

## Role of the NMDA-receptor in Prepulse Inhibition in the Rat

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**Abstract:** Kynurenic acid (KYNA) is an endogenous metabolite of tryptophan. Studies have revealed increased brain KYNA levels in patients with schizophrenia. Prepulse inhibition (PPI) is a behavioral model for sensorimotor gating and found to be reduced in schizophrenia. Previous studies have shown that pharmacologically elevated brain KYNA levels disrupt PPI in the rat. The aim of the present study was to investigate the receptor(s) involved in this effect. Rats were treated with different drugs selectively blocking each of the sites that KYNA antagonizes, namely the glutamate recognition site of the N-methyl-D-aspartate receptor (NMDAR), the  $\alpha 7^*$  nicotinic acetylcholine receptor ( $\alpha 7^*nAChR$ ) and the glycine site of the NMDAR. Kynurenine (200 mg/kg) was given to replicate the effects of increased levels of KYNA on PPI. In order to block the glutamate recognition site of the NMDAR, CGS 19755 (10 mg/kg) or SDZ 220-581 (2.5 mg/kg) were administered and to antagonize the  $\alpha 7^*nAChR$  methyllycaconitine (MLA; 6 mg/kg) was given. L-701,324 (1 and 4 mg/kg) or 4-Chloro-kynurenine (4-Cl-KYN; 25, 50 and 100 mg/kg), a drug in situ converted to 7-Chloro-kynurenic acid, were used to block the glycine-site of the NMDAR. Administration of SDZ 220-581 or CGS 19755 was associated with a robust reduction in PPI, whereas L-701,324, 4-Cl-KYN or MLA failed to alter PPI. Kynurenine increased brain KYNA levels 5-fold and tended to decrease PPI. The present study suggests that neither antagonism of the glycine-site of the NMDA receptor nor antagonism of the  $\alpha 7^*nAChR$  disrupts PPI, rather with regard to the effects of KYNA, blockade of the glutamate recognition-site is necessary to reduce PPI.

**Keywords:** kynurenic acid, kynurenine, sensorimotor gating,  $\alpha 7^*$  nicotinic acetylcholine receptor, NMDA/glycine-site

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## Introduction

Kynurenic acid is an endogenous tryptophan metabolite, primarily synthesized in and released from astrocytes.<sup>1–3</sup> During the last decade, several studies implicate KYNA in the pathophysiology of various psychiatric conditions.<sup>4,5</sup> For example, studies of patients with schizophrenia have revealed elevated levels of KYNA in both the cerebrospinal fluid (CSF) and in the post mortem prefrontal cortex.<sup>6,7</sup> In addition, suicidal attempters with major depressive disorder<sup>8</sup> as well as patients with bipolar disorder<sup>9</sup> display elevated levels of KYNA in the CSF.

Kynurenic acid (KYNA, Fig. 1a) is an antagonist at glutamatergic and cholinergic receptors. In particular, low concentrations of KYNA block the glycine co-agonist site of the N-methyl-D-aspartate receptor (NMDAR, Fig. 2a;  $IC_{50} = 8–15 \mu M^{10,11}$ ) and the  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7nAChR$ , Fig. 2b;  $IC_{50} = 7 \mu M^{12}$ ). At higher concentrations, KYNA also blocks the glutamate recognition site of the NMDA receptor ( $IC_{50} = 200–500 \mu M^{10}$ ) and the AMPA/kainate receptors ( $IC_{50}$  in the millimolar range<sup>13</sup>). In addition, KYNA was recently found to stimulate the previously orphan G-protein coupled receptor GPR35 in the rat ( $EC_{50} = 7 \mu M^{14}$ ).

The physiological significance of brain KYNA has been demonstrated in a number of studies during the last decade.<sup>4</sup> However, it is unclear which receptor(s) participate in the various effects of KYNA in the brain. Previous studies have shown that acute and chronic pharmacological elevation of brain KYNA is associated with increased firing of rat midbrain dopamine (DA) neurons,<sup>15–21</sup> an effect recently shown to be mediated via blockade of the glycine-site of the NMDA receptor.<sup>16,18</sup> Furthermore, local administration of KYNA in the rat striatum decreases terminal DA release via specific blockade of  $\alpha 7nAChR$ .<sup>22</sup> A previous study has also shown that pharmacologically elevated levels of KYNA disrupts prepulse inhibition (PPI) in the rat,<sup>17,23</sup> although the specific receptor mechanism involved was not ascertained.

PPI is defined as the attenuation of the startle response to a startling stimulus (e.g. a pulse), when such a stimulus is briefly preceded by a stimulus of subthreshold intensity (prepulse). Disruptions of sensorimotor gating are considered to reflect dysfunctions in the ability to filter out extraneous stimuli that might interfere with information processing and

attention.<sup>24</sup> Deficits in PPI are frequently observed in patients with schizophrenia.<sup>25–28</sup> Interestingly, non-competitive NMDAR antagonist, e.g. PCP, ketamine or MK 801, disrupt PPI in rodents.<sup>29</sup> These effects on PPI are also in line with the NMDAR hypofunction hypothesis of schizophrenia,<sup>30–32</sup> based on the finding that NMDAR antagonists cause psychotic symptoms in healthy volunteers and worsen clinical symptoms in patients.<sup>33–36</sup>

The present study investigated which receptor(s) mediate the effects of KYNA on PPI. For this purpose we administered drugs selectively blocking the different receptor-sites known to be blocked by KYNA; Methyllycaconitine (MLA, Fig. 1b), a selective antagonist at the  $\alpha 7nAChR$ ; SDZ 220–581 and CGS 19755 (Fig. 1c and 1d, respectively), selective blockers of the glutamate recognition-site of the NMDA-receptor; L-701,324 (Fig. 1e) and 4-Cl-KYN (*in situ* converted to 7-Cl-KYNA, Fig. 1f) was given to selectively block the glycine-site of the NMDAR. A putative role of the GPR35 receptor in this regard was not tested due to its limited expression in the brain.<sup>14</sup>

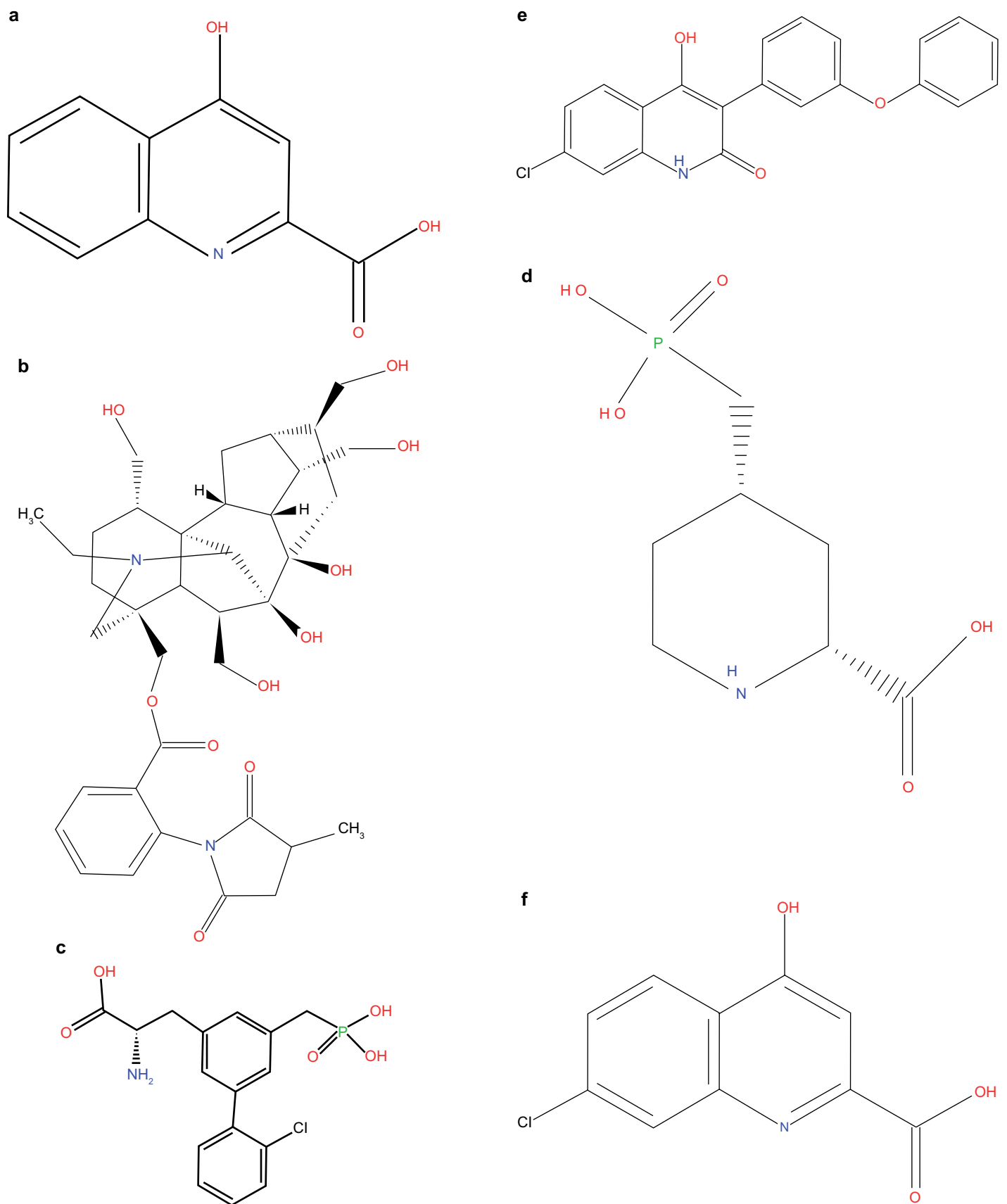
## Materials and Methods

### Animals

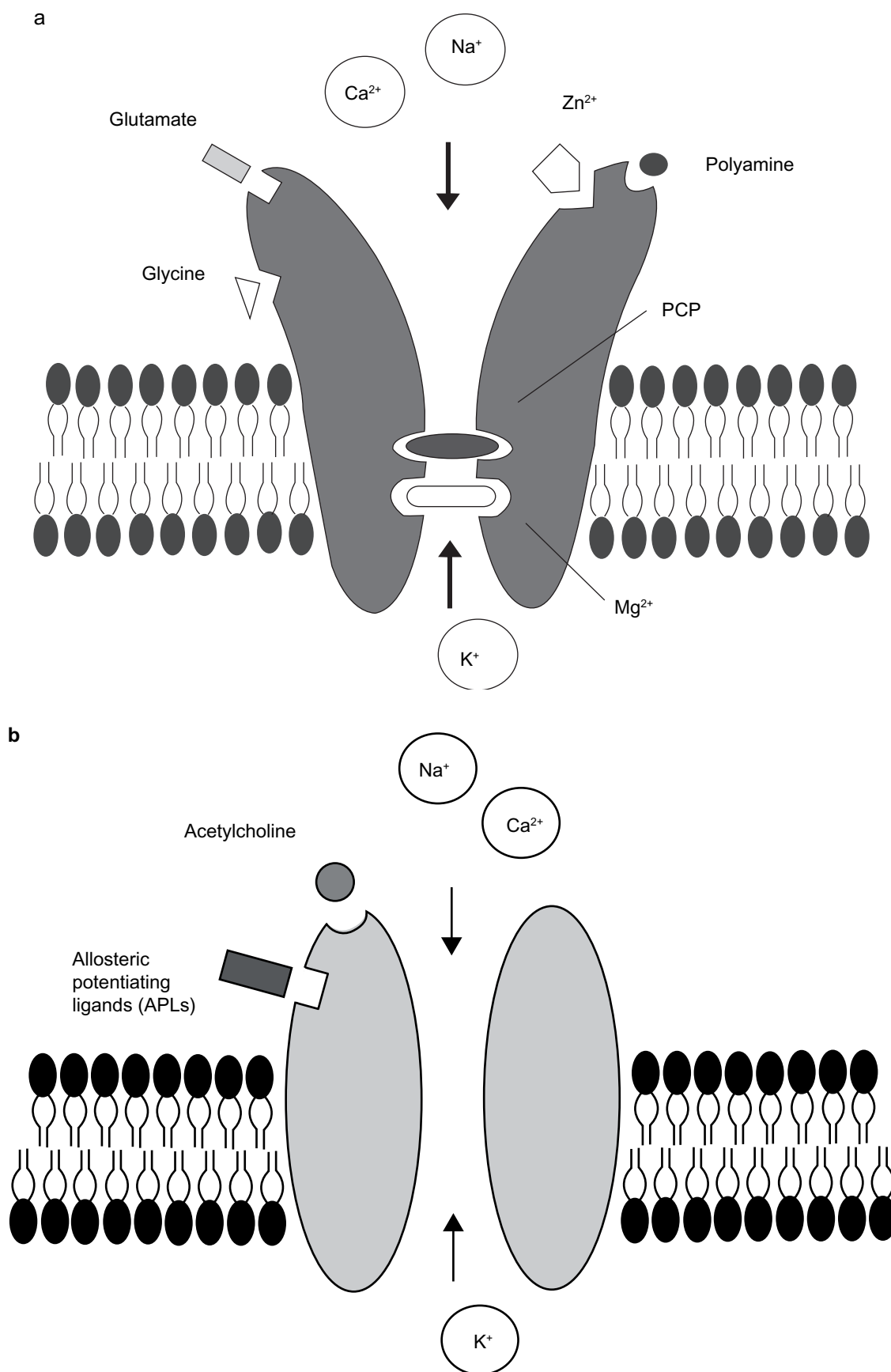
Experiments were performed on male Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden; weighing between 200–330 g). The animals were housed in groups of five with free access to food and water. Environmental conditions were checked daily and maintained under constant temperature (25 °C) and 40%–60% humidity in a room with a regulated, reversed 12 h light/dark cycle (lights off at 07.00 AM, lights on at 07.00 PM). Animals were handled at least 2 days before testing to reduce any subsequent handling stress. Experiments were approved by and performed in accordance with the guidelines of the Ethical Committee of Northern Stockholm, Sweden and all efforts were made to minimize the number of animals used and their suffering.

### Drugs

The following drugs were used: 4-Cl-KYN (kindly supplied by Vistagen Therapeutics, South San Francisco, CA, USA and dissolved in 7.5% (2-hydroxypropyl)- $\beta$ -cyclodextrin, 7-Cl-KYNA, CGS 19755 and SDZ 220–581 (Tocris, Avonmouth, UK); KYNA, L-kynurenic sulfate salt, L-701,324 and MLA (Sigma, St. Louis,



**Figures 1.** Kynurenic acid **a**) and selective inhibitors for  $\alpha 7$ nAChR (MLA, **b**), glutamate recognition-site of the NMDAR (SDZ 220-581, **c**; CGS 19755, **d**) and the glycine-site of the NMDAR (L-701,324, **e**; 7-Cl-KYNA, **f**).



**Figures 2.** Scheme of the NMDAR **a**) and  $\alpha 7nAChR$  **b**) and their allosteric- and ligand binding-sites.



MO, USA). The chemicals used were: zinc acetate and acetic acid (Sigma, St. Louis, MO, USA); sodium acetate (Riedel-de Haen, Germany) and acetonitrile (Labasco, Partille, Sweden). 4-Cl-KYN, L-kynurenine, L-701,324 and MLA were administered intraperitoneally (i.p.). SDZ 220-581 and CGS 19755 were administered subcutaneously (s.c.). All doses are expressed as free base.

## Apparatus

Two startle chambers were used for measuring the startle response (SR-LAB, San Diego Instruments, San Diego, California). Each chamber consisted of a Plexiglas cylinder (9-cm diameter) mounted on a frame, housed within a ventilated chamber (39 × 38 × 58 cm). Sudden movements within the cylinder were detected by a piezoelectric accelerometer attached below the cylinder. A loudspeaker (Supertweeter; Radio Shack, Fort Worth, Texas) mounted 24 cm above the cylinder provided the broadband background noise and acoustic stimuli. Presentations of the acoustic stimuli were controlled by the SR-LAB software and interface system, which also rectified, digitized (0-4095), and recorded responses from the accelerometer. As described previously,<sup>37</sup> sound levels [dB(A) scale] and accelerometer sensitivities within each chamber were calibrated regularly and found to remain constant over the test period.

## Experimental protocols

To elevate levels of endogenous brain KYNA, rats ( $n = 14$ ) were pretreated with kynurenine (200 mg/kg) i.p. 60 min before testing. Control rats ( $n = 13$ ) received vehicle i.p. 60 min before testing for comparison with animals treated with kynurenine. In order to block the glutamate recognition-site of the NMDAR, rats were pretreated with SDZ 220-581 (2.5 mg/kg,  $n = 12$ ) s.c. 30 min before testing or CGS 19755 (10 mg/kg,  $n = 12$ ) s.c. 45 min before testing. For these experiments, rats receiving saline ( $n = 12$ ) s.c. 30 min before testing, were used as controls. In a third experiment, rats were treated with drugs blocking the glycine-site of the NMDAR or the  $\alpha 7nAChR$ . In order to block the glycine-site of the NMDAR, in situ produced 7-Cl-KYNA or pretreatment with L-701,324 (1 mg/kg,  $n = 13$  or 4 mg/kg,  $n = 17$ ) i.p. 15 min before testing were used. To elevate 7-Cl-KYNA, rats were pretreated with

4-Cl-KYN (25 mg/kg,  $n = 15$ ; 50 mg/kg,  $n = 14$ ; or 100 mg/kg,  $n = 10$ ) i.p. 60 min before testing. For selective blocking of the  $\alpha 7nAChR$ , rats were treated with methyllycaconitine (MLA, 6 mg/kg,  $n = 15$ ) i.p. 10 min before testing. Controls in this study ( $n = 18$ ) received saline i.p. 15 min before testing. Pre-treatment times were based on previous studies.<sup>18,38,39</sup> All drug combinations were balanced across the two startle chambers. The experimental session consisted of a 5 min acclimatization period to a 65-dB background noise (continuous throughout the session), followed by a 20-min acoustic PPI test session. Seven days before any drug testing, animals were pre-exposed to the chambers and the testing session. The purpose of the preexposure was to acclimatize the animals to the testing chambers and startle/prepulse stimuli and to baseline-match the groups for subsequent testing (groups were matched for equivalent mean startle magnitude and percent PPI, as defined below). In the test session, a background noise (65 dB) was presented alone for 5 min and then continued throughout the remainder of the session. The test session used in all of the experiments contained five different trial types and had a duration of 20 min: a “pulse-alone” trial, in which a 40-msec 120-dB broadband burst was presented; three “prepulse-pulse” trials, in which 20-msec noises that were either 3, 6, or 12 dB above the background noise were presented 100 msec before the onset of the 120-dB pulse; and a “no stimulus” trial, which included only the background noise. All trial types were presented several times in a pseudo-random order for 60 trials (12 pulse-alone trials, 10 each of the remaining prepulse trial types, and eight no-stimulus trials). Five pulse-alone trials, which were not included in the calculation of PPI values, were presented at the beginning of the test session to achieve a relatively stable level of startle reactivity for the remainder of the session (based on the observation that the most rapid habituation of the startle reflex occurs within the first few presentations of the startling stimulus<sup>40</sup>). In addition, five pulse-alone trials occurred at the end of the session to assess startle habituation but were not included in the calculation of PPI. An average of 15 sec (range, 9–21 sec) separated consecutive trials. The whole session lasted approximately 24 min. A brief baseline session used to familiarize rats with the testing procedure and match groups for pharmacological studies consisted of 24 trials



(18 120-dB pulse-alone and six prepulse-pulse trials with a 12-dB prepulse intensity).

### Analysis of whole-brain kynurenic acid and 7-chloro-kynurenic acid

Immediately after the behavioral experiments, the rats were killed by decapitation. The brains were taken out rapidly and stored immediately at  $-70\text{ }^{\circ}\text{C}$  for subsequent analysis of KYNA and 7-Cl-KYNA. The brains were sonicated with homogenization medium (perchloric acid 0.4 mol/L,  $\text{Na}_2\text{S}_2\text{O}_5$  0.1%, and ethylenediaminetetraacetic acid 0.05%), which was added in the same amount as the weight of the brain before sonication. The samples were centrifuged at 4000 g for 10 min, and 40  $\mu\text{L}$  perchloric acid (70%) was added to the supernatant. Thereafter, the supernatant was centrifuged twice. For analysis of KYNA and 7-Cl-KYNA, an isocratic reversed phase high-performance liquid chromatography (HPLC) system was used, including a dual piston, high liquid delivery pump (Bischoff, Leonberg, Germany), a ReproSil-Pur C18 column (4  $\times$  150 mm, Dr. Maisch GmbH, Ammerbuch, Germany) and a fluorescence detector (Jasco Ltd, Hachioji City, Japan) with an excitation and emission wavelength of 344 nm and 398 nm, respectively (18 nm bandwidth). A mobile phase of sodium acetate (50 mM, pH 6.20, adjusted with acetic acid) and acetonitrile (7% or 10%, for KYNA or 7-Cl-KYNA, respectively) was pumped through the reversed-phase column at a flow rate of 0.5 mL/min. Samples of 30  $\mu\text{L}$  were manually injected (Rheodyne, Cotati, CA, USA). Zinc acetate (0.5 M, not pH adjusted) was delivered post column by a peristaltic pump (P-500, Pharmacia, Uppsala, Sweden) at a flow rate of 0.10 mL/min. The signals from the fluorescence detector were transferred to a computer for analysis utilizing Datalys Azur (Grenoble, France). The retention time of KYNA or 7-Cl-KYNA was about 7 or 16 min, respectively.

### Data and statistical analysis

For each pulse-alone and prepulse-pulse trial, the startle response to the 120-dB burst was recorded. Two measures were then calculated from these data for each animal. First, startle magnitudes were calculated as the average response to the pulse-alone trials within each of the four blocks and analyzed with mixed-design analyses of variance (ANOVAs), with

block as the repeated measure and pretreatment and/or treatment as between-subject factors. Data from the first and last blocks of five pulse-alone trials are not presented, because the startle data from the middle two blocks when PPI was assessed were representative of the treatment effects, and no reliable effects on startle habituation were observed. Second, the amount of PPI was calculated as a percentage score for each prepulse + pulse trial type:  $\%PPI = 100 - \left( \frac{\text{startle response for prepulse + pulse trial}}{\text{startle response for pulse-alone trial}} \right) \times 100$ . All data were first analyzed in a three-factor ANOVA with blocks (first and second halves of the session) and prepulse as within subject factors and treatment as a between subject factor. When the block factor did not interact with another factor, only the two-factor ANOVA (treatment and prepulse intensity) are reported. The main effect of prepulse intensity was always significant and is not reported specifically. Post hoc comparisons of means were carried out with Tukey's test. Each experiment was analyzed separately.

All data are presented as mean  $\pm$  SEM. Statistically significant differences regarding concentrations of KYNA and 7-Cl-KYNA were established using Kruskal-Wallis analysis of variance followed by Mann-Whitney U-test. Alpha was set at 0.05.

### Results

Rats administered kynurenine (200 mg/kg) displayed a 5-fold increase in whole brain KYNA levels (123.1  $\pm$  18.8 nM,  $n = 14$ ) compared to controls (23.3  $\pm$  2.7 nM,  $n = 13$ ; Table 1). This elevation of brain KYNA was associated with a tendency to decrease PPI at all prepulse intensities ( $F(1,25) = 2.56$ ,  $p = 0.12$ ; Fig. 3) and a trend toward decreasing in startle magnitude ( $F(1,25) = 3.37$ ,  $p = 0.078$ ).

Treatment with drugs blocking the glutamate recognition site of the NMDA receptor, i.e. SDZ 220-581 (2.5 mg/kg;  $F(1,22) = 12.33$ ,  $p < 0.01$ ) or CGS 19755 (10 mg/kg;  $F(1,22) = 16.47$ ,  $p < 0.001$ ), was found to clearly reduce PPI (Fig. 4). While SDZ 220-581 had no effect on startle magnitude, CGS 19755 significantly decreased startle magnitude ( $F(1,22) = 5.32$ ,  $p < 0.05$ ).

In order to investigate if blockade of the glycine-site of the NMDA receptor or blockade of the  $\alpha 7\text{nAChR}$  disrupts PPI, three different drugs were used, i.e. L-701,324, 4-Cl-KYN and MLA. Administration of

**Table 1.** Whole brain concentrations of KYNA or 7-Cl-KYNA in rats pretreated with kynurenine (i.p., 1.5 h, n = 14) or 4-Cl-KYN (i.p., 1.5 h, n = 10–15).<sup>1</sup>

Treatment	KYNA, nM	7-Cl-Kyna, nM
Control <sup>2</sup>	23.26 ± 2.67	-
Kynurenine, 200 mg/kg	123.10 ± 18.76***	-
4-Cl-Kynurenine, 25 mg/kg	-	9.23 ± 1.60
4-Cl-Kynurenine, 50 mg/kg	-	18.44 ± 3.14
4-Cl-Kynurenine, 100 mg/kg	-	52.10 ± 8.84

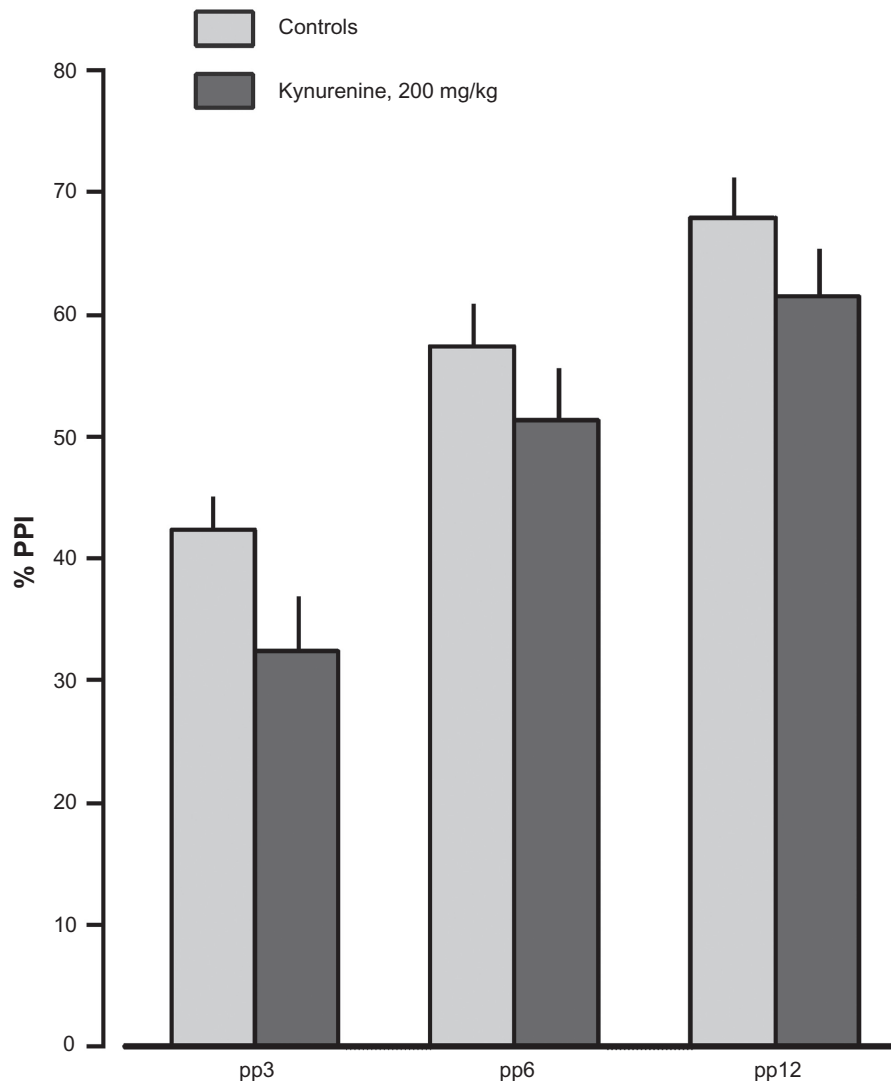
<sup>1</sup>Values represent mean ± SEM. Statistics: \*\*\*p < 0.001 vs. control (Mann-Whitney U-test).

<sup>2</sup>n = 13.

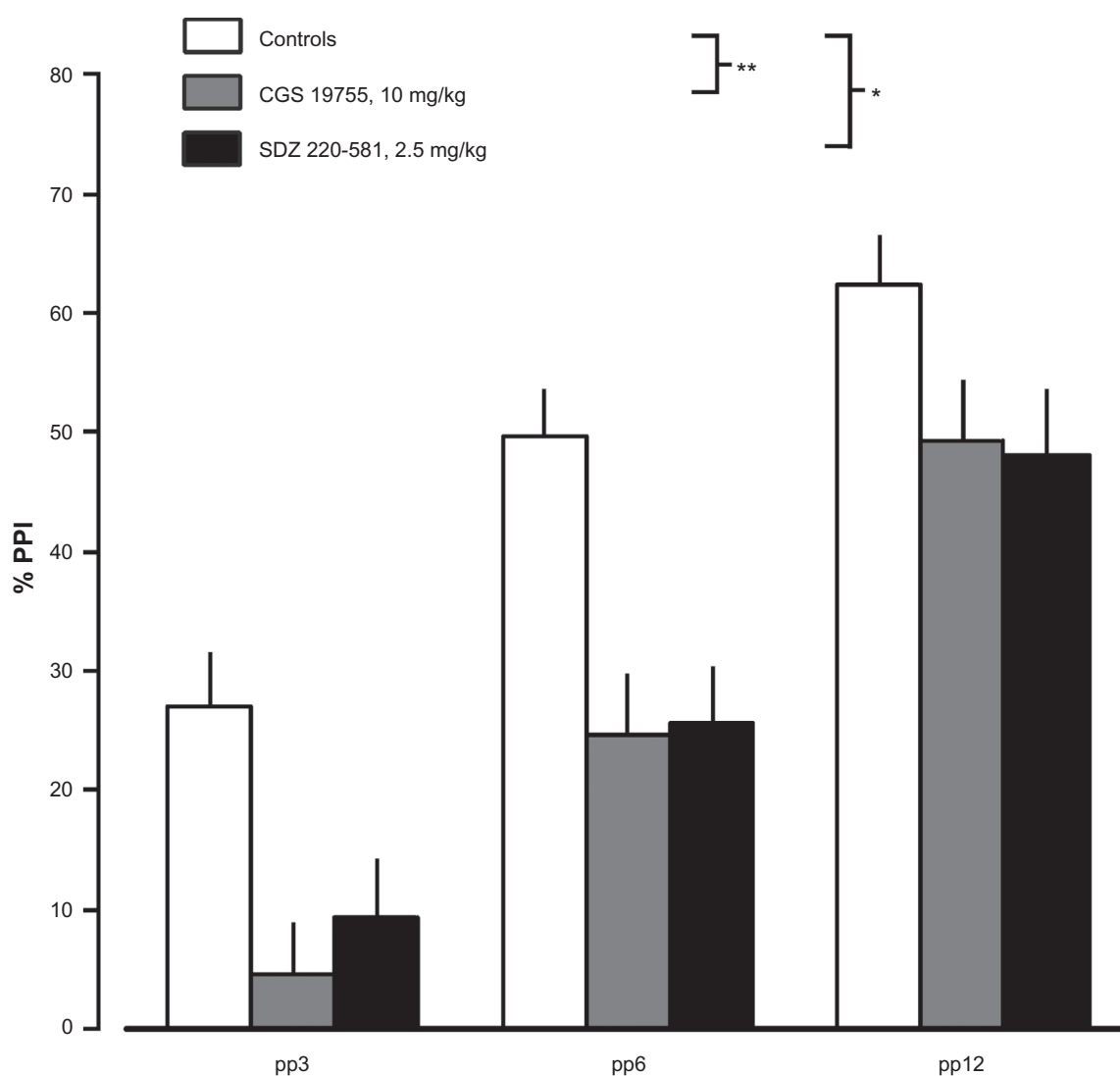
L-701,324 (1 and 4 mg/kg;  $F(2,45) = 2.22$ ,  $p = 0.12$ ), 4-Cl-KYN (25; 50 and 100 mg/kg; NS), which in situ was converted to 7-Cl-KYNA (a potent and selective antagonist at the glycine-site of the NMDA receptor) or MLA (6 mg/kg) were not associated with disrupted PPI ( $F(1,31) = 2.84$ ,  $p = 0.102$ ; Figs. 5 and 6). MLA and 4-Cl-KYN did not affect startle magnitude; whereas L-701,324 (4 mg/kg) reduced startle ( $F(2,45) = 3.20$ ,  $p = 0.0503$ ).

## Discussion

Present results show that pharmacological elevation of brain KYNA is associated with a tendency to disrupt PPI in the rat. The reason for not reaching statistical significance may reflect the fact that kynurenine



**Figures 3.** Effects of kynurenine (200 mg/kg, i.p., 60 min, n = 14) or vehicle (i.p. 60 min, n = 13) on prepulse inhibition (PPI). Values represent mean ± SEM for each group.



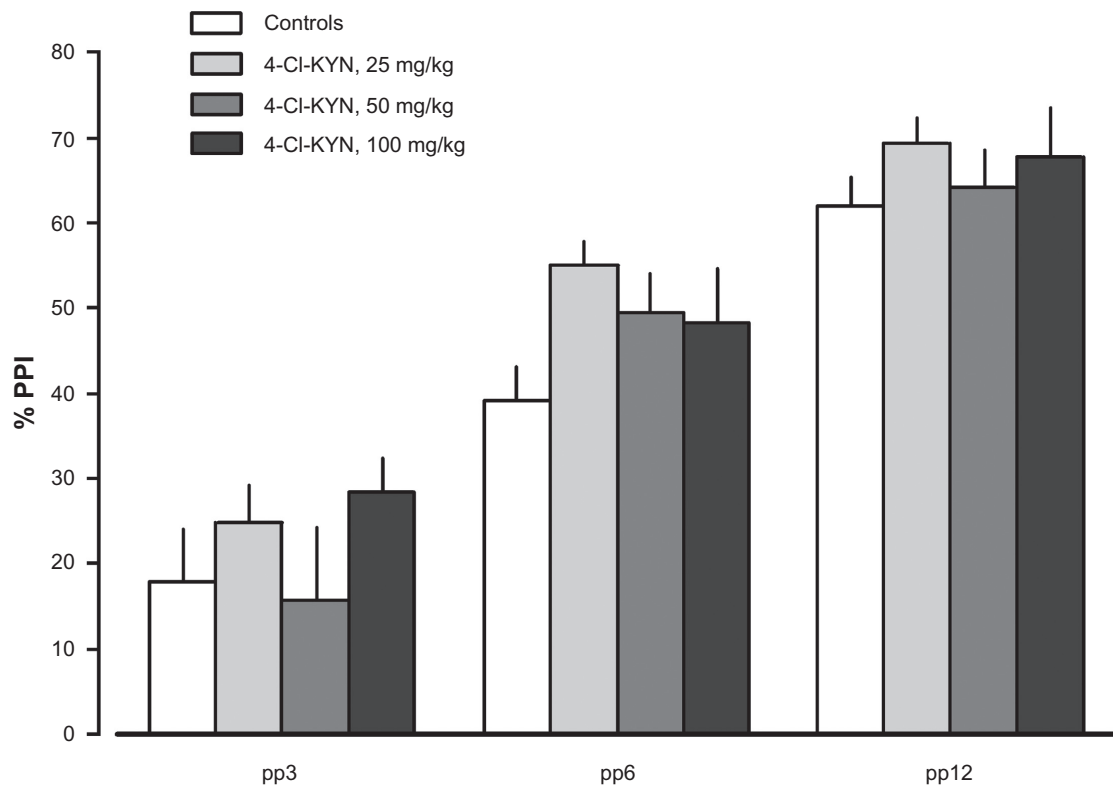
**Figures 4.** Effects of CGS 19755 (10 mg/kg, s.c., 45 min, n = 12), SDZ 220–581 (2.5 mg/kg, s.c., 30 min, n = 12) or saline (n = 12) on PPI. Values represent mean  $\pm$  SEM for each group. Statistics: \*p < 0.01 vs. saline, \*\*p < 0.001 vs. saline.

**Table 2.** Effects on startle magnitude.

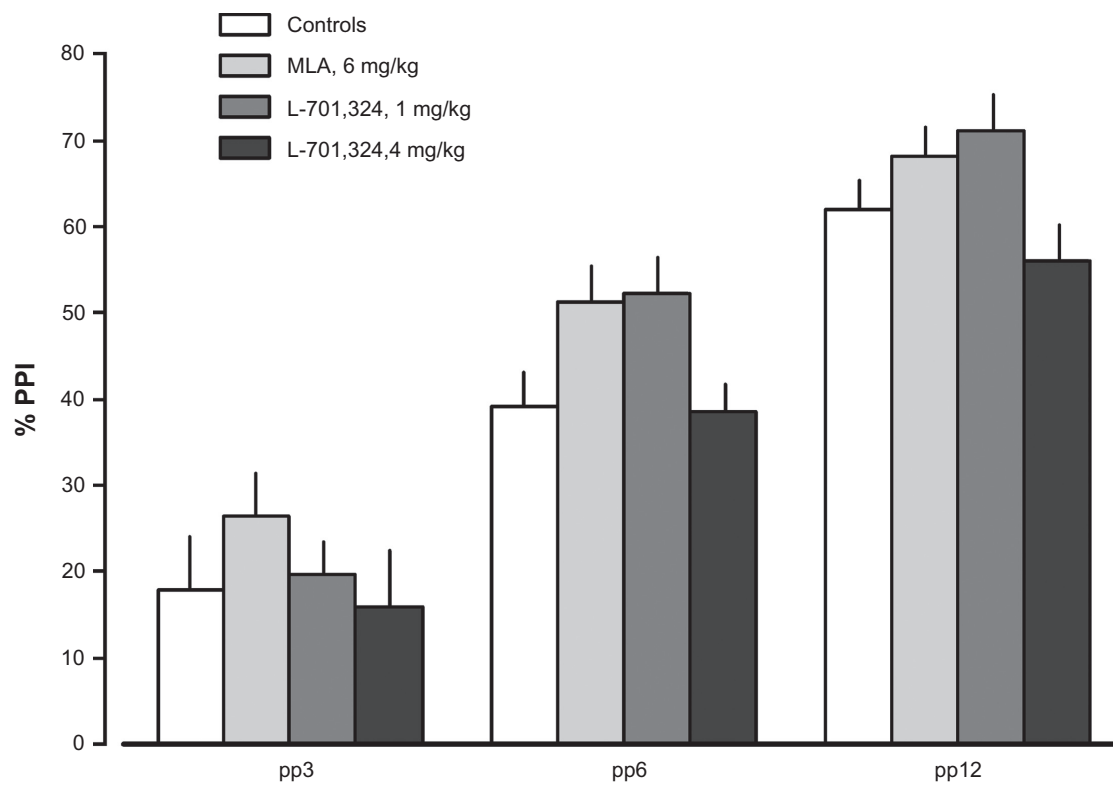
Vehicle	Kynurenine, 200 mg/kg		
281.82 $\pm$ 41.68	203.75 $\pm$ 17.95		
Saline	CGS 19755, 10 mg/kg	SDZ 220–581, 2.5 mg/kg	
347.16 $\pm$ 43.07	235.87 $\pm$ 26.20*	358.42 $\pm$ 62.10	
Saline	4-Cl-KYN, 25 mg/kg	4-Cl-KYN, 50 mg/kg	4-Cl-KYN, 100 mg/kg
284.80 $\pm$ 31.54	342.44 $\pm$ 81.18	294.48 $\pm$ 49.01	288.82 $\pm$ 44.57
	L-701, 324, 1 mg/kg	L-701, 324, 4 mg/kg	
	290.15 $\pm$ 50.24	189.21 $\pm$ 18.75	
	MLA, 6 mg/kg		
	264.59 $\pm$ 26.20		

Values represent mean  $\pm$  SEM. \*p < 0.05 vs. control, F(1,22) = 5.32.





**Figures 5.** Effects of 4-Chloro-kynurenine (25, 50 or 100 mg/kg, i.p., 60 min; n = 15, n = 14 and n = 10, respectively; *in situ* converted to 7-Cl-KYNA) or saline (n = 18) on PPI. Values represent mean  $\pm$  SEM for each group.



**Figures 6.** Effects of L-701,324 (1 or 4 mg/kg, i.p., 15 min, n = 13 and n = 17, respectively), MLA (6 mg/kg, i.p., 10 min, n = 15) or saline (n = 18) on PPI. Values represent mean  $\pm$  SEM for each group.



does not selectively increase KYNA but also increases other neuroactive metabolites of the kynurenine pathway, e.g. 3-hydroxykynurenine and quinolinic acid.<sup>41</sup> In a previous study we showed that both kynurenine as well as PNU 156561A, a drug blocking kynurenine 3-hydroxylase and thereby shunting the synthesis towards KYNA, significantly reduce PPI in Sprague Dawley rats.<sup>23</sup> A limitation with the present study is the fact that pharmacological tools, aiming at selectively increase KYNA, are lacking, simply because such tools are not available.

The results of the present study also show that administration of CGS 19755 or the highest dose of L-701,324 reduce startle. Such an effect was previously observed in rats with a 60-fold increase in brain KYNA levels<sup>23</sup> and thought to be related to sedation. In the present study no signs of sedation was observed in rats treated with CGS 19755 or L-701,324. The reduced PPI following administration of SDZ 220-581 or CGS 19755 confirms that blockade of the glutamate recognition site of the NMDA receptor is associated with disrupted PPI.<sup>38,42</sup> In contrast, blockade of either the  $\alpha 7$ nAChR or the NMDAR/glycine-site had no effect on PPI. These effects are in line with previous studies showing that systemic administration of antagonists of the NMDAR/glycine-site do not affect PPI.<sup>42,43</sup> However, local administration of 7-Cl-KYNA, intracerebroventricularely, or into the nucleus accumbens, has been found to reduce PPI.<sup>39,44,45</sup> A benefit of using systemic administration of 4-Cl-KYN is the *in situ* production of 7-Cl-KYNA. 4-Cl-KYN utilizes the same enzymatic machinery as kynurenine and hence 7-Cl-KYNA will be produced in the same regions and micro-compartments in which KYNA is produced. The effects of locally produced 7-Cl-KYNA, derived from systemic administration of 4-Cl-KYN, should thus better correspond to the effects seen by increased levels of KYNA. A recent study supports this view, since it has been shown that the increased firing of midbrain dopamine cells, following *in situ* produced 7-Cl-KYNA, are almost identical to the enhanced dopaminergic firing following pharmacologically elevated levels of KYNA. Thus, the absence of an effect on PPI following elevated levels of 7-Cl-KYNA reliably suggest that the glycine-site of the NMDAR is not primarily involved in the modulation of PPI. Of note,

4-Cl-KYN is developed for the treatment of neurological pain and the absence of a disruptive effect on PPI following administration of this agent suggest a lower risk of side effects related to cognition. Previous studies analyzing a putative involvement of the  $\alpha 7$ nAChR on PPI are conflicting as it has been shown that administration of  $\alpha$ -bungarotoxin, another  $\alpha 7$ nAChR antagonist, or removal of hippocampal cholinergic afferents, disrupts PPI.<sup>46–48</sup> However, the tendency to an increased PPI in the present study following administration of MLA, an antagonist of  $\alpha 7$ nAChR, is more consistent with a previous study by Schreiber et al,<sup>49</sup> and by the finding that PPI is normal in  $\alpha 7^*$  null mutant mice.<sup>50</sup>

KYNA plays a significant physiological role in the brain (c.f. Introduction). In addition, elevation of brain KYNA is associated with cognitive dysfunctions in rodents,<sup>51–53</sup> deficits also present in psychiatric disorders, i.e. schizophrenia, bipolar disorder and major depressive disorder. In order to design novel therapeutic drugs, specifically aiming at preventing the effects of KYNA, the specific receptor involved in the variety of effects induced by KYNA must be ascertained. The present study suggests that neither antagonism of the glycine-site of the NMDAR nor antagonism of the  $\alpha 7$ nAChR disrupts PPI. Rather, with regard to the effects of KYNA, blockade of the glutamate recognition-site is necessary to reduce PPI.

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## Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors report no conflicts of interest: Klas Linderholm, none; Susan Powell, none; Elin Olsson, none; Maria Holtze, none; Sophie



Erhardt, none; R. Snodgrass, employee and stockholder of VistaGen Therapeutics, Inc.

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