P, the significance levels apply to comparison with the same type of receptor activated by P4S in the absence of 3 $\alpha$ 5 $\alpha$ P. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; †, not significant.



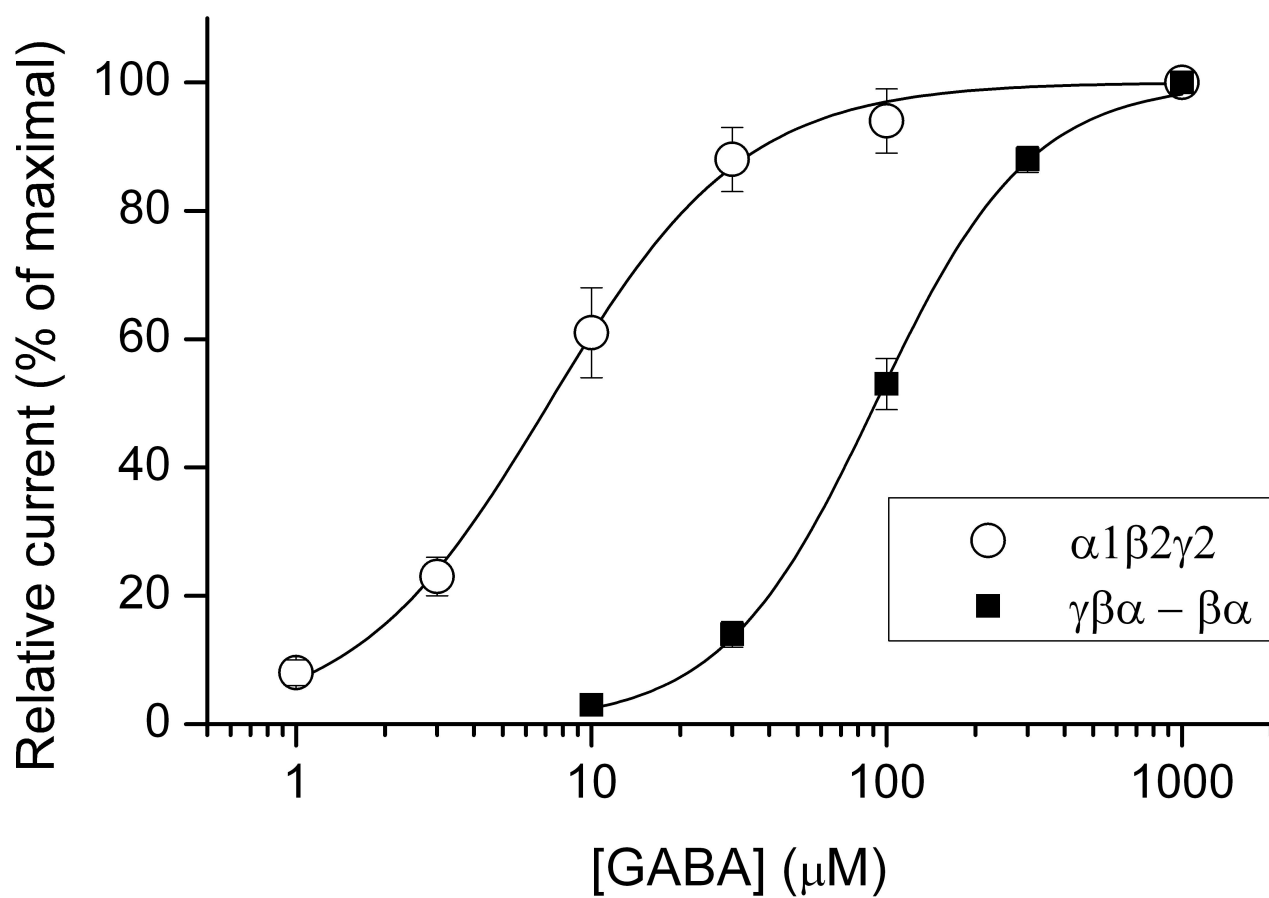
Receptor	P4S	3 $\alpha$ 5 $\alpha$ P	CT1 (ms)	Fraction CT1	CT2 (ms)	Fraction CT2	CT3 (ms)	Fraction CT3
$\gamma\beta\alpha$ - $\beta\alpha$	1 mM	-	0.42 $\pm$ 0.34	0.10 $\pm$ 0.04	42 $\pm$ 33	0.90 $\pm$ 0.04	-	-
$\gamma\beta\alpha$ - $\beta\alpha$	1 mM	1 $\mu$ M	0.21 $\pm$ 0.08 <sup>na,†</sup>	0.40 $\pm$ 0.10 <sup>na,*</sup>	1.2 $\pm$ 0.3 <sup>na,*</sup>	0.39 $\pm$ 0.07 <sup>na,†</sup>	9 $\pm$ 3 <sup>na,†</sup>	0.21 $\pm$ 0.06 <sup>na,*</sup>
$\gamma\beta\alpha$ Q241W - $\beta\alpha$	1 mM	-	0.14 $\pm$ 0.04	0.17 $\pm$ 0.06	3.6 $\pm$ 3.1	0.33 $\pm$ 0.23	15 $\pm$ 6	0.50 $\pm$ 0.24
$\gamma\beta\alpha$ Q241W - $\beta\alpha$	1 mM	1 $\mu$ M	0.21 $\pm$ 0.09 <sup>†,†</sup>	0.39 $\pm$ 0.05 <sup>***,*</sup>	1.6 $\pm$ 0.7 <sup>†,†</sup>	0.35 $\pm$ 0.08 <sup>†,†</sup>	13 $\pm$ 8 <sup>†,†</sup>	0.26 $\pm$ 0.07 <sup>†,†</sup>
$\gamma\beta\alpha$ - $\beta\alpha$ Q241W	1 mM	-	0.27 $\pm$ 0.12	0.19 $\pm$ 0.10	2.9 $\pm$ 2.4	0.20 $\pm$ 0.19	29 $\pm$ 12	0.61 $\pm$ 0.19
$\gamma\beta\alpha$ - $\beta\alpha$ Q241W	1 mM	1 $\mu$ M	0.18 $\pm$ 0.03 <sup>†,†</sup>	0.35 $\pm$ 0.04 <sup>**,*</sup>	1.4 $\pm$ 0.4 <sup>†,*</sup>	0.29 $\pm$ 0.07 <sup>†,†</sup>	18 $\pm$ 7 <sup>†,*</sup>	0.36 $\pm$ 0.07 <sup>**,*</sup>
$\gamma\beta\alpha$ Q241W - $\beta\alpha$ Q241W	1 mM	-	0.23 $\pm$ 0.10	0.24 $\pm$ 0.05	2.5 $\pm$ 0.3	0.42 $\pm$ 0.11	9 $\pm$ 3	0.34 $\pm$ 0.15
$\gamma\beta\alpha$ Q241W - $\beta\alpha$ Q241W	1 mM	1 $\mu$ M	0.27 $\pm$ 0.03 <sup>†,na</sup>	0.28 $\pm$ 0.06 <sup>†,na</sup>	2.6 $\pm$ 1.0 <sup>†,na</sup>	0.37 $\pm$ 0.07 <sup>†,na</sup>	8 $\pm$ 3 <sup>†,na</sup>	0.35 $\pm$ 0.09 <sup>†,na</sup>

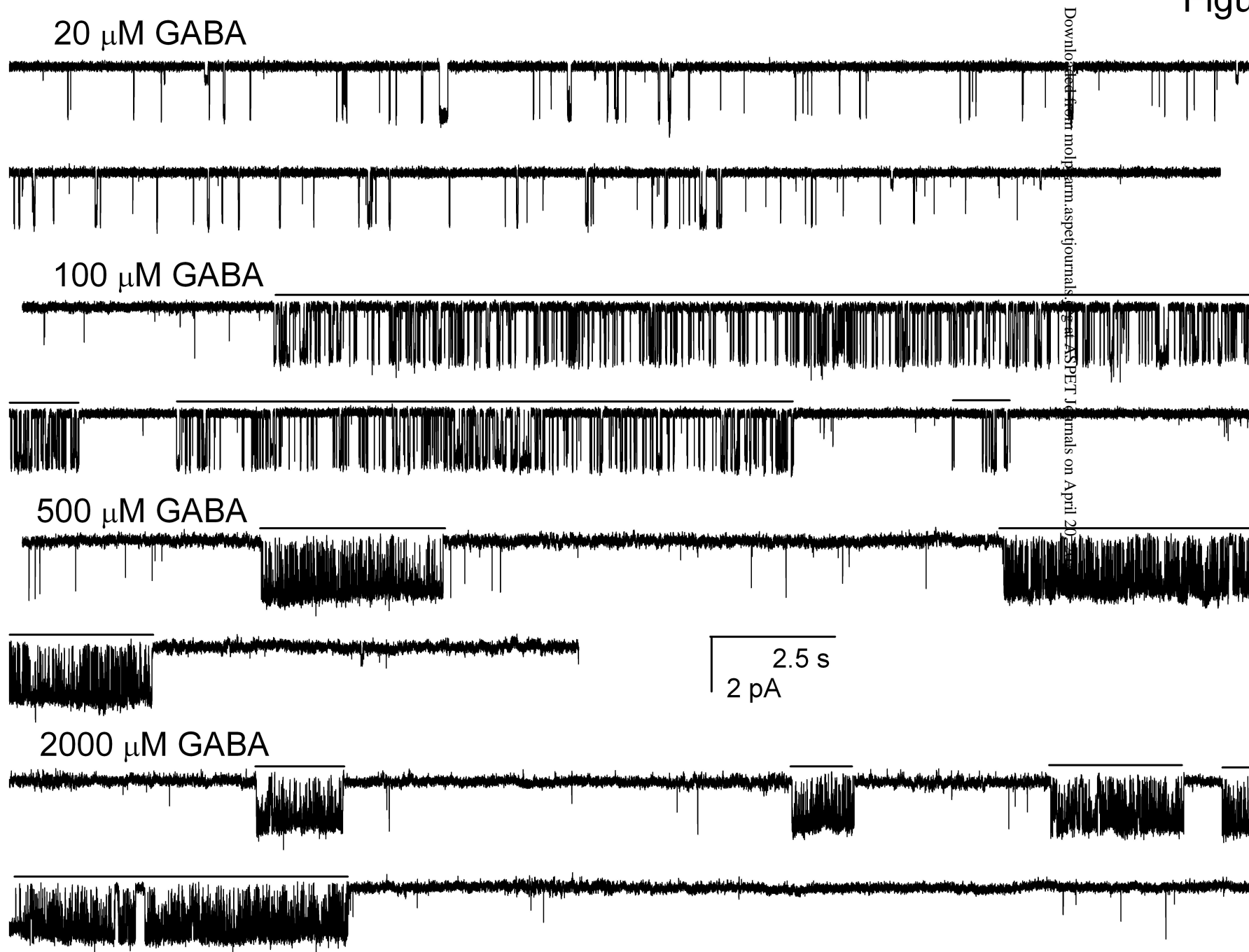
**Table 6. The effect of the  $\alpha$ Q241W mutation on channel activation by P4S in the absence and presence of 3 $\alpha$ 5 $\alpha$ P: closed times.** The closed time histograms were fitted to sums of two ( $\gamma\beta\alpha$  -  $\beta\alpha$  with P4S) or three exponentials. The table gives the mean durations (CT1-3) and relative contributions (fraction CT1-3) for the closed time components. For data obtained in the presence of P4S + 3 $\alpha$ 5 $\alpha$ P, the significance levels apply to comparison with the same type of receptor activated by P4S (t-test), and to the  $\gamma$ 2L $\beta$ 2 $\alpha$ 1Q241W -  $\beta$ 2 $\alpha$ 1Q241W receptor activated by P4S + 3 $\alpha$ 5 $\alpha$ P (ANOVA with Dunnett's correction). Due to lack of clusters, statistical comparison for the effect of steroid was not carried out for the wild-type concatemeric receptor. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001; †, not significant; na, not applicable.

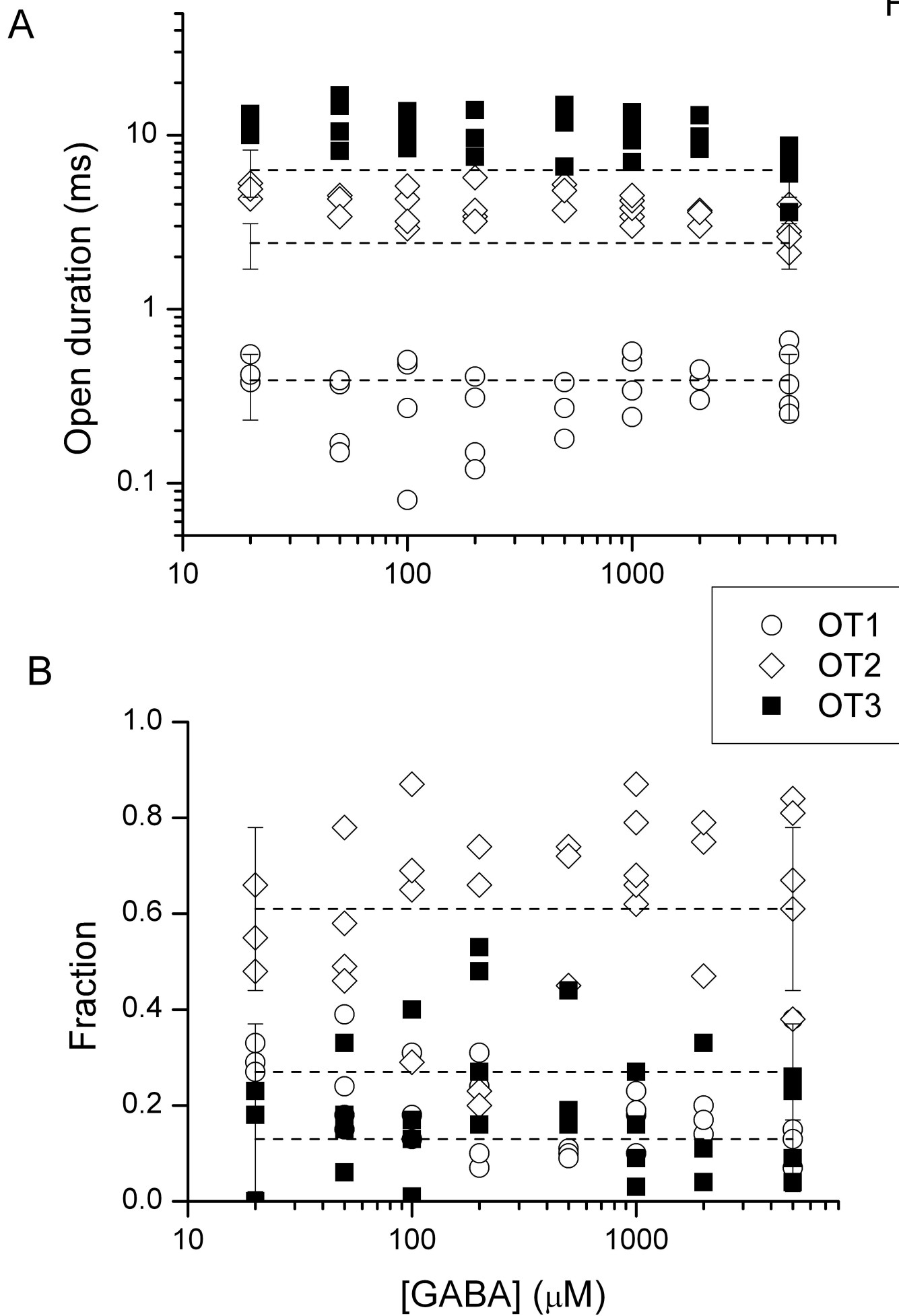
A

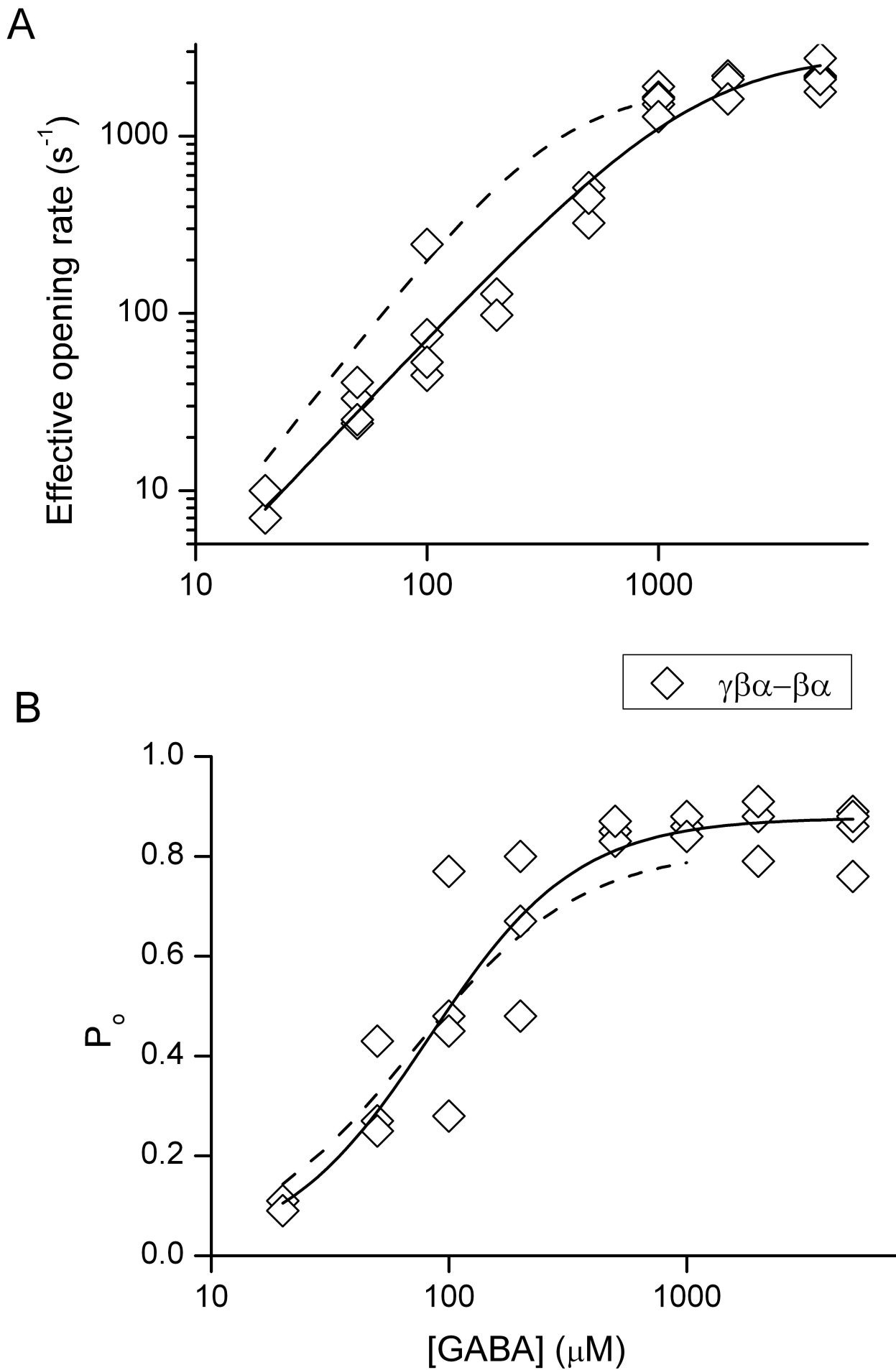


B









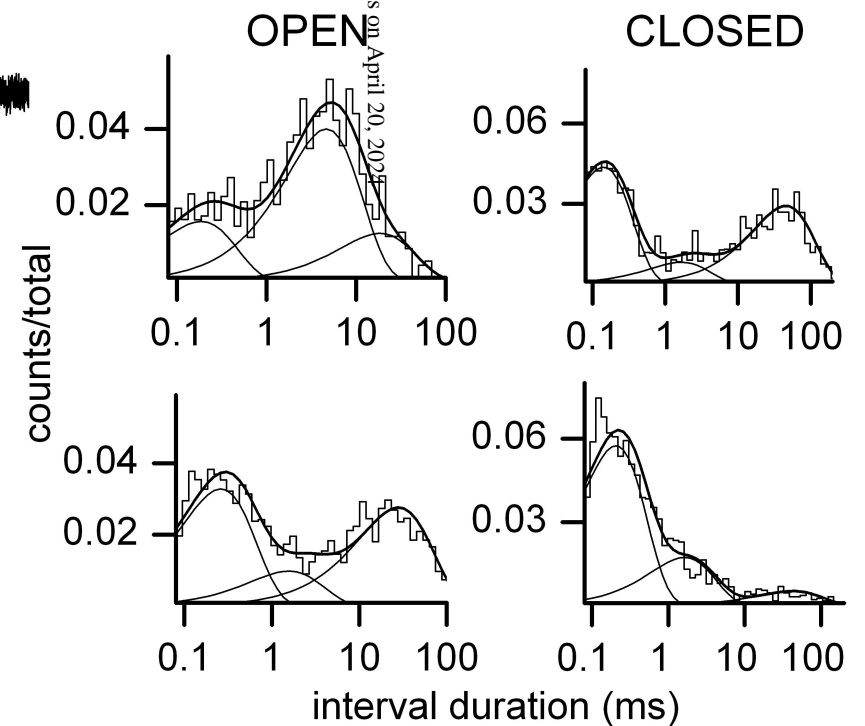
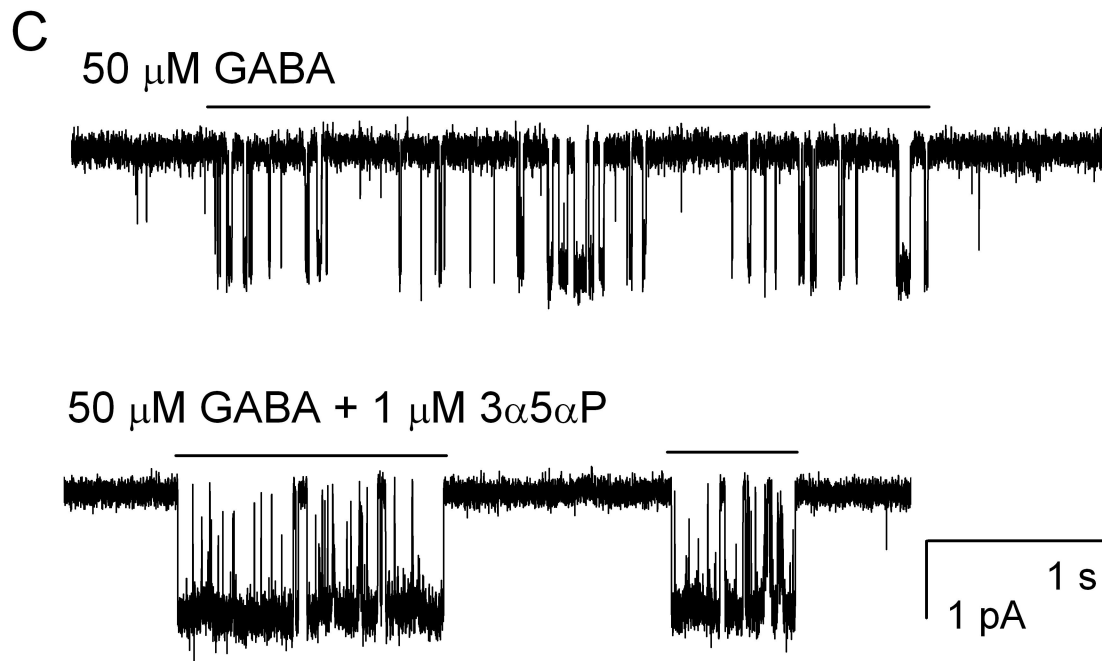
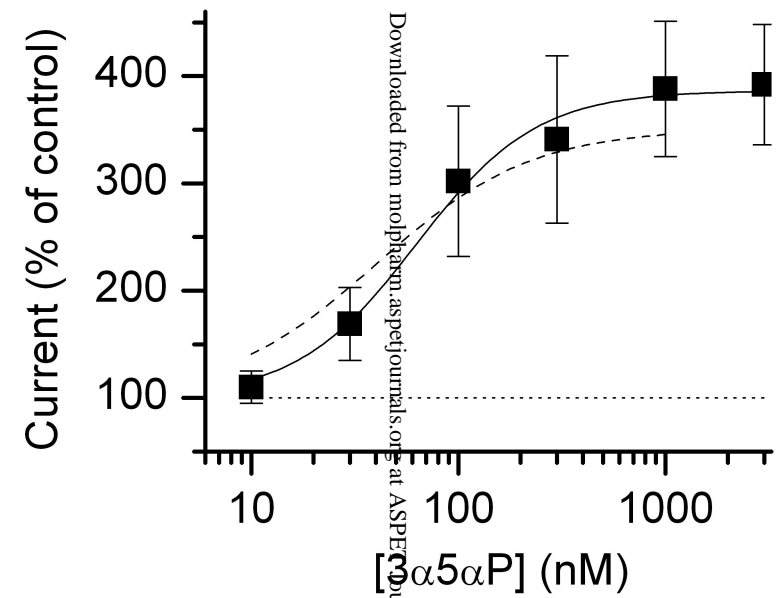
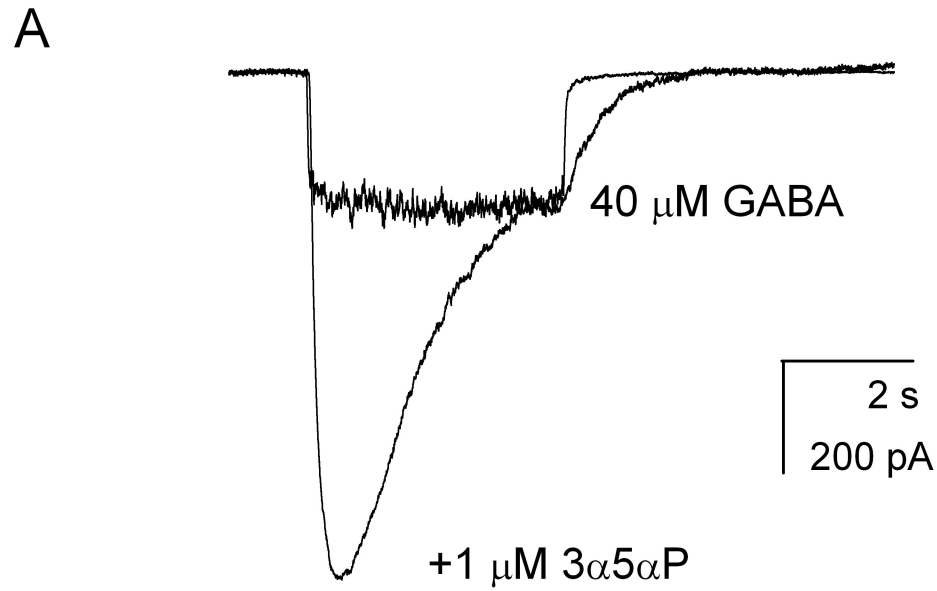


Figure 6

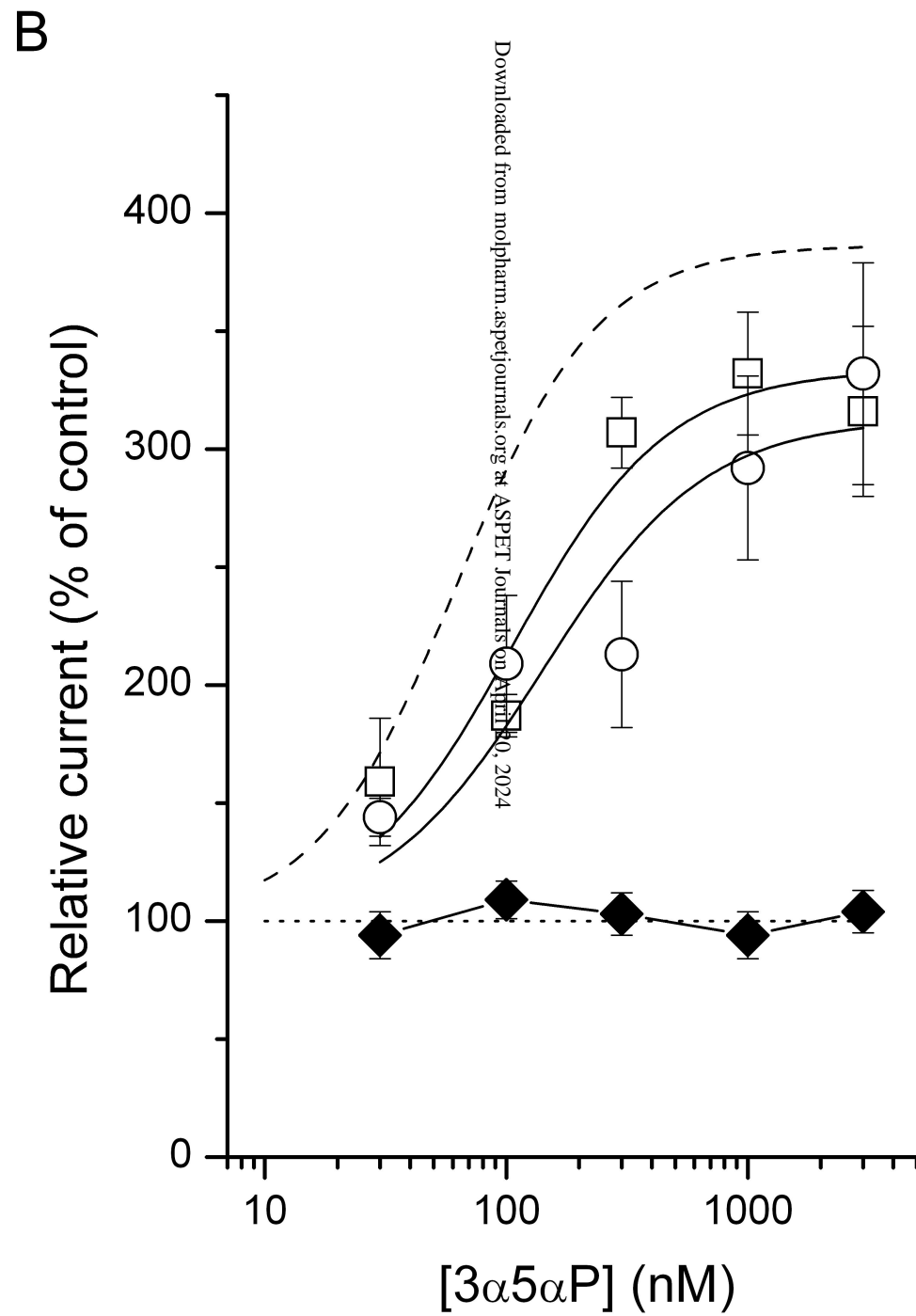
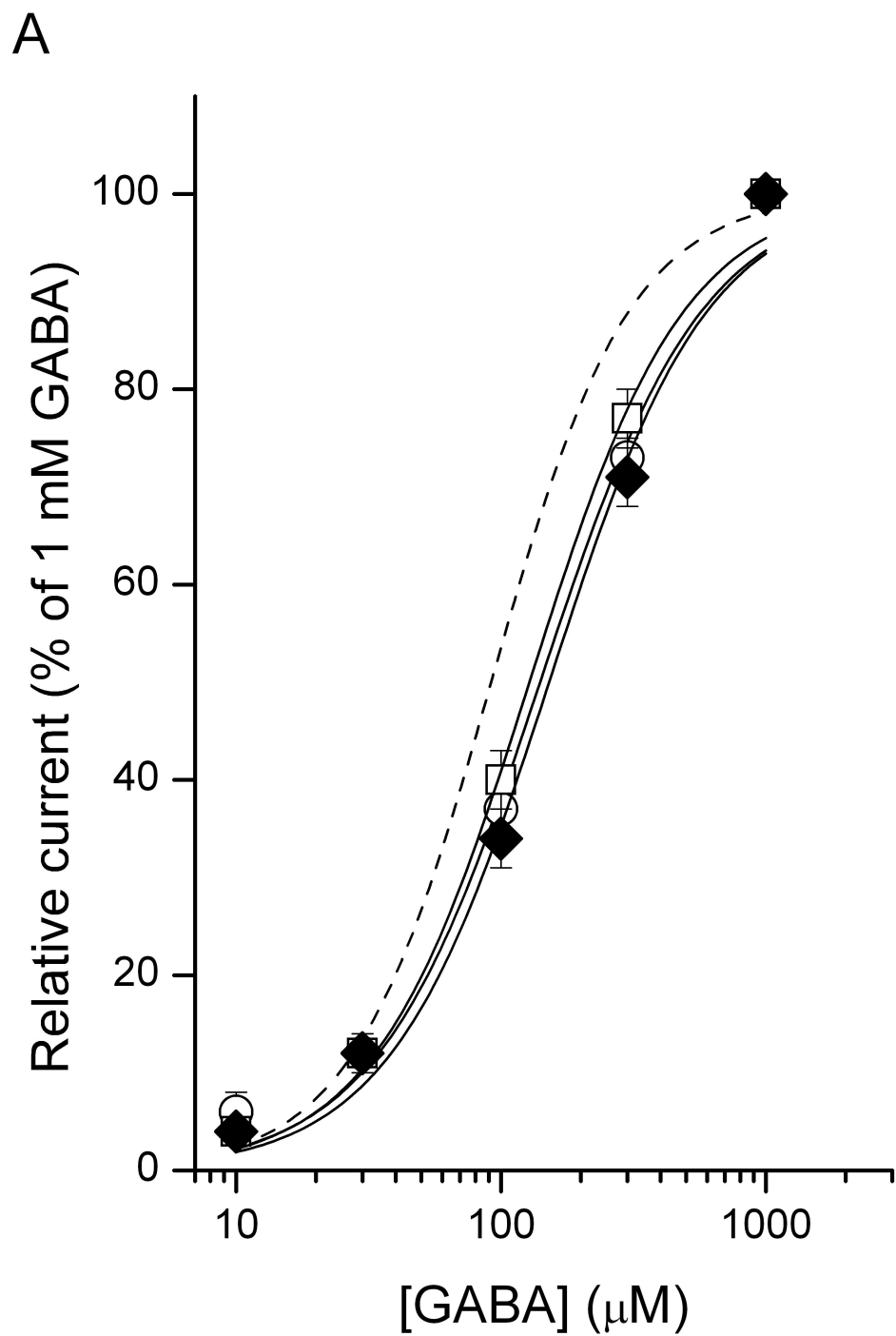
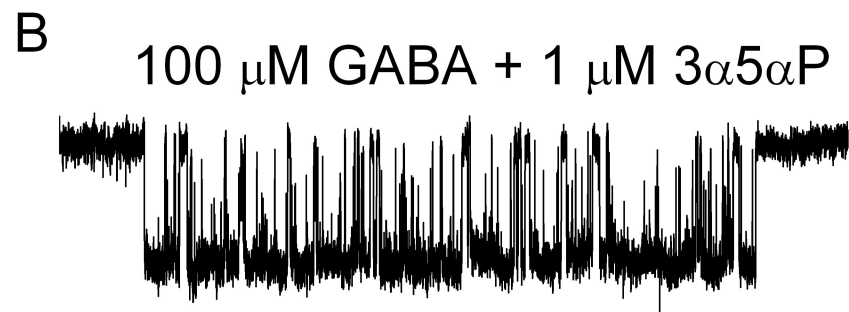


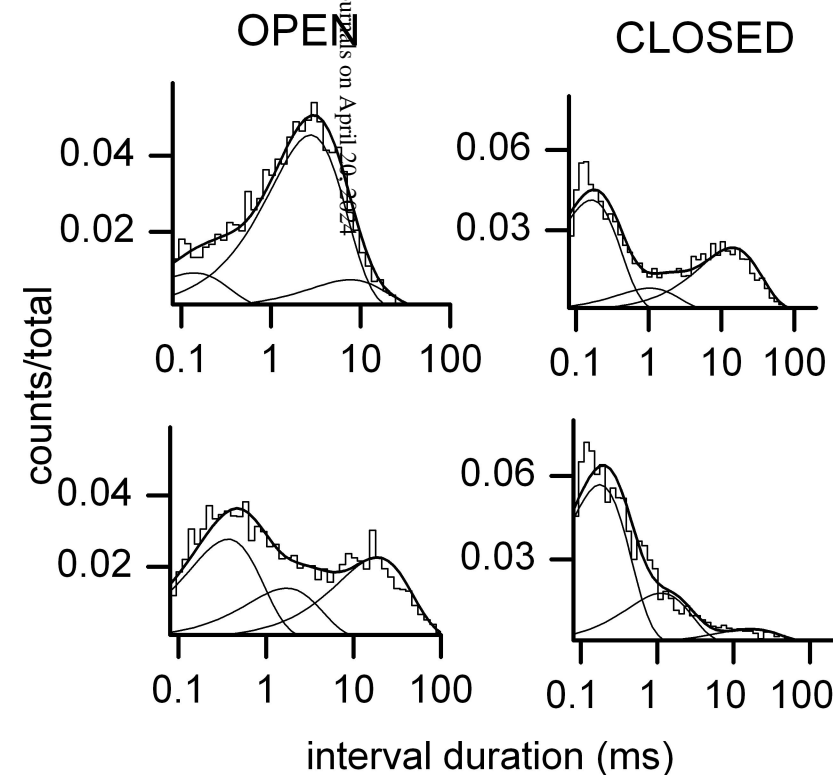


Figure 7

$\gamma\beta\alpha$ Q241L -  $\beta\alpha$



1 pA 1 s



A  $\gamma\beta\alpha-\beta\alpha$ B  $\gamma\beta\alpha Q241W-\beta\alpha$ C  $\gamma\beta\alpha-\beta\alpha Q241W$ D  $\gamma\beta\alpha Q241W-\beta\alpha Q241W$ 