

## **Differential modulation of glutamatergic transmission by 3,5-dibromo-L-phenylalanine**

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Running title:

**3,5-dibromo-L-phenylalanine modulates glutamate transmission**

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Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), gamma-aminobutyric acid (GABA), N-methyl-D-aspartate (NMDA), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[f]quinoxaline-7-sulfonamide (NBQX), dizocilpine (MK-801), phencyclidine (PCP), 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe), tetrodotoxin (TTX)

## Abstract

An increasing body of evidence supports the hypothesis that diminished function of N-methyl-D-aspartate (NMDA) receptors and the associated increase in glutamate release and over-stimulation of (RS)--amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors are critical elements of the pathophysiology of schizophrenia. Here we describe a halogenated derivative of the aromatic amino acid L-phenylalanine, that: 1) activates NMDA receptors; 2) depresses presynaptic glutamate release; and 3) blocks AMPA/kainate receptors. The experiments were conducted in rat cerebrocortical cultured neurons by using the patch-clamp technique. 3, 5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) augmented NMDA miniature excitatory postsynaptic currents (mEPSCs) and activated the steady-state current, effects that were eliminated by NMDA receptor antagonists AP-5 and MK-801. 3,5-DBr-L-Phe was a partial agonist at the glutamate-binding site of NMDA receptors with an  $EC_{50}$  of  $331.6 \pm 78.6 \mu M$  and with an efficacy of  $30.5 \pm 4.7 \%$  compared with NMDA. 3,5-DBr-L-Phe depressed both amplitude and frequency of AMPA/kainate mEPSCs. The  $IC_{50}$  of 3,5-DBr-L-Phe to inhibit AMPA/kainate mEPSC frequency was  $29.4 \pm 4.3 \mu M$ . 3,5-DBr-L-Phe significantly decreased paired-pulse depression of AMPA/kainate EPSCs and attenuated current activated by AMPA with higher efficacy at lower concentration of AMPA. 3, 5-DBr-L-Phe neither affected gamma-aminobutyric (GABA) miniature inhibitory postsynaptic currents nor elicited action potentials.

By enhancing NMDA receptor function, reducing glutamate release and blocking AMPA/kainate receptors 3,5-DBr-L-Phe represents a new type of polyvalent modulator of glutamatergic synaptic transmission with potential therapeutic applications.

A variety of *in vitro*, *in vivo*, and clinical evidence indicates that disturbed glutamatergic synaptic transmission may be a critical element in the pathophysiology of several brain disorders including schizophrenia (Konradia and Heckersa, 2003; Moghaddam and Jackson, 2003). Abnormal glutamatergic neurotransmission has been reported in animal models of schizophrenia as well as in human postmortem and glutamate receptor antagonist studies (Hirsh and Weinberger, 2003; Konradia and Heckersa, 2003; Moghaddam and Jackson, 2003). Non-competitive antagonists of NMDA receptors (dizocilpine [MK-80], phencyclidine [PCP]) increase the locomotor activity of animals and induce stereotyped behavior characteristic of human schizophrenia (Lipska and Weinberger, 2000). Similarly, NMDA receptor antagonists produce in otherwise healthy humans positive and negative symptoms of schizophrenia and exacerbate those in schizophrenic patients (Krystal et al., 1994; Jentsch and Roth, 1999). In addition to decreased NMDA receptor function, changes in glutamatergic transmission characteristic of schizophrenia include increased glutamate release and overstimulation of non-NMDA (AMPA/kainate) receptors (Moghaddam et al., 1997). Consistent with this hypothesis are findings showing an increase in the number of NMDA receptor subunits NR1 in cortical regions and a decrease in the expression of AMPA/kainate receptors in the hippocampus of schizophrenics (Meador-Woodruff and Healy, 2000). The observed decrease in AMPA/kainate receptors may be a down-regulation secondary to increased glutamate release, which in turn results from decreased stimulation of the GABA-ergic system due to hypofunction of NMDA receptors (Moghaddam et al., 1997; Greene 2001).

Therefore, in order to possess antipsychotic properties, potential pharmacological candidates with glutamatergic action should either augment NMDA receptor function,

diminish glutamate release, or block postsynaptic AMPA/kainate receptors. Consistent with this contention, agonists of the glycine-binding site of NMDA receptors alleviate not only negative, but also positive symptoms of schizophrenia and improve cognitive function (Heresco-Levy and Javitt, 2004). Antagonists of AMPA/kainate receptors and agonists for group II metabotropic glutamate receptors reverse the psychotic effects of PCP, inhibit conditioned avoidance responses and amphetamine-induced hyperactivity (Moghaddam and Adams, 1998; Takahata and Moghaddam, 2003; Lorrain et al., 2003). Despite the availability of many types of antipsychotic drugs the search for new efficacious agents without unwanted side effects remains a high priority in the treatment of schizophrenia (Hyman and Fenton, 2003; Moghaddam, 2003).

Here, we describe a compound that simultaneously produces all three desirable actions on glutamatergic synaptic transmission. During investigation of the cellular mechanisms of brain dysfunction seen in phenylketonuria, we found that L-Phe selectively depresses glutamatergic synaptic transmission by a combination of pre- and postsynaptic actions (Glushakov et al., 2002; Glushakov et al., 2003). Specifically, L-Phe depresses glutamatergic synaptic transmission by: 1) competing for the glycine-binding site of NMDA receptors; 2) competing for the glutamate-binding site of AMPA/kainate receptors; and 3) attenuating glutamate release. The relative contribution of each individual mechanism to the antiglutamatergic effect of L-Phe can be modified by creating halogenated derivatives of the molecule (Kagiyama et al., 2004). This provides the opportunity to select/synthesize ligands with specific patterns of glutamatergic action. The halogenated derivative of L-Phe, 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) acts as partial agonist of NMDA receptors, inhibits glutamate release and depresses

AMPA/kainate receptor activity, and, importantly, does not influence activity of voltage-operated ionic channels.

## Materials and Methods

All experiments were approved by the University of Florida Animal Care and Use Committee.

**Neuronal Cultures.** Cerebral cortices were dissected from newborn rats and treated with 0.25% trypsin to dissociate the cells. Dissociated cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% plasma derived horse serum (PDHS) and were plated in poly-L-lysine-coated, 35 mm Nunc plastic tissue culture dishes ( $3.0 \times 10^6$  cells/dish/2ml media). Cultures were maintained in an atmosphere of 5% CO<sub>2</sub>/95% air.

**Electrophysiological recordings:** Voltage- and current-clamp recordings of membrane ionic currents and potentials were conducted by using Axopatch 200B and Axoclamp 1B amplifiers (Axon Instruments, Foster City, CA). The perforated nystatin- and gramicidin-based patch-clamp recording techniques were used in some cases to reduce nonspecific rundown of intracellular processes. Neurons were used for electrophysiological recordings between 12 and 27 days *in vitro*. If a neuron showed either a marked change in holding current or a noticeable alteration in amplitude or shape of capacitance transients during the experiment, the data from that neuron were discarded. Patch microelectrodes were pulled from 1.5 mm borosilicate glass tubing using a two-stage vertical pipette puller (Narishige, East Meadow, NY). When filled with recording solution, patch microelectrodes had a resistance of 3-5 M $\Omega$ . For rapid application of agonist-containing solutions to neurons, the SF-77B system (Warner Instrument Corp., Hamden, CT) was used. Otherwise a slower exchange of extracellular solution was done by a home-made system. The slow onset of activation of 3-5-DBr-L-Phe-induced current

seen in some figures is related to slow exchange of extracellular solution and not to a gradual onset of 3,5-DBr-L-Phe-induced activation of NMDA receptors.

The miniature EPSCs were recorded in TTX-containing (0.3-1  $\mu$ M) extracellular solution at  $V_h = -60$  mV. In case of NMDA receptor-mediated mEPSC recordings  $Mg^{2+}$  was omitted from the extracellular solution. In order to isolate the NMDA component of glutamate receptor-mediated currents, the non-NMDA (AMPA/kainate) receptor antagonist NBQX (10-20  $\mu$ M) was added to extracellular solutions. Conversely, to isolate the non-NMDA receptor-mediated currents, the experiments were performed in the presence of NMDA receptor channel blocker, MK-801 (5-10  $\mu$ M), or in the presence of the NMDA receptor antagonist, AP-5 (20  $\mu$ M). Strychnine (1  $\mu$ M) and picrotoxin (100  $\mu$ M) were added to the extracellular solution to block glycine and GABA receptors, respectively. The basic extracellular solution contained (in mM): NaCl 140, KCl 4,  $CaCl_2$  2,  $MgCl_2$  1, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, and glucose 11. The pH of the extracellular solution was adjusted to 7.4 using NaOH. The main solution for filling the patch electrodes contained (in mM): Cs gluconate 135, NaCl 5, KCl 10,  $MgCl_2$  1,  $CaCl_2$  1, EGTA 11, HEPES 10,  $Na_2ATP$  2,  $Na_2GTP$  0.2 mM. The pH of the intracellular solution was adjusted to 7.4 using CsOH. To record GABA receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs), picrotoxin (100  $\mu$ M) in the extracellular solution and Cs gluconate (135 mM) in the intrapipette solution were replaced with NBQX (5  $\mu$ M) and KCl (135 mM), respectively. Various concentrations of 3,5-DBr-L-Phe, NMDA, AMPA, glycine were added to the extracellular solution according to the protocols described. 3,5-DBr-L-Phe was obtained



from Showa Denko K.K., Kawasaki, Japan. All other compounds were purchased from Sigma Chemical Co., St Louis, MO and Tocris Cookson Inc., Ellisville, MO.

The digitized data were analyzed off-line using the Mini-Analysis Program (Synaptosoft, Leonia, NJ) or pCLAMP9 (Axon Instruments, Union City, CA). Miniature EPSCs were identified and confirmed by analyzing the rise time, decay time, and waveform of each individual spontaneous event.

**General data analysis.** Values are reported as mean  $\pm$  SEM. Prior to parametric testing, the assumption of normality was validated using the Kolmogorov-Smirnov test with Lilliefors's correction (SSPS v10, SPSS, Inc., Chicago, IL). Multiple comparisons among groups were analyzed using ANOVA (two or one way repeated measures with 2 or 1 way replication where appropriate) followed by Student-Newman-Keuls testing. Single comparisons were analyzed using a 2-tailed Student's *t* test. A  $P < 0.05$  was considered significant.

## Results

### *Effect of 3,5-DBr-L-Phe on NMDA receptor function*

The NMDA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in rat cultured neurons were recorded in  $Mg^{2+}$ -free extracellular solution in the presence of AMPA/kainate, glycine and GABA receptor blockers and were analyzed as fluctuating background inward current (noise). 3,5-DBr-L-Phe, added to the extracellular solution, increased this current in a concentration-dependent manner (Figure 1A). The augmenting effect of 3,5-DBr-L-Phe was detectable at concentrations as low as 10  $\mu M$  and reached its maximum at concentrations of 1000  $\mu M$ . The effect of 3,5-DBr-L-Phe at low concentrations (less than 30  $\mu M$ ) appeared as potentiation of the mEPSCs, whereas at higher concentrations 3, 5-DBr-L-Phe activated the steady-state inward current (Figure 1A). The concentration of 3,5-DBr-L-Phe required to produce half-maximal effect ( $EC_{50}$ ) was  $331.6 \pm 78.6 \mu M$ . The concentration-response relationships for 3,5-DBr-L-Phe to activate total steady-state and fluctuating background inward currents are shown in Figure 1B and 1C, respectively. Compared to NMDA-activated current, 3,5-DBr-L-Phe-activated current had a similar onset and reverted to base line on washout of 3,5-DBr-L-Phe (see Figure 1A). The NMDA receptor antagonist, AP-5, and the NMDA receptor channel blocker, MK-801 (not shown), completely blocked current activated by 3,5-DBr-L-Phe (Figure 1D).

In order to elucidate whether 3,5-DBr-L-Phe interacts with the glutamate- or glycine-binding sites on NMDA receptors we measured its effects in the presence of different concentrations of glycine, glutamate and NMDA added to the extracellular solution. A 100-fold increase in concentration of glycine from 0.1  $\mu M$  to 10  $\mu M$  did not

prevent the augmenting effect of 3,5-DBr-L-Phe on NMDA receptor mediated current (Figure 2A). 3,5-DBr-L-Phe (100  $\mu$ M) activated NMDA receptor-mediated current of  $67.4 \pm 16.4$  and  $144.4 \pm 32.7$  pA in the presence of 0.1 and 10  $\mu$ M of glycine, respectively. In order to obtain further evidence whether or not 3,5-DBr-L-Phe interacts with the glycine-binding site of NMDA receptors, the dose-response curves for glycine to modulate NMDA current were generated in the absence and presence of 3,5-DBr-L-Phe. These dose-response curves overlapped (Fig 2B) indicating that augmentation of NMDA receptor current by 3,5-DBr-L-Phe cannot be explained by its interaction with the glycine-binding site of NMDA receptors. The effect of 3,5-DBr-L-Phe on NMDA receptor function, however, did depend on the concentration of glutamate or NMDA. At low concentrations of glutamate and NMDA, total NMDA receptor-mediated current activated by both glutamate or NMDA and 3,5-DBr-L-Phe was bigger than the current recorded in response to application of glutamate or NMDA only. In contrast, combined current, activated by both 3,5-DBr-L-Phe and higher concentration of glutamate or NMDA, was either smaller or similar to the current recorded in the presence of glutamate or NMDA only (Figure 2C,D). Comparison of the concentration-response relationships for 3,5-DBr-L-Phe and NMDA to activate NMDA receptor-mediated currents showed that efficacy for 3,5-DBr-L-Phe was  $30.5 \pm 4.7$  % compared with NMDA (Figure 2E,F). Similar efficacy for 3,5-DBr-L-Phe was observed when instead of NMDA glutamate was used as agonist (data not shown). These results demonstrate that 3,5-DBr-L-Phe acts as a partial agonist of the glutamate-binding site of NMDA receptors.

***Effect of 3,5-DBr-L-Phe on glutamate release and AMPA/kainate receptor function***

To isolate the non-NMDA receptor-mediated EPSCs, experiments were performed in the presence of NMDA receptor channel blocker, MK-801 (5-10  $\mu$ M), or in the presence of the NMDA receptor antagonist, AP-5 (20  $\mu$ M). Strychnine (1  $\mu$ M) and picrotoxin (100  $\mu$ M) were added to the extracellular solution to block glycine and GABA receptors, respectively. Further addition of the non-NMDA receptor antagonist, NBQX (10  $\mu$ M), completely abolished all postsynaptic currents, indicating that the recorded mEPSCs were mediated through activation of a non-NMDA receptor (AMPA/kainate) subtype of glutamate receptors. A typical record of membrane ionic current under such conditions is illustrated in Fig. 3A. In contrast to NMDA receptor-mediated currents, 3,5-DBr-L-Phe depressed both the frequency and amplitude of AMPA/kainate receptor-mediated mEPSCs. The frequency and amplitude of AMPA/kainate receptor-mediated mEPSCs were depressed from  $2.4 \pm 0.7$  Hz and  $20.0 \pm 6.6$  pA in control to  $0.6 \pm 0.2$  Hz and  $12.8 \pm 3.9$  pA in the presence of 3,5-DBr-L-Phe (100  $\mu$ M), respectively (Fig. 2B,C). The effect of 3,5-DBr-L-Phe had a fast onset and was reversible on washout. A clear reduction in the frequency and amplitude of detected mEPSCs was seen within seconds after application of 3,5-DBr-L-Phe. The depressant effect of 3,5-DBr-L-Phe on AMPA/kainate-mediated mEPSCs was concentration-dependent. Analysis of the concentration-response curve for the effect of 3,5-DBr-L-Phe to inhibit mEPSC frequency using a non-linear logistic regression technique showed that the concentration of 3,5-DBr-L-Phe required to depress non-NMDA receptor-mediated mEPSCs by half ( $IC_{50}$ ) was  $29.4 \pm 4.3$   $\mu$ M. The finding that 3,5-DBr-L-Phe attenuates both the frequency and the amplitude of AMPA/kainate receptor-mediated mEPSCs indicates that 3,5-DBr-L-Phe may produce both pre- and postsynaptic effects.

In order to determine whether 3,5-DBr-L-Phe changes the probability of glutamate release the extent of paired-pulse depression for AMPA/kainate receptor-mediated component of EPSCs and its changes following application of 3,5-DBr-L-Phe was established. Low-density areas in dishes with cultured cells (2-5 neurons in 400  $\mu\text{m}$  diameter view-field) were selected for the experiments. Synaptic responses were evoked by applying voltage pulses (0.2-1 ms, 20-90 V) to an extracellular electrode (a patch electrode filled with the extracellular solution) positioned in the vicinity of the presynaptic neuron soma or neurite (Figure 4A). As a quantitative estimate of paired-pulse depression, the paired pulse ratio, i.e. the ratio of the amplitude of the second EPSC in the pair to that of the first (EPSC2/EPSC1) was estimated. Application of 100  $\mu\text{M}$  3,5-DBr-L-Phe decreased the amplitude of single evoked EPSCs (1st response in a paired-pulse experiment) in cerebrocortical neurons by  $47.5 \pm 2.9\%$  (Figure 4B). While the EPSC kinetics did not appear to change, 3,5-DBr-L-Phe decreased the paired pulse ratio by  $41.4 \pm 15.0\%$ . These data suggest that a reduced probability of synaptic vesicle release may contribute to the inhibitory effect of 3,5-DBr-L-Phe on excitatory transmission at these synapses.

In order to assess whether 3,5-DBr-L-Phe may also depress activity of postsynaptic AMPA/kainate receptors, we investigated the effect of 3,5-DBr-L-Phe on current activated by AMPA. AMPA (3  $\mu\text{M}$ ) activated a rapidly rising inward current (i.e.,  $I_{\text{AMPA}}$ ) that was depressed by 3,5-DBr-L-Phe (Figure 4C). The effect of 3,5-DBr-L-Phe on  $I_{\text{AMPA}}$  was concentration-dependent and reversible upon washout of the drug. The concentration-response relationships for 3,5-DBr-L-Phe to depress  $I_{\text{AMPA}}$  obtained at two different concentrations of AMPA are shown in Figure 4D. The increase in the

concentration of AMPA from 3  $\mu$  to 10  $\mu$ M markedly shifted the concentration-response relationship for 3,5-DBr-L-Phe to depress  $I_{\text{AMPA}}$  rightward (Figure 4D). The  $IC_{50}$  values of 3,5-DBr-L-Phe in the presence of 3  $\mu$ M and 10  $\mu$ M AMPA were  $294.4 \pm 8.9$   $\mu$ M and  $1140.1 \pm 54.6$   $\mu$ M, respectively. These results indicate that 3,5-DBr-L-Phe, most likely, acts as a competitive inhibitor of the glutamate-binding site on the AMPA receptors.

***Effect of 3,5-DBr-L-Phe on GABA-ergic transmission and elicited action potentials***

GABA receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the presence of MK-801 (5  $\mu$ M), NBQX (10  $\mu$ M), and strychnine (1  $\mu$ M) to block NMDA, AMPA/kainate and glycine receptors, respectively. A typical record of membrane ionic current under such conditions is illustrated in Fig. 5A. 3,5-DBr-L-Phe (100  $\mu$ M) significantly changed neither the frequency nor the amplitude of GABA receptor-mediated mIPSCs. The frequency and amplitude of GABA receptor-mediated mIPSCs were depressed from  $1.0 \pm 0.2$  Hz and  $25.1 \pm 2.3$  pA in control to  $0.8 \pm 0.2$  Hz and  $24.2 \pm 1.6$  pA in the presence of 3,5-DBr-L-Phe (100  $\mu$ M), respectively (Fig. 5A,B). Likewise, 3,5-DBr-L-Phe (100  $\mu$ M) did not cause any changes in elicited action potentials (Figure 5C), indicating lack of the effect of 3,5-DBr-L-Phe on voltage-operated ionic channels.

## Discussion

The results of the current study clearly demonstrate that 3,5-DBr-L-Phe produces a unique combination of actions on excitatory glutamatergic synaptic transmission in rat cerebrocortical cultured neurons. Specifically, 3,5-DBr-L-Phe activates NMDA receptor-mediated current, depresses activity of non-NMDA (AMPA/kainate) subtype of glutamate receptors, and diminishes pre-synaptic glutamate release. Such differential modulation of glutamatergic synaptic transmission may be beneficial in brain disorders characterized by alterations in the glutamate system.

## Mechanism of action

Current activated by 3,5-DBr-L-Phe was blocked by the specific NMDA receptor antagonists, AP-5 and MK-801. These and other experimental evidence indicate that 3,5-DBr-L-Phe acts as agonist of NMDA receptors. NMDA receptors require two co-agonists, glutamate and glycine for their activation. The binding sites for glycine and glutamate are located on subunits 1 and 2, respectively (Laube et al., 1997). Earlier, we have demonstrated that L-Phe depressed NMDA receptor function by competing for the glycine-binding site of NMDA receptors and depressed non-NMDA receptor activity by competing for the glutamate-binding site of these receptors (Glushakov et al., 2002; Glushakov et al., 2003). Similar to L-Phe, 3,5-DBr-L-Phe is a competitive antagonist of AMPA/kainate receptors also. Surprisingly, halogenation of the L-Phe molecule not only reversed its effect (activation vs. depression), but also changed the site of its interaction with the NMDA receptors (glutamate-binding site vs. glycine-binding site). Thus, the activating effect of 3,5-DBr-L-Phe on NMDA receptor function did not depend on

concentration of glycine. Increases in the concentration of glycine augmented the 3,5-DBr-L-Phe-activated current as would be expected from an increased concentration of co-agonist. On the other hand, the activating effect of 3,5-DBr-L-Phe on NMDA receptor function depended on the concentration of glutamate or NMDA added to the extracellular solution. The total current activated by 3,5-DBr-L-Phe and low concentrations of the agonists was bigger than the current activated by glutamate or NMDA only. The opposite changes in NMDA receptor-mediated current took place at higher concentrations of glutamate or NMDA. Importantly, the maximal current activated by 3,5-DBr-L-Phe was approximately 30 % of the maximal current activated by NMDA or glutamate. Together, these findings support the notion that 3,5-DBr-L-Phe acts as a partial NMDA receptor agonist at the glutamate-binding site.

Our results indicate that 3,5-DBr-L-Phe has a postsynaptic effect on non-NMDAR (AMPA/kainate)-mediated events as well. 3,5-DBr-L-Phe depressed the amplitude of non-NMDAR-mediated mEPSCs and the current activated by exogenous AMPA. Increases in the concentration of AMPA shifted the concentration-response curve for the effect of 3,5-DBr-L-Phe on  $I_{\text{AMPA}}$  rightward. This finding suggests that the glutamate-binding site on the AMPA receptor is a possible target for the inhibitory action of 3,5-DBr-L-Phe. The postsynaptic effects of 3,5-DBr-L-Phe on non-NMDA receptors may be further augmented by the presynaptic action of 3,5-DBr-L-Phe. While the postsynaptic effects of 3,5-DBr-L-Phe can reduce the frequency of AMPA/kainate receptor-mediated mEPSCs by decreasing the mEPSC amplitude of some events below the threshold for detection, the predominant depression of mEPSC frequency over mEPSC amplitude strongly suggests a presynaptic action based on a decreased probability of glutamate



release. The 3,5-DBr-L-Phe-induced decrease in the frequency of AMPA/kainate mEPSCs in combination with the decreased extent of the paired-pulse depression for elicited AMPA/kainate EPSCs in the presence of 3,5-DBr-L-Phe independently support the possibility that 3,5-DBr-L-Phe also depresses of neurotransmitter release.

Elucidation of the mechanism(s) of 3,5-DBr-L-Phe-induced depression of neurotransmitter release requires further investigation. Because mEPSCs, unlike elicited EPSCs, do not depend on  $\text{Ca}^{2+}$  entry, a reduction of  $\text{Ca}^{2+}$  influx into the presynaptic terminal cannot explain the action of 3,5-DBr-L-Phe. In addition, absence of any effect of 3,5-DBr-L-Phe on elicited action potentials indicates that this agent is unlikely to affect initiation of  $\text{Ca}^{2+}$  influx into the presynaptic terminal by action potentials. The failure of 3,5-DBr-L-Phe to affect either elicited action potentials or GABA receptor-mediated mIPSCs further shows that the effects of 3,5-DBr-L-Phe are quite specific to glutamatergic transmission. In contrast, the interaction of 3,5-DBr-L-Phe with the glutamate-binding sites on both NMDA and AMPA/kainate receptors shows that among the glutamate binding sites within the glutamatergic system 3,5-DBr-L-Phe is less specific. If the limited selectivity for glutamate binding sites extends to other glutamate receptors, 3,5-DBr-L-Phe may potentially modulate the activity of metabotropic glutamate receptors. A number of studies demonstrate in fact that activation of the metabotropic glutamate receptors, mGlu2/3, depresses glutamate release (Battaglia et al., 1997; Crtmell and Schoepp, 2000). However, in our experiments (data not shown) a selective group II metabotropic glutamate receptor antagonist, LY 3414953, did not influence the effect of 5-DBr-L-Phe on the frequency of the AMPA/kainate mEPSCs indicating that other mechanisms mediate the depressant effect of 3,5-DBr-L-Phe on

glutamate release. Elucidation of the mechanisms whereby 3,5-DBr-L-Phe depresses neurotransmitter release requires detailed investigation and will be a subject for our future experiments.

### ***Potential application of 3,5-DBr-L-Phe***

Given the fact that decreased function of NMDA receptors and accompanying increased neurotransmitter release with overactivation of AMPA/kainate receptors are important contributors to the pathophysiology of schizophrenia (Hirsh and Weinberger, 2003; Konradia and Heckers, 2003; Jentsch and Roth, 1999; Moghaddam et al., 1997; Meador-Woodruff and Healy, 2000; Greene, 2001), 3,5-DBr-L-Phe may represent the prototype of a new class of antipsychotic drugs. An important requirement for therapeutic agents with glutamatergic actions is their ability to modulate synaptic transmission, without producing excitotoxicity. 3,5-DBr-L-Phe as a partial agonist of the glutamate-binding site on NMDA receptors should allow normalization of the depressed NMDA receptor activity, while preventing overactivation of NMDA receptors by excess glutamate release. In fact, in our preliminary studies we found that 3,5-DBr-L-Phe decreased cell death in neuronal cultures exposed to oxygen glucose deprivation.

Along with activation of NMDA receptors 3,5-DBr-L-Phe depresses glutamate release and postsynaptic AMPA/kainate receptors. Agents that not only augment NMDA receptor function, but also regulate glutamate release and activity of AMPA/kainate receptors should potentially be more efficacious in producing antipsychotic effects. Because of the structural relationship between L-Phe and dopamine, there is the possibility of modulation of dopamine receptors by 3,5-DBr-L-Phe as well. L-Phe is the

direct precursor of tyrosine, which in turn is metabolized to dopamine. Finally, 3,5-DBr-L-Phe does not alter activity of voltage-operated ionic channels that contribute to generation of action potentials, therefore, it is unlikely that agents with such profile of action would produce significant unwanted effects. Specific investigation of the role of 3,5-DBr-L-Phe in modulation of other neurotransmitter systems and investigation of their antipsychotic properties in animal models of schizophrenia will provide further insight into the possibility of using it as prototype of a new class of antipsychotic agents.

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## Footnote

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## Figure legends

**Figure 1.** 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) activates NMDA receptor-mediated currents in rat cerebrocortical neurons in concentration-dependent manner. **A:** Example of NMDA receptor-mediated mEPSCs recorded from a single neuron in the presence of different concentrations of 3,5-DBr-L-Phe. Horizontal bars denote 3,5-DBr-L-Phe applications. NMDA receptor-mediated mEPSCs were recorded in TTX-containing (0.3  $\mu$ M),  $Mg^{2+}$ -free extracellular solution at holding membrane potential of -60 mV. NBQX (10  $\mu$ M), strychnine (1  $\mu$ M) and picrotoxin (100  $\mu$ M) were added to the extracellular solution to block AMPA/kainate, glycine and GABA receptors, respectively. **B** and **C:** Concentration-response relationships for 3,5-DBr-L-Phe to activate total NMDA receptor-mediated current ( $I_{3,5-DBr-L-Phe}$ ) and fluctuating background currents (mEPSCs), respectively. Amplitude of total NMDA receptor current was calculated by subtracting mean value of the current in the absence of 3,5-DBr-L-Phe from the current recorded in the presence of 3,5-DBr-L-Phe and plotted against the concentration of 3,5-DBr-L-Phe. NMDA receptor-mediated mEPSCs were analyzed as background noise current by calculating standard deviation (SD) from mean baseline amplitude. Data expressed as mean  $\pm$  S.E.M. for 5-14 cells. **D:** 3,5-DBr-L-Phe-activated current is blocked by NMDA receptor specific antagonists. Representative example of depression of 3,5-DBr-L-Phe-activated current by NMDA receptor antagonist AP-5. Horizontal bars denote 3,5-DBr-L-Phe (100  $\mu$ M) and AP-5 (20  $\mu$ M) applications. Similar results were obtained from total of 6 neurons. NMDA receptor-mediated currents were recorded under the same conditions as described in Fig. 1A.



**Figure 2.** Properties of 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe)-activated current.

**A and B:** Activating effect of 3,5-DBr-L-Phe on NMDA receptor-mediated current does not depend on concentration of glycine. Example of the effect of 3,5-DBr-L-Phe (100  $\mu$ M) on NMDA mEPSCs recorded from the single neuron in the presence of different concentrations of glycine (**A**). Horizontal bars denote 3,5-DBr-L-Phe (100  $\mu$ M) and glycine applications. Panel B depicts the concentration-response curves for glycine (Gly) to modulate NMDA (10  $\mu$ M)-activated current ( $I_{\text{NMDA}}$ ) in the absence and presence of 3,5-DBr-L-Phe (100  $\mu$ M). Peak  $I_{\text{NMDA}}$  was normalized to  $I_{\text{NMDA}}$  recorded at 10  $\mu$ M Gly and plotted against the concentration of glycine [Gly]. Endogenous Gly in primary neuronal cultures explains the non-zero  $I_{\text{NMDA}}$  in the absence of added Gly. **C and D:** Activating effect of 3,5-DBr-L-Phe on NMDA receptor-mediated current depends on concentration of glutamate or NMDA in extracellular solution. **C:** Examples of glutamate (L-Glu, 0.3 and 10  $\mu$ M)-activated currents recorded from the same neuron in the absence and presence of 3,5-DBr-L-Phe (300  $\mu$ M). Horizontal bars denote L-Glu (0.3  $\mu$ M, left pair, and 10  $\mu$ M, right pair) and 3,5-DBr-L-Phe (300  $\mu$ M) applications. 3,5-DBr-L-Phe exposure was initiated 45 s before the start of glutamate application. **D:** Effect of 3,5-DBr-L-Phe on current activated by different concentrations of glutamate or NMDA (agonists). Amplitude of total current ( $I_{\text{agonist}}$ ) was normalized to control values ( $I_{\text{agonist}}$  in the absence of 3,5-DBr-L-Phe) and plotted against the concentration of glutamate or NMDA. The total current was measured from baseline (zero current before application of 3,5-DBr-L-Phe, dashed lines) to peak value of the current activated in the presence of both 3,5-DBr-L-Phe and glutamate or NMDA. In case of NMDA-activated current concentration of 3,5-DBr-L-Phe was 100  $\mu$ M. Data expressed as mean $\pm$ S.E.M. for 3-5

cells. **E** and **F**: Concentration-response relationships for NMDA and 3,5-DBr-L-Phe to activate NMDA receptor mediated current. **E**: Examples of NMDA (1 mM) and 3,5-DBr-L-Phe (1 mM) activated-currents recorded from the same neuron. **F**: Concentration-response curves to NMDA and 3,5-DBr-L-Phe were carried out in rat cerebrocortical cultured neurons in the presence of 10  $\mu$ M glycine. Peak  $I_{\text{NMDA}}$  and  $I_{3,5\text{-DBr-L-Phe}}$  were normalized to  $I_{\text{NMDA}}$  recorded at 1 mM NMDA and plotted against the concentration of NMDA and 3,5-DBr-L-Phe, respectively. **C-F**: Currents were recorded at a  $V_H$  of -30 mV and the extracellular solutions contained  $\text{Mg}^{2+}$  (1 mM).

**Figure 3.** 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) depresses AMPA-kainate receptor-mediated mEPSCs in rat cerebrocortical cultured neurons in concentration-dependent manner. **A**: Representative traces of AMPA-kainate mEPSCs recorded from a cortical neuron under the following conditions: control; in the presence of 3,5-DBr-L-Phe (100  $\mu$ M); after washout of 3,5-DBr-L-Phe. AMPA/kainate receptor-mediated currents were recorded in TTX-containing (0.3  $\mu$ M) extracellular solution at holding membrane potential of -60 mV. MK-801 (10  $\mu$ M), strychnine (1  $\mu$ M) and picrotoxin (100  $\mu$ M) were added to the extracellular solution to block NMDA, glycine and GABA receptors, respectively. **B** and **C**: Concentration-response relationships for 3,5-DBr-L-Phe to attenuate AMPA/kainate receptor-mediated mEPSC frequency and amplitude, respectively. Data were normalized to control values and plotted against the concentration of 3,5-DBr-L-Phe. Data are expressed as mean $\pm$ SEM of 6-7 cells. Intervention vs. Control: \*,  $P < 0.01$ . Curve fitting and estimation of value of  $\text{IC}_{50}$  for the frequency of AMPA/kainate mEPSCs was made according to the 4-parameter logistic

equation. The  $IC_{50}$  for the effect of 3,5-DBr-L-Phe on the amplitude of AMPA/kainate mEPSCs was not determined because the small number of mEPSCs in the presence of 3,5-DBr-L-Phe concentrations higher than 100  $\mu$ M made it impossible to adequately determine the average amplitude of non-NMDAR-mediated mEPSCs.

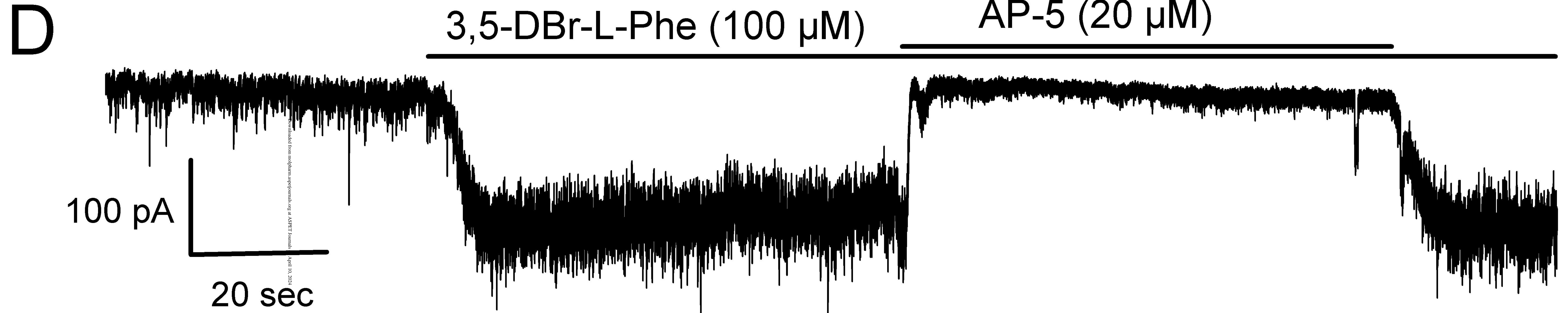
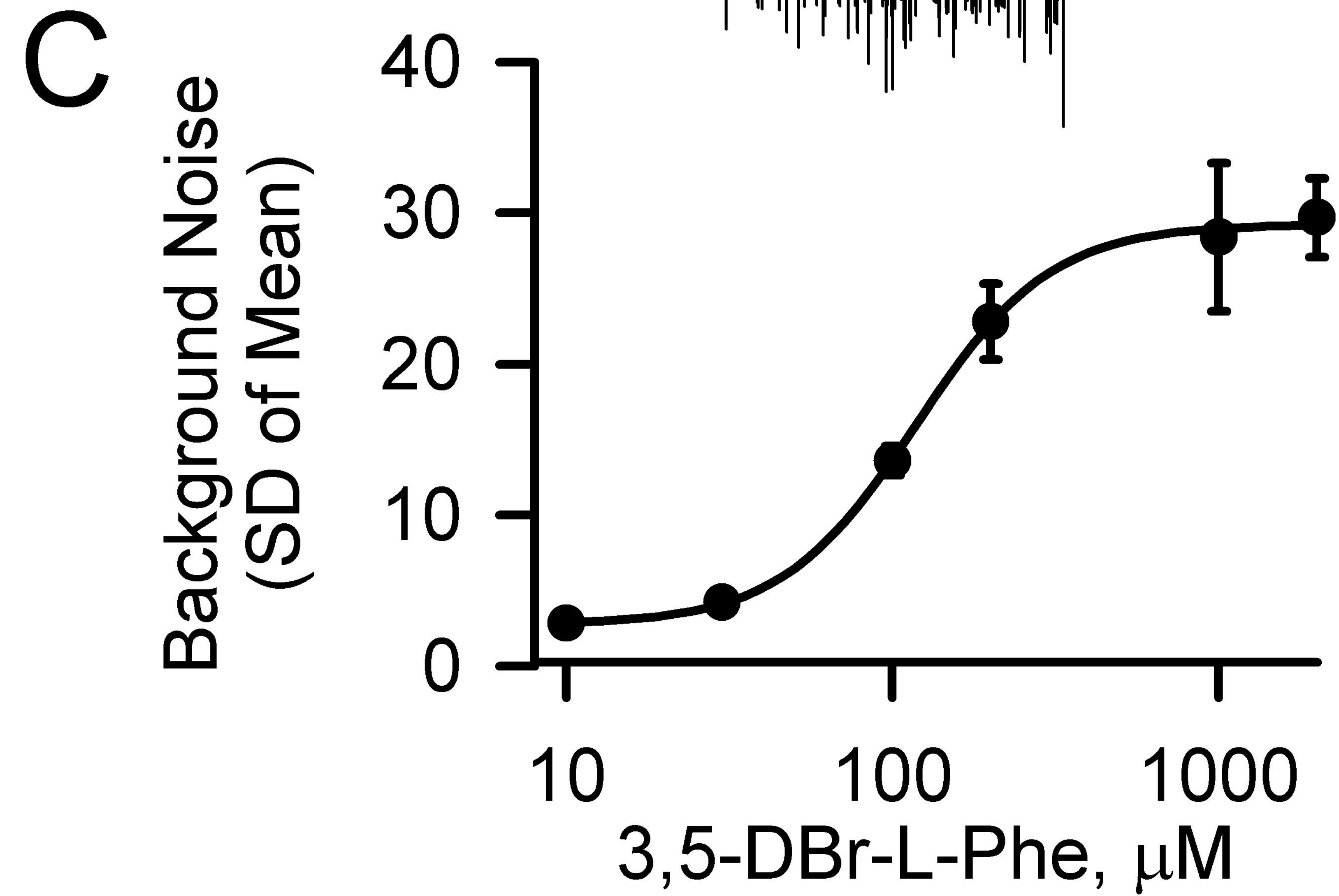
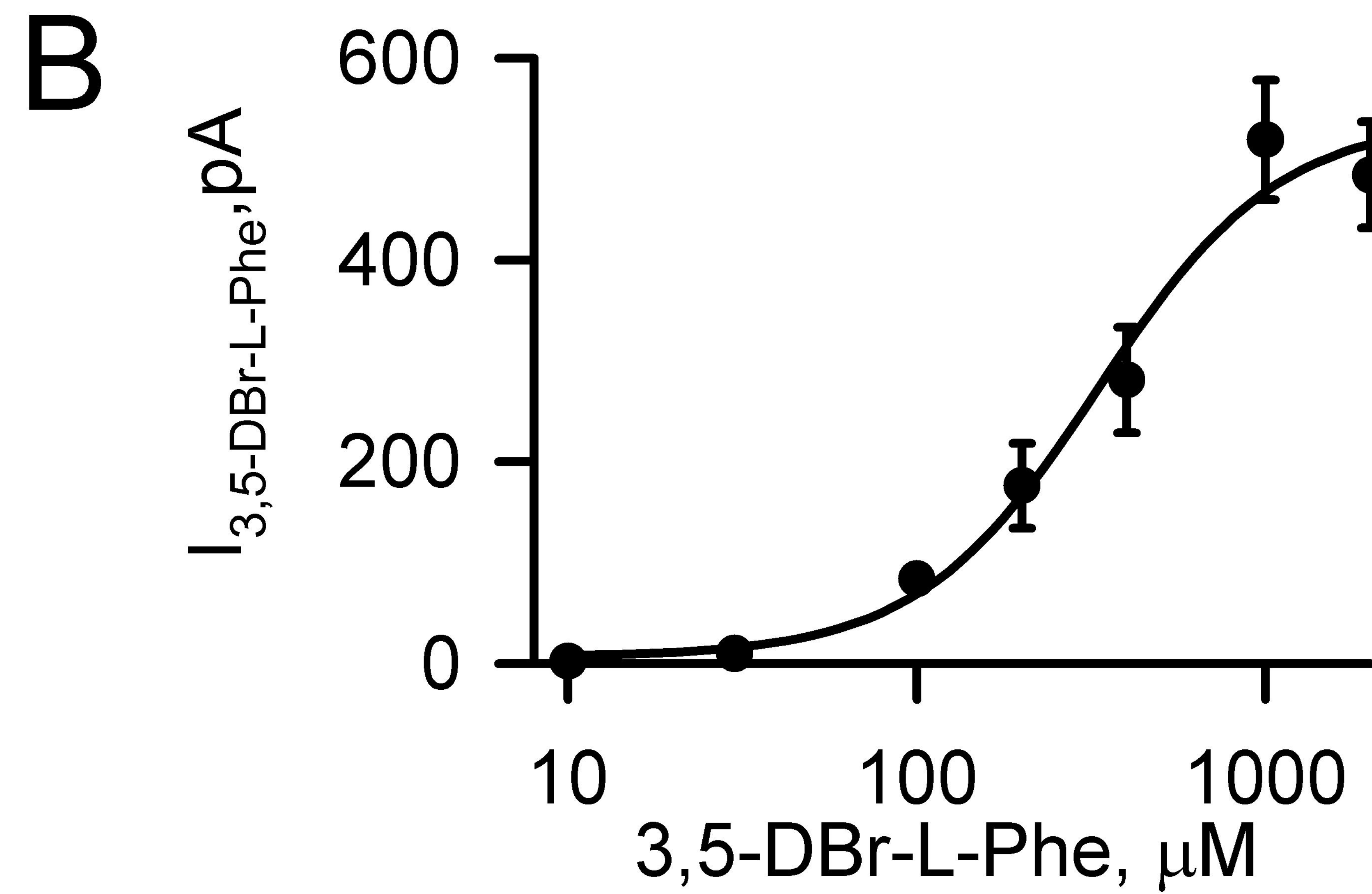
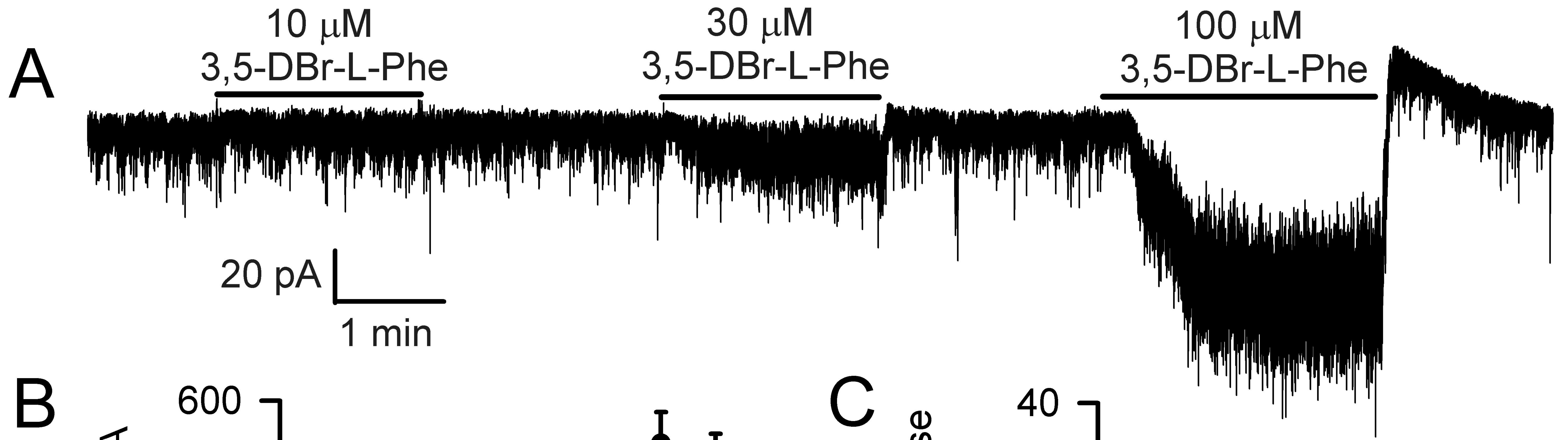
**Figure 4.** 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) causes depression of glutamate release and activity of postsynaptic AMPA-kainate receptors. **A:** Effect of 3,5-DBr-L-Phe on the evoked EPSCs in rat cerebrocortical cultured neuron. Examples of average EPSCs (20 traces average) in control conditions (open circle) and in the presence of 3,5-DBr-L-Phe (filled circle). Synaptic responses were evoked by applying two sub-threshold electric stimuli (0.4-1 ms, 50-90 V, 250 ms apart) to an extracellular electrode (a patch electrode filled with the extracellular solution) positioned in the vicinity of the presynaptic neuron. Sweeps were recorded at 10 s intervals. After 20 sweeps, 100  $\mu$ M 3,5-DBr-L-Phe was added. Neuron was held in whole-cell mode at  $V_h = -60$  mV in  $Mg^{2+}$  (1 mM) containing extracellular solution. Strychnine (1  $\mu$ M) and picrotoxin (100  $\mu$ M) were added to the extracellular solution to block glycine and GABA receptors, respectively. **B:** Values of the 2nd/1st amplitude ratio of the paired EPSC responses. The amplitude of the 1st and 2nd EPSCs were measured against the baseline; each point represents an average of five subsequent sweeps. Data expressed as mean  $\pm$  S.E.M. for 7 cells. \*,  $P < 0.01$  compared to control.

**C and D:** The depressant effect of 3,5-DBr-L-Phe on AMPA-activated current ( $I_{AMPA}$ ) depends on concentration of AMPA. **C:** Examples of AMPA-activated currents recorded from the same rat cortical neuron before application of 3,5-DBr-L-Phe (Control), during

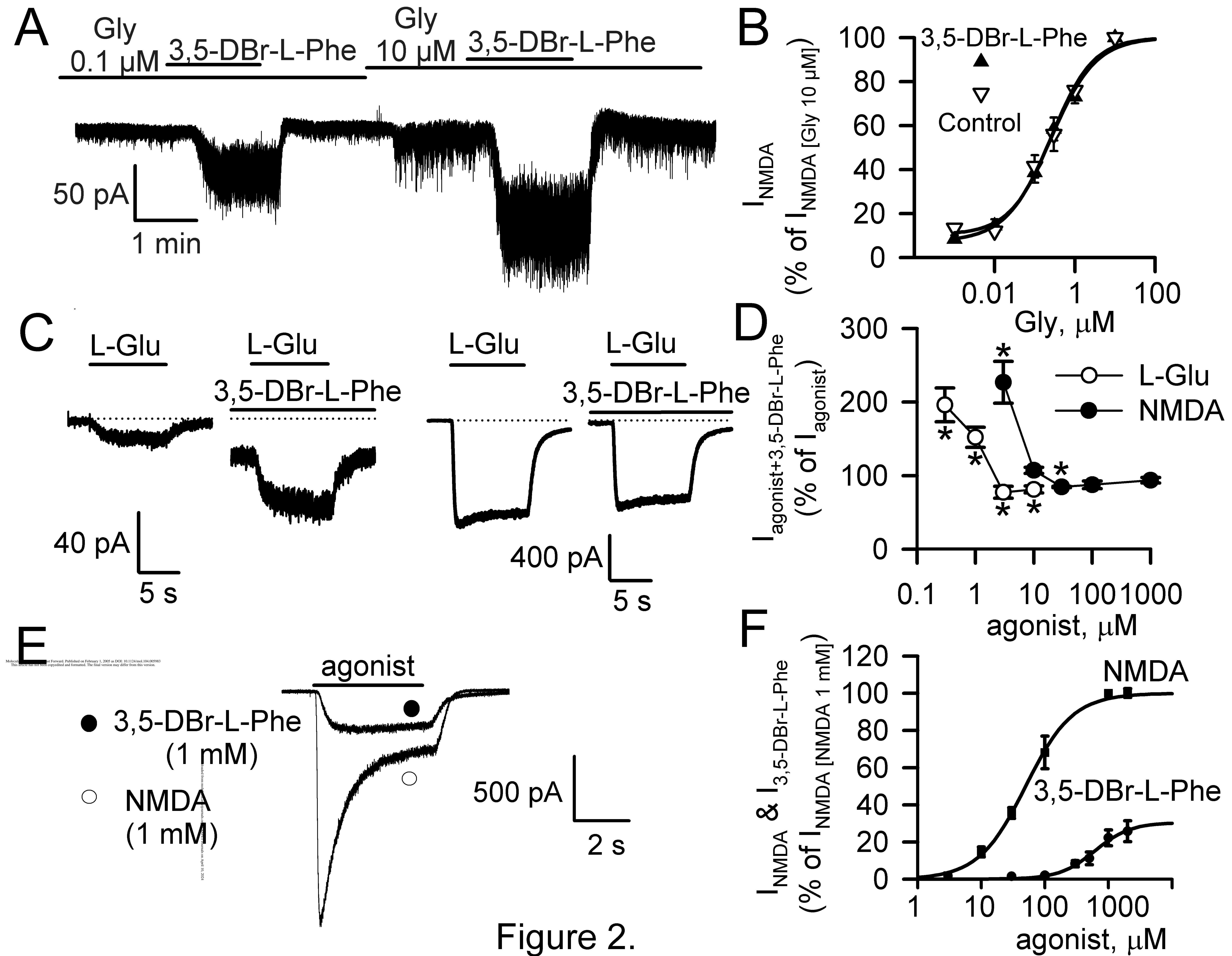
exposure to DBrPhe (300  $\mu$ M) and after washout of 3,5-DBr-L-Phe (Wash). 3,5-DBr-L-Phe exposure was initiated 45 s before the start of AMPA application. Horizontal bar denotes AMPA (3  $\mu$ M) application. **D:** Concentration-response relationships for 3,5-DBr-L-Phe to attenuate AMP-activated currents. Concentrations of AMPA are shown in Figure. Peak  $I_{\text{AMPA}}$  was normalized to control values (in the absence of 3,5-DBr-L-Phe) and plotted against the concentration of 3,5-DBr-L-Phe. Data expressed as mean $\pm$ S.E.M. for 3-5 cells. \*,  $P < 0.05$  compared to control.

**Figure 5.** 3,5-dibromo-L-phenylalanine (3,5-DBr-L-Phe) does not significantly affect gamma-aminobutyric (GABA) receptor-mediated mIPSCs and elicited action potentials in rat cerebrocortical cultured neurons. **A:** Representative GABA receptor-mediated mIPSCs recorded from the same neuron before (control), during (100  $\mu$ M), and after (wash) application of 3,5-DBr-L-Phe. GABA receptor-mediated mIPSCs were recorded in TTX-containing (0.3  $\mu$ M) extracellular solution at holding membrane potential of -60 mV. NBQX (10  $\mu$ M), MK-801 (10  $\mu$ M) and strychnine (1  $\mu$ M) were added to the extracellular solution to block AMPA/kainate, NMDA and glycine receptors, respectively. **B:** Histograms summarizing the effects of 3,5-DBr-L-Phe (100  $\mu$ M) on the amplitude and frequency of GABA receptor-mediated mIPSCs. Summary data are expressed as mean $\pm$ SEM of 5 cells.

**C:** Examples of action potentials elicited by depolarizing the membrane with inward current pulses of 2 ms duration and 2 nA amplitude in control (before application of 3,5-DBr-L-Phe), in the presence of 3,5-DBr-L-Phe (100  $\mu$ M) and after wash-out of the drug. Similar responses were recorded from 5 of 5 neurons.

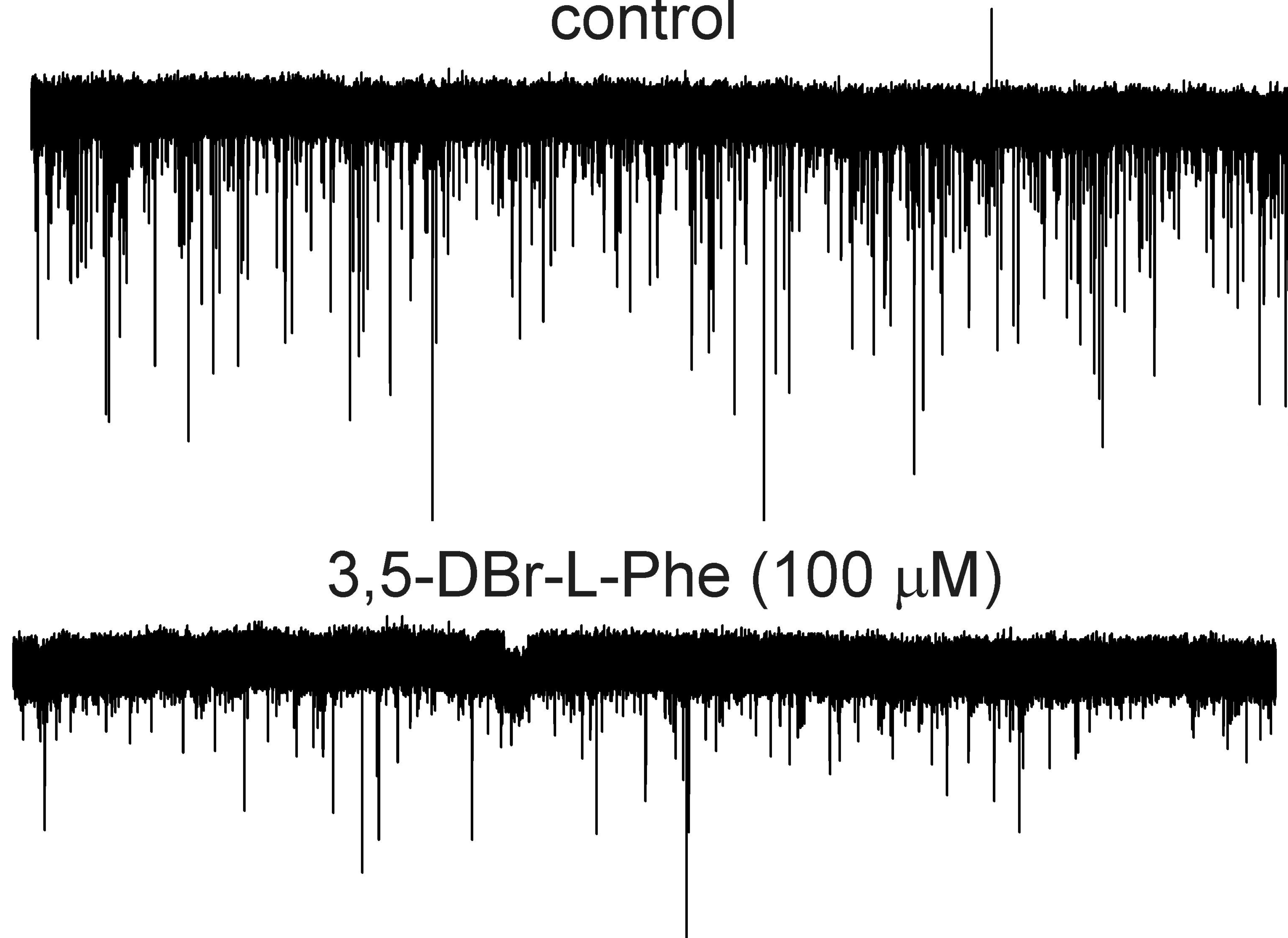




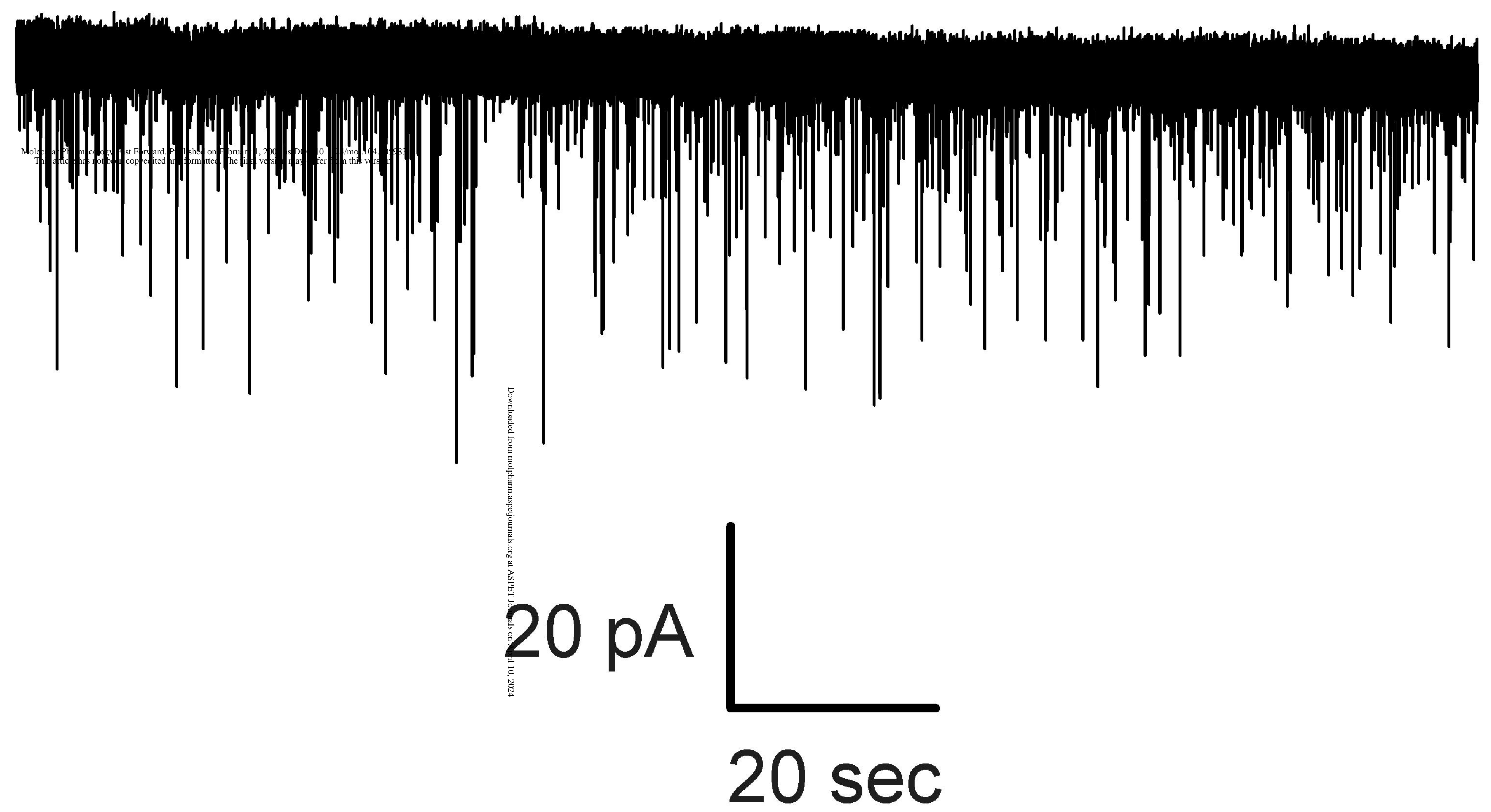


A

control



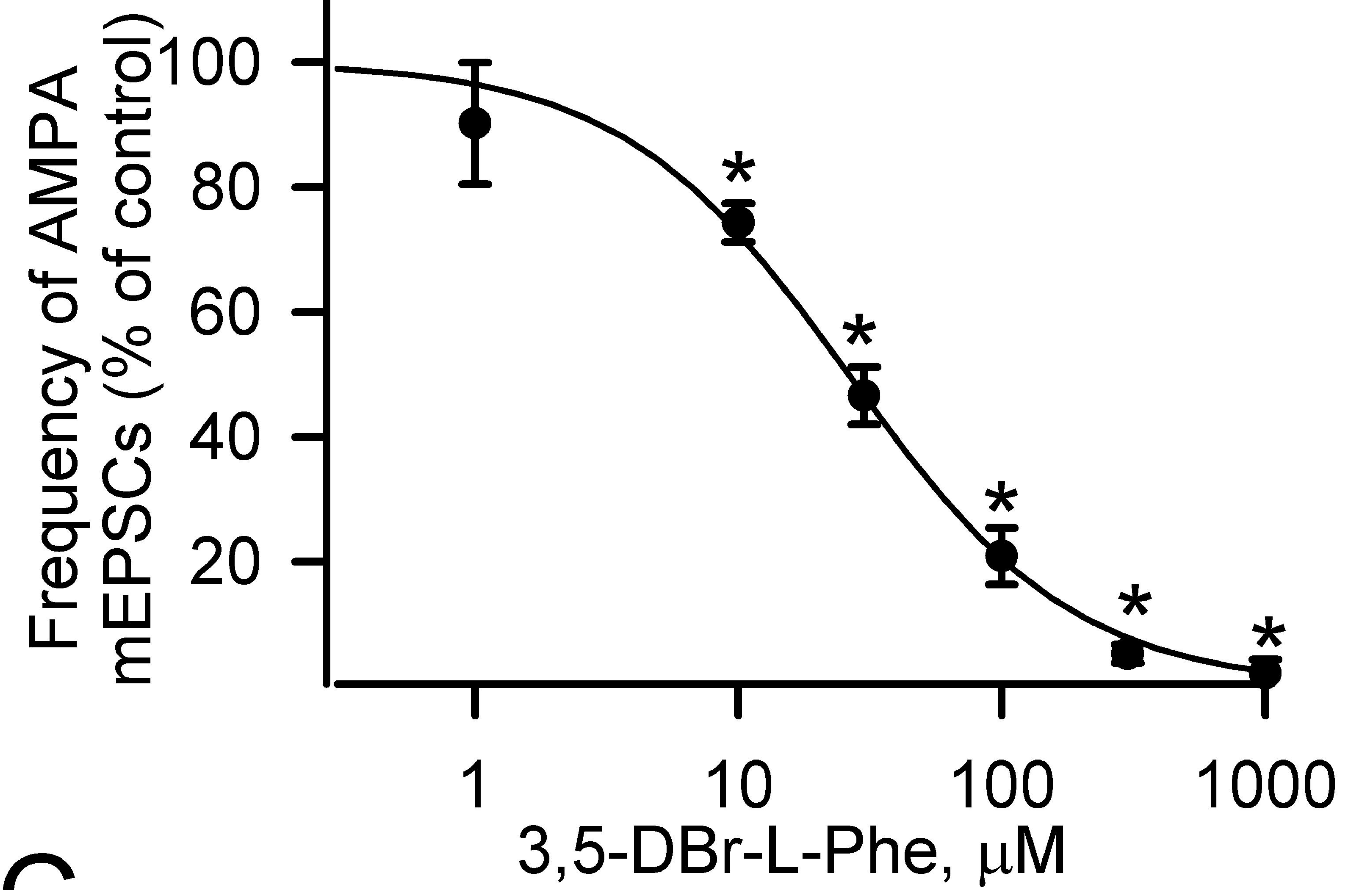
wash out



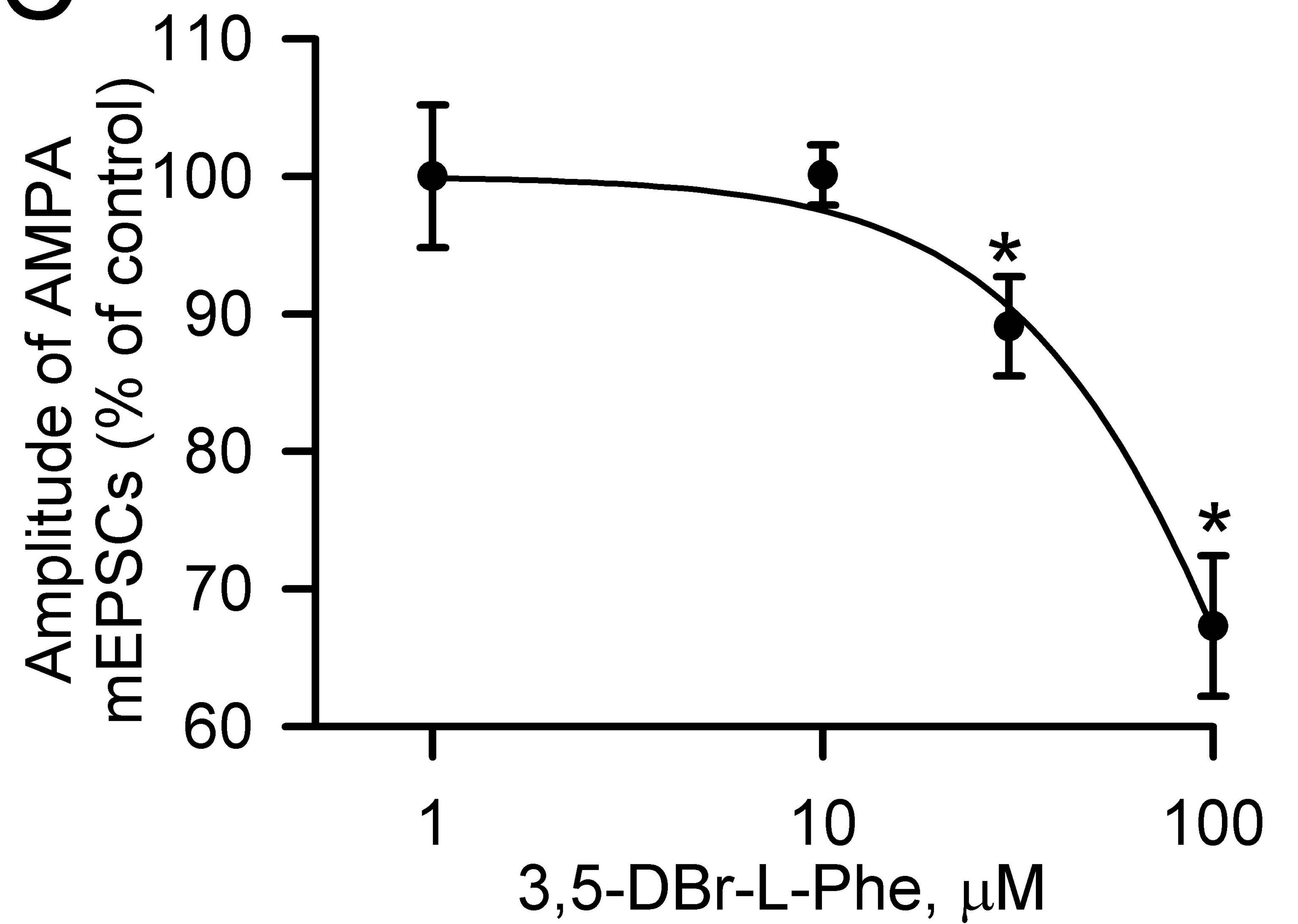
20 pA

20 sec

B

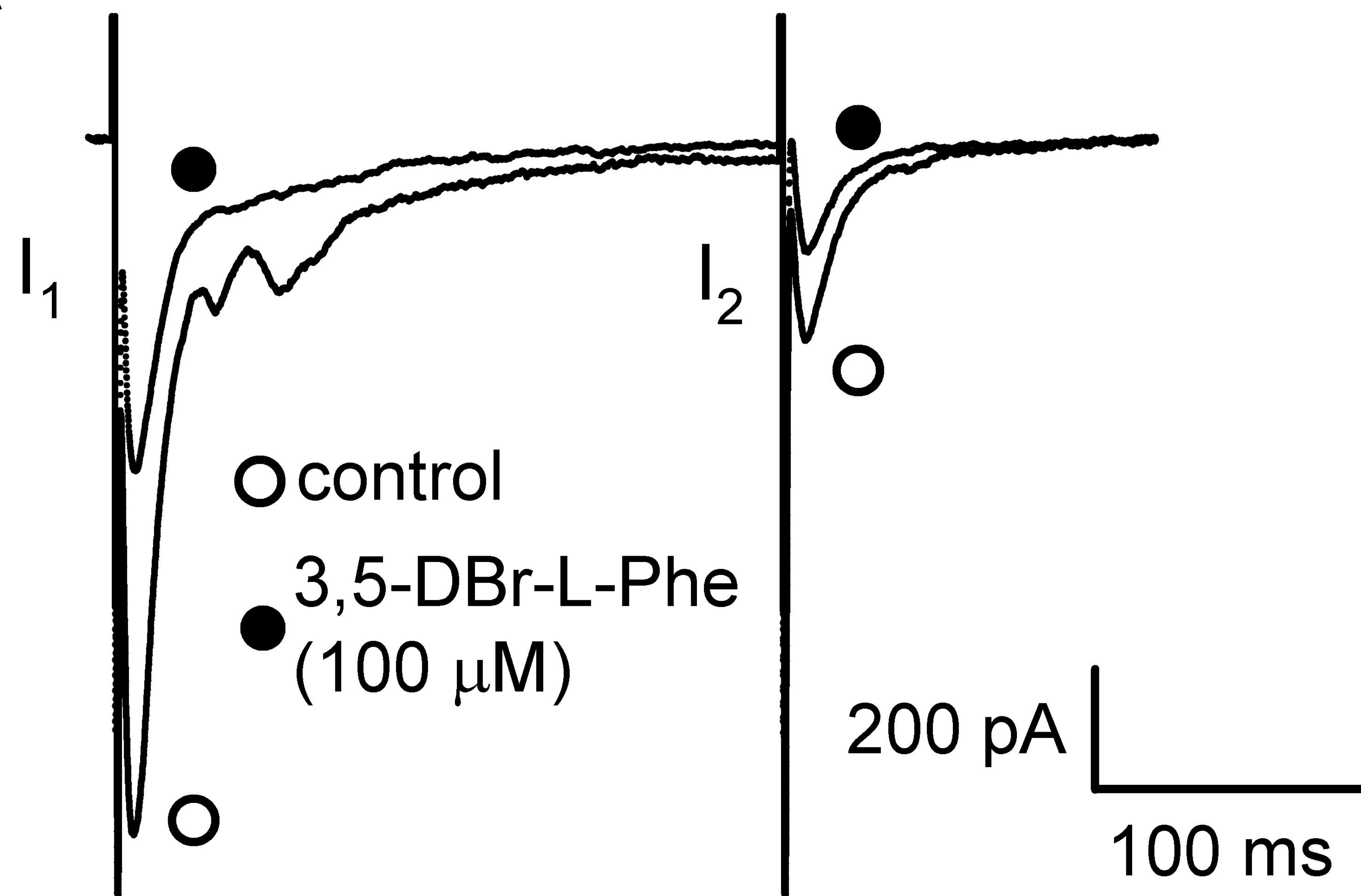


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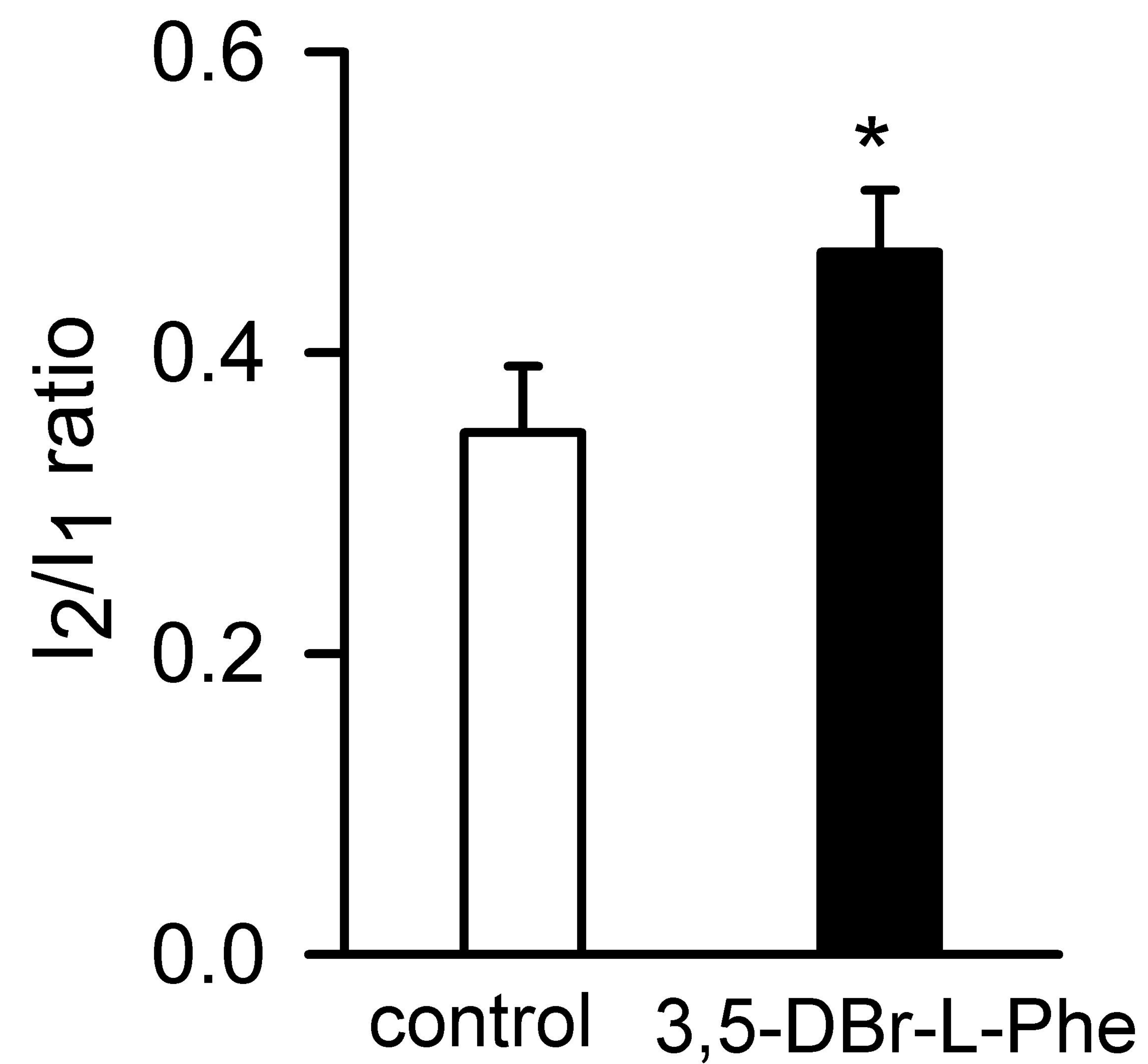




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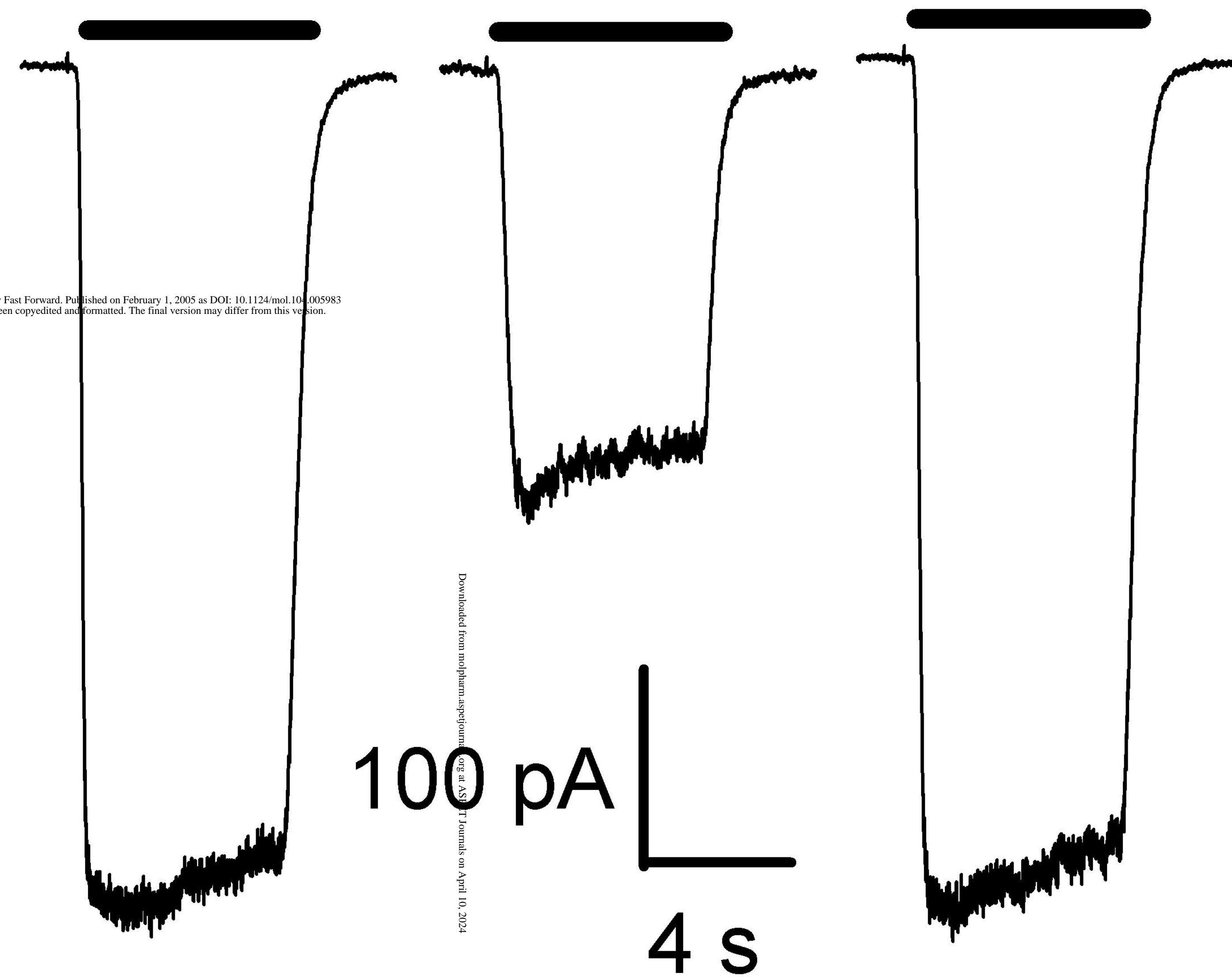


B



C

Control 3,5-DBr-L-Phe Wash



D

