The IGF-derived tripeptide Gly-Pro-Glu is a weak NMDA receptor agonist

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In the central nervous system, fast excitatory neurotransmission is mediated by ionotropic glutamate receptors, including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) kainate, and N-methyl-d-aspartate (NMDA) receptors (Watkins and Evans 1981). Glutamate, however, acts as a universal agonist at all glutamate receptors, due in part to its high degree of flexibility. Other amino acids and small peptides can activate N-methyl-D-aspartate (NMDA) receptors, albeit usually with lower affinity and efficacy. Here, we examined the action of glycine-proline-glutamate (GPE), a naturally occurring tripeptide formed in the brain following cleavage of IGF-I. GPE is thought to have biological activity in the brain, but its mechanism of action remains unclear. With its flanking glutamate and glycine residues, GPE could bind to either the agonist or coagonist sites on NMDA receptors, however, this has not been directly tested. Using whole cell patch-clamp recordings in combination with rapid solution exchange, we examined both steady-state currents induced by GPE as well as the effects of GPE on synaptically evoked currents. High concentrations of GPE evoked inward currents, which were blocked either by NMDA receptor competitive antagonists or the voltage-dependent channel blocker Mg2+. GPE also produced a slight attenuation in the NMDA- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-mediated excitatory postsynaptic currents without altering the paired-pulse ratio. Our results suggest that GPE can activate NMDA receptors but at concentrations well above the expected concentration of GPE in the brain. Therefore, it is unlikely that endogenous GPE interacts with glutamate receptors under normal conditions.

portance of IGF-I signaling given its upregulation following ischemic injury (Yamaguchi et al. 1991). Although GPE was initially considered a nonbioactive byproduct, exogenous application of GPE has been shown to provide neuroprotection following ischemic-reperfusion injuries (Guan 2011). Although the mechanism of action of GPE is not well-understood (Sara et al. 1993; Yamamoto and Murphy 1995), radioligand-binding studies have suggested that GPE binds to NMDA receptors, and in Müller glia GPE mediates mitogenic effects in an NMDA receptor-dependent manner (Ikeda et al. 1995; Sara et al. 1989). Ischemic injuries can induce excitotoxicity that can be ameliorated by NMDA receptor antagonists such as AP-5 and MK-801 (Lai et al. 2014); therefore, we sought to determine whether GPE acts at NMDA receptors, potentially as a novel antagonist. Using whole cell voltage-clamp recordings in primary cultures of mouse hippocampal neurons, we report that GPE functions not as an antagonist but rather as a weak NMDA receptor agonist.

MATERIALS AND METHODS

Cell culture. Mouse (strain C57BL/6J) hippocampal neurons were cultured on isolated glial microislands as described previously (Tovar et al. 2009). Briefly, wild-type neonatal (postnatal days 0–1) male mice were decapitated, brains were removed, and hippocampi were dissected. Microislands were generated by plating at 125,000 cells/35 mm dish. After 7 days, the cultures were treated with 200 μM glutamate for 30 min to kill any surviving neurons, leaving a glial feeder layer. Neurons were then plated on the glial feeder layer at 25,000 cells/35 mm dish. Cultures were maintained in a tissue culture incubator (37°C, 5% CO2) in minimum essential media with 2 mM GlutaMAX (Invitrogen), 5% heat-inactivated fetal calf serum (Lonza), and 1 ml/l MUTO+ Serum Extender (BD Biosciences) supplemented with glucose to a final concentration of 21 mM. All animal use and procedures were reviewed and approved by the Oregon Health and Science University Institutional Animal Care and Use Committee and conformed with National Institutes of Health (NIH) policies on animal care and use.

Electrophysiology. Whole cell voltage-clamp recordings were made from microislands at 3–16 days after neuronal plating [days in vitro (DIV)]. The magnesium-free extracellular solution contained (in mM): 158 NaCl, 2.4 KCl, 1.3 CaCl2, 10 HEPES, and 10 D-glucose (pH 7.4, 320 mosmol). Glass recording pipettes were pulled to a resistance of 2–6 MΩ and filled with a solution containing (in mM): 140 K-glucionate, 4 CaCl2, 8 NaCl, 2 MgCl2, 10 EGTA, 10 HEPES, 4 Na-ATP, and 0.2 Na2GTP (pH 7.39, 319 mosmol). All salts were purchased from Sigma-Aldrich or Fluka. Antagonists were purchased from Ascent Scientific, and GPE [IGF (1–3)] was obtained from Bachem. We used a custom-built multibarrel, piezoelectric flow pipe system for rapid solution exchange. To optimize solution exchange rates, flow pipes were placed within 200–300 μm of the cell body. Transitions between flow pipes were digitally controlled by a transistor-transistor logic (TTL) pulse, allowing for rapid translation between solutions. For steady-state experiments, GPE was delivered in...
the presence of 2.5 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and 10 μM gabazine [SR-95531; 6-imino-3-(4-methoxyphenyl)-1(6H)-pyridazinebutanoic acid hydrobromide] to block AMPA and GABA_A receptors and 500 nM TTX to block synaptic activity. To examine the effects of GPE at the glycine-binding site, a zero-added glycine solution was prepared with HPLC-grade water.

Autaptic excitatory postsynaptic currents (EPSCs) were recorded in microislands (5–16 DIV) that contained a single neuron. To isolate NMDA receptor-mediated EPSCs, we recorded in the presence of 2.5 μM NBQX and 10 μM gabazine; AMPA receptor-mediated EPSCs were recorded in the presence of 10 μM 3-(R)-2-carboxypropyl-1-phosphonic acid [(R)-CPP; 10 μM gabazine]. Brief depolarization [command voltage (V_c) = +30 mV, 0.5 ms] to the soma resulted in an unclamped action potential followed by a post- synaptic EPSC (Tovar et al. 2009). EPSCs were evoked at 0.1 Hz and allowed to reach steady amplitudes (>80 evoked EPSCs) before data acquisition. For paired-pulse experiments, AMPA receptor-mediated EPSCs were evoked with stimuli delivered at 100-ms intervals. Inhibitory neurons that triggered autaptic inhibitory postsynaptic currents (IPSCs) were excluded from the analysis. Input resistance was continuously monitored with a 10-mV hyperpolarizing step before each stimulus. Data were acquired using an Axopatch-IC amplifier and AxoGraph X acquisition software. In all cells, the series resistance was <10 MΩ and was compensated by at least 80% by amplifier circuitry. Data were low-pass filtered at 5 kHz and digitized at 10 kHz.

Analysis. All data were analyzed using AxoGraph X, Microsoft Excel, and IGOR Pro (version 6.22A). For direct agonist application and EPSC experiments, traces are displayed as averages of at least 10 traces. Unless otherwise noted, agonist-induced currents were measured 250–500 ms after the onset of application (window 50–100 ms). Flow pipe solenoid artifacts were blanked. All data are reported as means ± SE unless otherwise noted. Significance was tested using paired Student’s t-tests or ANOVA with a Tukey post hoc correction for multiple comparisons where applicable. In all experiments, α was set at P < 0.05.

Fluorometric assay. To test for possible contamination by free glutamate in GPE-containing solutions, we used a fluorometric assay (Molecular Probes) designed to detect L-glutamic acid (McElroy et al. 2000). This assay relies on the oxidation of glutamate by glutamate oxidase as the oxidized amine group in GPE is bound to produce a fluorescent molecule (resorufin). GPE cannot be oxidized by glutamate oxidase to H2O2, which then reacts with the Amplex Red reagent set at 10 kHz. Analysis. All data were analyzed using AxoGraph X, Microsoft Excel, and IGOR Pro (version 6.22A). For direct agonist application and EPSC experiments, traces are displayed as averages of at least 10 traces. Unless otherwise noted, agonist-induced currents were measured 250–500 ms after the onset of application (window 50–100 ms). Flow pipe solenoid artifacts were blanked. All data are reported as means ± SE unless otherwise noted. Significance was tested using paired Student’s t-tests or ANOVA with a Tukey post hoc correction for multiple comparisons where applicable. In all experiments, α was set at P < 0.05.

RESULTS

High concentrations of GPE activate NMDA receptors. To test whether GPE directly activates NMDA receptors, we performed whole cell voltage-clamp recordings on cultured hippocampal neurons in the presence of an AMPA receptor antagonist (2.5 μM NBQX) and a saturating concentration of glycine (20 μM). Rapid application of GPE produced a dose-dependent inward current (30 μM: 4.7 ± 2.5 pA, n = 3 cells; 500 μM: 114.6 ± 18.3 pA, n = 12 cells; Fig. 1, A–C), that was completely blocked by the competitive NMDA receptor antagonist (R)-CPP (10 μM; Fig. 1, B and C). The GPE-induced current was also blocked by 1 mM Mg2+ at −70 mV but not +40 mV (Fig. 1, D and E), consistent with the voltage-dependent behavior of NMDA receptors (Mayer et al. 1984). These data suggest that high concentrations of GPE are capable of directly activating NMDA receptors by binding to the glutamate-binding site.

GPE has low glutamate contamination and does not act on the glycine-binding site. To rule out the possibility that the GPE-containing solutions contained excessive amounts of contaminating glutamate, we used a fluorometric assay (see MATERIALS AND METHODS) to measure the level of glutamate in GPE-containing solutions. We performed the fluorometric assay on a range of GPE concentrations (250 μM to 4 mM). According to this assay, 500 μM GPE contained 55.8 nM contaminating glutamate (Fig. 2A), which is very close to the detection limit of this assay. Therefore, we conservatively...

Fig. 1. Glycine-proline-glutamate (GPE) induced an N-methyl-D-aspartate (NMDA)-mediated inward current. A: whole cell recording with direct application of 30 μM GPE produced a just detectable inward current in hippocampal neurons in culture. The black bars represent time of drug delivery. B and C: 500 μM GPE induces a large inward current that was completely blocked by the competitive NMDA receptor antagonist 3-(R)-2-carboxypropyl-1-phosphonic acid [(R)-CPP; n = 7 cells; P < 0.001]. D and E: the GPE-induced inward current was blocked by 1 mM MgCl2 at negative holding potentials (−70 mV, red trace; 500 μM GPE: −109.7 ± 28.9 pA; 500 μM GPE + 1 mM MgCl2: 27.7 ± 10.6 pA; n = 7 cells; P = 0.006) but not at positive holding potentials (+40 mV, blue trace; 500 μM GPE: 68.7 ± 25.7 pA; 500 μM GPE + 1 mM MgCl2: 72.1 ± 27.5 pA; n = 5 cells; P = 0.44). Data are presented as means ± SE. *P < 0.05, Student’s t-test, paired comparison. ns, Not significant.
assumed a contamination value yielded by two of the higher concentrations tested (2 and 4 mM), which both yielded a contamination of ~0.02%. Therefore, using these estimates, a 500 μM GPE solution contains at most 105 nM glutamate. Although low, this concentration of glutamate cannot be attributed to contaminating glutamate but could also act at the glycine-binding site of the NMDA receptor. These data indicate that the action of GPE on NMDA receptors cannot be attributed to contaminating glutamate or activation of the glycine site. GPE, with both glutamate and glycine residues, ostensibly activated NMDA-dependent inward currents of comparable size (control: 0.817 ± 0.65 nA; GPE: 0.753 ± 0.70 nA; n = 8 cells; P = 0.124). a.u., Arbitrary units.

**DISCUSSION**

Here, we show that the naturally occurring IGF cleavage product, Gly-Pro-Glu, activated NMDA-dependent inward currents through interactions at the glutamate-binding site.

![Figure 2](image-url)

**Fig. 2.** GPE-induced currents could not be attributed to contaminating glutamate or activation of the glycine site. A: L-glutamic acid fluorometric assay was used to measure the level of contaminating glutamate in 500 μM GPE. Standard curve for glutamate is shown with the power function fit (red line), and x-axis is plotted on a logarithmic scale. Red cross indicates the concentration of free glutamate detected in 500 μM GPE (55.8 nM). B: comparison of the current induced by 3 mM GPE (black trace) and the corresponding level of contaminating glutamate (Glu; 620 nM; red trace) assuming 0.02% contamination. C and D: whole cell recording in conditions of zero-added glycine. Application of 1 mM glutamate alone (black trace) and 1 mM glutamate + 500 μM GPE (red trace) evoked inward currents of comparable size (control: 0.817 ± 0.65 nA; GPE: 0.753 ± 0.70 nA; n = 8 cells; P = 0.124). a.u., Arbitrary units. The addition of 100 nM glycine resulted in a 168.5 ± 14.7% potentiation (n = 3; P = 0.04; data not shown) of the glutamate-evoked current. Thus we estimate that the contaminating glycine is 20 nM, sufficiently low to test for an action of GPE on the glycine site. The low level of contaminating glycine allowed us to assay simultaneously for either agonistic or antagonistic effects of GPE at the glycine site. The current produced by a saturating concentration of glutamate (1 mM) in zero-added glycine (1.27 ± 0.69 nA) was not different from the current evoked by 1 mM glutamate + 500 μM GPE (1.26 ± 0.71 nA, n = 12 cells; P = 0.9; Fig. 2, C and D), indicating that GPE has no significant action at the glycine site.

**Effect of GPE on NMDA- and AMPA-mediated synaptic responses.** To address whether GPE could affect synaptic transmission, we recorded autaptic NMDA receptor-mediated EPSCs in the presence of 2.5 μM NBQX and 10 μM gabazine to block AMPA and GABA<sub>A</sub> receptors, respectively. The peak amplitude of EPSCs in the presence of 500 μM GPE was slightly reduced (92.92 ± 1.78% of control, n = 9 cells; P = 0.004; Fig. 3, A and B), whereas 30 μM GPE had no significant effect (94.7 ± 2.1% control, n = 4 cells; P = 0.09; data not shown). Application of 500 μM GPE was also accompanied by a small inward current (~48.9 ± 10.94 pA, n = 9 cells; P = 0.002; Fig. 3A, inset) consistent with a direct action on NMDA receptors. Thus the small reduction of the NMDA receptor-mediated EPSC may reflect slight desensitization of NMDA receptors. GPE also produced a slight reduction in the AMPA receptor-mediated EPSCs recorded in 10 μM (R)-CPP and 10 μM gabazine (92.3 ± 1.87% of control, n = 9 cells; P = 0.017, paired Student’s t-test) but failed to elicit a shift in the holding current (~0.88 ± 10.32 pA, n = 9 cells; P = 0.93). Furthermore, GPE failed to alter the paired-pulse ratio (99.81 ± 0.91% of control, n = 9; P = 0.75), suggesting that GPE did not alter release probability. Although statistically significant, these small NMDA receptor-independent effects on EPSCs are unlikely to be biologically significant.

![Figure 3](image-url)

**Fig. 3.** GPE produced a small attenuation in the NMDA receptor-mediated excitatory postsynaptic current (EPSC). A and B: representative whole cell recording of synaptically evoked EPSC. Application of 500 μM GPE (red trace) caused a small but significant attenuation in the amplitude of the EPSC (92.92 ± 1.78% of control, n = 9 cells; P = 0.004). Inset: bath application of GPE also produced a negative shift in the holding current (average: ~48.9 ± 10.94 pA), consistent with GPE activation of postsynaptic NMDA receptors. *P < 0.05, Student’s t-test, paired comparison.
However, high concentrations of GPE were required. Using a fluorometric assay, we confirmed that GPE does not have appreciable contaminating glutamate (maximum contamination 0.02%). However, this assay cannot detect other potential contaminants, namely proline, which has been shown in some studies to interact with NMDA receptors (Cohen and Nadler 1997; Henzi et al. 1992). The biological action of GPE is mediated by interactions at the glutamate-binding site, as GPE failed to act at the glycine-binding site. Steady-state application of high concentrations of GPE did reduce the NMDA and AMPA receptor EPSC, however, these effects were small (<10%), independent of NMDA receptor activation, and therefore unlikely to cause neuroprotection by block of NMDA receptors.

The membrane action of GPE. GPE is a naturally occurring tripeptide produced both systemically and in brain following NH$_2$-terminal cleavage of IGF-I (Sara et al. 1993; Yamamoto and Murphy 1995). Thus there has been considerable interest in the biological role of IGF cleavage products, including GPE. Although the biological role of GPE is poorly understood, an action of GPE on NMDA receptors had been suggested by earlier studies (Ikeda et al. 1995; Sara et al. 1989). Binding at the glutamate site is interesting given the relatively large size of GPE compared with glutamate. However, other small peptides can activate NMDA receptors at either the glutamate- or glycine-binding sites, albeit with lower affinity (Moskal et al. 2005; Westbrook et al. 1986). In radioligand-binding studies, the tripeptide GPE displaced [$^3$H]glutamate binding with an IC$_{50}$ of $\sim$17 $\mu$M (Sara et al. 1989).

Our results using whole cell voltage-clamp recording in cultured hippocampal neurons indicates that GPE only activates functional NMDA receptors at much higher concentrations. The apparently higher affinity measured in earlier binding studies may reflect either binding to desensitized receptors or glutamate contamination. Structurally, the agonist-binding domain on ionotropic glutamate receptors resembles a clampshell (Armstrong and Gouaux 2000; Armstrong et al. 2003; Mayer and Armstrong 2004). The degree to which the lobes of the clampshell are able to close around an agonist is directly related to the degree of channel activation (Armstrong and Gouaux 2000; Armstrong et al. 2003; Inanobe et al. 2005; Mayer and Armstrong 2004). Furthermore, NMDA receptor agonists that contain carbocyclic rings function as partial agonists because of the smaller degree of clamshell closure (Inanobe et al. 2005; Moskal et al. 2005). These data suggest that the presumably rigid GPE functions either as a partial agonist due to incomplete clampshell closure or as a weak NMDA receptor agonist. However, due to the low affinity of GPE, we cannot distinguish between these two possibilities.

Possible functional roles of GPE. IGF-I can reach the brain from the systemic circulation but is also produced in the brain in response to cortical injury including cortical contusions and ischemia-reperfusion, where it may play a role in neuroprotection (de Pablo et al. 1995; Guan et al. 1993; Gustafson et al. 1999; Lee et al. 1992; Lin et al. 2009; Sandberg Nordqvist et al. 1996). Exogenous administration of GPE has also been reported to provide neuroprotection against ischemic-reperfusion injuries in rats (Guan et al. 2004; Shapira et al. 2009; Sizonenko et al. 2001). However, contrary to our results, neuroprotection is usually associated with inhibition, not activation, of NMDA receptors. Furthermore, in organotypic slice cultures, the neuroprotective effects of GPE were achieved at low micromolar concentrations (Guan et al. 2004; Sara et al. 1989; Saura et al. 1999), much lower than were required to activate NMDA receptors in our experiments. The concentration of IGF-I and GPE following cortical contusions is not known; however, even the most abundant intracellular proteins only reach a concentration of $\sim$1 mM (Edmonds et al. 2000), and there is no evidence that GPE is concentrated into synaptic vesicles. It therefore seems unlikely that the neuroprotective effects of GPE are mediated by interactions with NMDA receptors. Consistent with this idea, Sizonenko et al. (2001) suggested that glial cells mediate the neuroprotective effects of GPE, as [$^3$H]GPE was localized to glial cells. Additionally, the distribution of [$^3$H]GPE does not match the distribution of NMDA receptors in the hippocampus (Monaghan and Cotman 1985; Saura et al. 1999). Therefore, despite the ability of GPE to activate NMDA receptors at high concentrations, the neuroprotective effects of GPE are likely mediated by another mechanism.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

C.E.V., K.R.T., and G.L.W. conception and design of research; C.E.V. and K.R.T. performed experiments; C.E.V. and K.R.T. analyzed data; C.E.V. and K.R.T. interpreted results of experiments; C.E.V. prepared figures; C.E.V. and G.L.W. drafted manuscript; C.E.V. and G.L.W. edited and revised manuscript; G.L.W. approved final version of manuscript.

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**GPE ACTION ON NMDA RECEPTORS**

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