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The herbicide glyphosate is a weak inhibitor of acetylcholinesterase in rats

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ABSTRACT

The current work evaluated the inhibitory potency of the herbicide glyphosate (GLP) on acetylcholinesterase (AChE) activity in male and female rat tissues. The AChE activity in brain was higher ($p<0.05$) than those observed in kidney (females: 2.2-fold; males: 1.9-fold), liver (females: 6-fold; males: 6.9-fold) and plasma (females: 14.7-fold; males: 25.3-fold). Enzyme activities were higher in presence of 10 mM GLP compared to those measured at an equimolar concentration of the potent AChE inhibitor dichlorvos (DDVP). Moreover, IC50s for GLP resulted between $6x10^4$- and $6.8x10^5$-fold higher than those observed for DDVP. In conclusion, GLP is a weak inhibitor of AChE in rats.

Key words: glyphosate – dichlorvos – acetylcholinesterase – rats.

Highlights:

The inhibitory potency of glyphosate against acetylcholinesterase was studied in rats.

A higher enzyme activity was measured in brain compared to plasma, liver and kidney.

Glyphosate IC50s were overly high compared to those observed for dichlorvos.

The herbicide glyphosate was found as a weak inhibitor of acetylcholinesterase in rats.

Lower risk of inhibition of this enzyme by environmental levels of glyphosate.

1. INTRODUCTION

Organophosphates (OPs) are widely used in agriculture, horticulture, veterinary medicine and household. Most OPs are easily absorbed by oral, dermal, conjunctival, gastrointestinal and respiratory routes, and share the ability to inhibit cholinesterases in insects and mammals (Marrs, 1993). Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are the most relevant members within the cholinesterase family of enzymes. The inhibition of AChE leads to high concentrations of acetylcholine in cholinergic synapses of both central and peripheral nervous systems. Clinical signs of OP poisoning are consequence of excessive stimulation of muscarinic (i.e.: respiratory depression, salivation, bronchospasm, bradycardia) and nicotinic (i.e.: muscular fasciculation) receptors (Marrs, 1993). The definitive diagnosis of OP toxicity involves measurement of AChE activity in red blood cells and/or BChE in serum of animals or human beings exposed to these pesticides (Tafuri and Roberts, 1987). Moreover, measurement of AChE inhibition has been used as a biomarker of effect following exposure to OP pesticides (Lionetto *et al*., 2013).

The widespread use of OPs for pest control gives rise to large amounts of these compounds released to the environment (Lionetto *et al*., 2013). Consequently, non-target organisms (including human beings) could be exposed to these pesticides through environmental contamination or occupational use. Glyphosate (GLP) [N-(phosphonomethyl) glycine], the active ingredient of many broad-spectrum herbicide formulations, is one of the most commonly OP pesticides worldwide delivered to the environment. Inhibition of AChE activity was reported in brain and muscle of several fish species (*Rhamdia quelen*, *Prochilodus lineatus*, *Cyprinus carpio*, *Cnesterodon decemmaculatus*) and in other aquatic organisms like the tadpole *Rhinella arenarum* (Lajmanovich *et al*., 2011) exposed to sub-lethal levels of GLP or GLP-based herbicides dissolved in their aquatic habitats (Glusczak *et al*., 2007; Modesto and Martínez, 2010; Cattaneo *et al*., 2011; Menéndez-Helman *et al*., 2012). Interestingly, most of these studies included environmental concentrations of GLP (from 0.2 to 5 mg/L) and AChE inhibition was in the range of 20 to 30 % with respect to its basal levels of activity. Furthermore, a slight inhibition (between 13 to 20 %) of AChE activity was also observed when human erythrocytes were incubated with GLP at high concentrations (0.25 to 5 mM), which would be found in plasma only after acute exposure to this herbicide (Kwiatkowska *et al*., 2014). Based on these observations, the present research was designed to gain further insights on the effects of GLP against AChE in mammals. Thus, the aim of the current work was to evaluate *in vitro* the inhibitory potency of GLP on AChE

activity in plasma, brain, liver and kidney from rats. The *in vitro* assays included, as a positive control, the study of the effects of the insecticide dichlorvos (DDVP), a potent AChE inhibitor.

2. MATERIALS AND METHODS

2.1. Animals and sample collection

Healthy Wistar rats (5 females and 5 males), weighting 265 ± 35 g, were maintained under temperature controlled conditions (25°C), and a normal photoperiod of 12 h of darkness and 12 h of light. Animal procedures and management protocols were carried out according to internationally accepted animal welfare guidelines (AVMA, 2007) and approved by the Animal Welfare Committee of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires, Tandil, Argentina (Internal Protocol: 06/11; approval date: November 10th , 2011). Rats were starved overnight and then killed under anaesthesia in agreement with these institutional and internationally accepted animal welfare guidelines.

After sacrifice, the thorax was immediately opened through a longitudinal incision along the breastbone. Rapidly, a blood sample was collected from the heart into glass tubes containing heparin. Then, the abdomen was opened to remove the liver and kidneys. After that, the skull was opened with scissors and the brain carefully removed. Livers, kidneys and brains were rinsed with ice-cold KCl 1.15%, covered with aluminium foils and immediately frozen and stored in liquid N_2 during 1 or 2 days until used for preparation of subcellular fractions. Blood samples were centrifuged at 2000 x *g* during 15 min and plasma stored at -70 ºC until the time of analysis.

Tissue handle, homogenization and preparation of subcellular fractions were carried out as previously was described (Larsen et al., 2012). An aliquot of each tissue preparation was used to determine protein content using bovine serum albumin as a control standard (Lowry, 1951).

2.2. Measurement of AChE activity

AChE activity was monitored in an UV/Vis Spectrophotometer T80+ (PG Instruments Limited, Leicestershire, UK) following the Ellman's method (Ellman *et al*., 1961). Incubations were carried out in the absence (controls) and in presence of GLP (2.5, 5, 10, 20 and 40 mM, dissolved in 10 µL of water) or DDVP ($1.5x10^{-7}$, $1.5x10^{-5}$, $1.5x10^{-3}$, 0.15, 10 and 20 mM, dissolved in 10 µL of methanol). Control incubations contained also the same amount of water or methanol. GLP, DDVP and the substrate (acetylthiocholine iodide) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Data and statistical analysis

AChE activity was expressed as nmol of substrate hydrolysed per min per mg of protein. Data are expressed as mean ± standard deviation (SD). The concentrations (mM) of GLP or DDVP that produced a 50% decrease in AChE activity (IC50s) were determined from best-fit plots of the mean (±SD) percentages of inhibition versus the natural logarithm of the concentrations assayed for each xenobiotic. The statistical analysis of data was carried out with Instat 3.00 software (Graph Pad Software, Inc., San Diego, USA). A normality test was performed for testing if the data was sampled from populations that follow Gaussian distributions. This assumption was tested using the Kolmogorov and Smirnov method. Data was analysed by means of parametric (one-way) ANOVA. Where significant overall differences ($p<0.05$) were observed, further analysis among experimental groups was performed using Tukey multiple-range test.

3. RESULTS

Basal AChE activities in rat tissues are shown in Figure 1 (inserted Tables). The enzyme activity measured in brain was significantly higher ($p<0.05$) than those observed in kidney (females: 2.2-fold; males: 1.9-fold), liver (females: 6-fold; males: 6.9-fold) and plasma (females: 14.7-fold; males: 25.3-fold). A sex-related difference was only observed in plasma, being the AChE activity 108% higher ($p<0.05$) in females compared to males.

Both GLP and DDVP, incubated at an equimolar concentration of 10 mM, inhibited AChE activity in the different tissues studied (Figure 1). The percentages of inhibition ranged between 17% (kidney) to 37% (plasma from male rats) for GLP and 42% (liver) to 97% (plasma from female rats) for DDVP.

Best-fit plots of the mean percentages of inhibition of AChE activities in brain and plasma versus the natural log concentrations of GLP or DDVP are shown in Figure 2. These graphics were useful for the assessment of GLP and DDVP IC50s in both tissues.

4. DISCUSSION

The widespread use of pesticides in agriculture and animal husbandry increases the risk of environmental contamination by different xenobiotics potentially noxious for non-target organisms including human beings. Since its introduction in 1974, the use of GLP has been globally expanded. In this regard, around two-thirds of the total volume of GLP-based herbicides has been delivered to the environment during the last decade (Myers *et al*., 2016). Thus, the presence of GLP, aminomethylphosphonic acid (AMPA, the major GLP metabolic product) and adjuvants of GLP formulations (i.e.: non-ionic surfactants such as alcohol ethoxylates and alkylamine ethoxylates) in soils, surface/groundwater and precipitation appear to be of major concern (Peruzzo *et al*., 2008; Aparicio *et al*., 2013; Battaglin *et al*., 2014). This scenario pointed to the need for further research on the potential deleterious effects of GLP-based herbicides for non-target species. Several studies revealed certain biochemical modifications taking place in different tissues without evidences of clinical toxicity in laboratory animals exposed to sub-lethal levels of GLP-based herbicides (Benedetti *et al*., 2004; Beuret *et al*., 2005; Larsen *et al*., 2012; Larsen *et al*., 2014). In this context, research efforts devoted to the search for biomarkers of exposure would be of particular relevance. For instance, AChE and/or BChE activities in blood are well-known markers of OP-related biological effects, as they give early warning of exposure to these pesticides before adverse clinical health effects occur in humans and animals living in sprayed environments (Skrzypczak *et al*., 2011). As GLP is an OP compound, this research aimed to establish whether or not AChE inhibition would be used as a biomarker of effect in a non-target species exposed to this herbicide.

To the best of our knowledge, there is scarce information on the inhibitory potency of GLP against cholinesterases in mammals. Up to 20 % inhibition of AChE activity was observed *in vitro* in human erythrocytes incubated with 5 mM of either GLP or its main metabolites and impurities of GLP-based herbicides (Kwiatkowska *et al*., 2014). This previous observation is in agreement with the results of the current work; when GLP was incubated at 10 mM, the inhibition of AChE activity ranged between 17 and 37 % in the different tissues studied (see Figure 1). Compared to DDVP, GLP was found as a weak inhibitor of AChE in rat tissues. In fact, when both xenobiotics were incubated at an equimolar concentration of 10 mM, the percentages of inhibition were lower for GLP compared to those observed for DDVP in all tissues studied. Moreover, the IC50 values for the herbicide in brain and plasma resulted between $6x10^4$- and $6.8x10^5$-fold higher than those observed for DDVP (see Figure 2). Previously published data also showed that GLP is a weak inhibitor of AChE in human serum (IC50:

714.3 mM) (El-Demerdash *et al*., 2001) and erythrocytes (Kwiatkowska *et al*., 2014). In the current work, the IC50s for GLP in both plasma and brain were excessively higher compared to the peak plasma concentration of the herbicide measured in a toxicokinetic study performed in rats (4.62 mg/L=0.03 mM) or in women exposed to genetically modified foods in Canada (73.6 µg/L=$5x10^{-4}$ mM) (Aris and Leblanc, 2011).

An interesting outcome of this research was the observation of a higher plasmatic AChE activity in females compared to males. This observation disagrees with previously published data showing a higher enzyme activity in males compared to females (Smith *et al*., 2015) or a lack of a sex-related difference (Alves-Amaral *et al*., 2010). In addition, the IC50 for DDVP in rat plasma was around 7-fold higher in males compared to females. Therefore, notwithstanding their lower activity in plasma, male rats appear to be less sensitive to a DDVP-dependent inhibition of AChE.

In conclusion, this research shows that the herbicide GLP is a weak inhibitor of AChE in rats under *in vitro* conditions. In rat plasma and brain, the concentrations required to produce a 50% inhibition of the enzyme's activity are overly high compared to those needed for other OPs such as DDVP. High systemic concentrations of GLP would be only expected after accidental ingestion of large amounts of the herbicide causing acute poisoning. Therefore, environmental levels of GLP would not involve a risk of toxicity by inhibiting the function of this enzyme in non-target mammalian species.

5. ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: Effects of glyphosate (GLP) and dichlorvos (DDVP) on acetylcholinesterase (AChE) activity in rat brain, kidney, liver and plasma. Values (mean ± SD; 5 females and 5 males) are expressed as fold-change with respect to the basal (control) AChE activity measured in each tissue. Xenobiotics were incubated at an equimolar concentration (10 mM). The inserted tables show basal AChE activities (mean ± SD; 5 females and 5 males) measured in the different tissues studied.

Values with different letters on bars and in tables are significantly different at $p<0.05$.
§: Significantly different vs. males ($p<0.05$).

Figure 2: Inhibitory effects of glyphosate (GLP) and dichlorvos (DDVP) on acetylcholinesterase activity (AChE) in rat brain (upper panel) and plasma (lower panel). Data (mean ± SD; 5 females and 5 males) represent the percentages of inhibition of AChE activity vs. the natural logarithmic concentrations of each xenobiotic. Inserts show the concentration (mM) of GLP or DDVP that produced a 50% decrease in AChE avtivity (IC50s).

Figure
Fig. 1

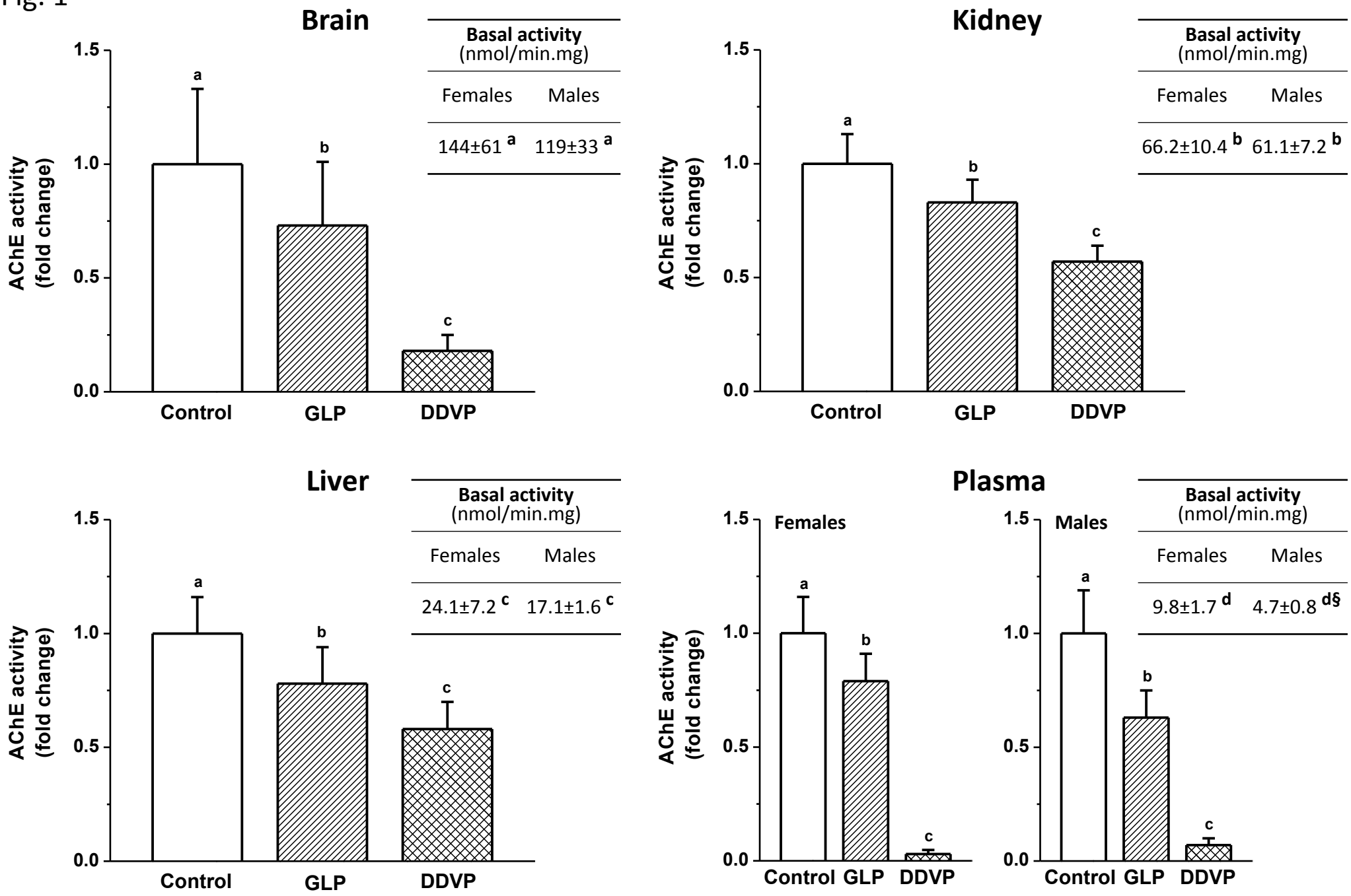
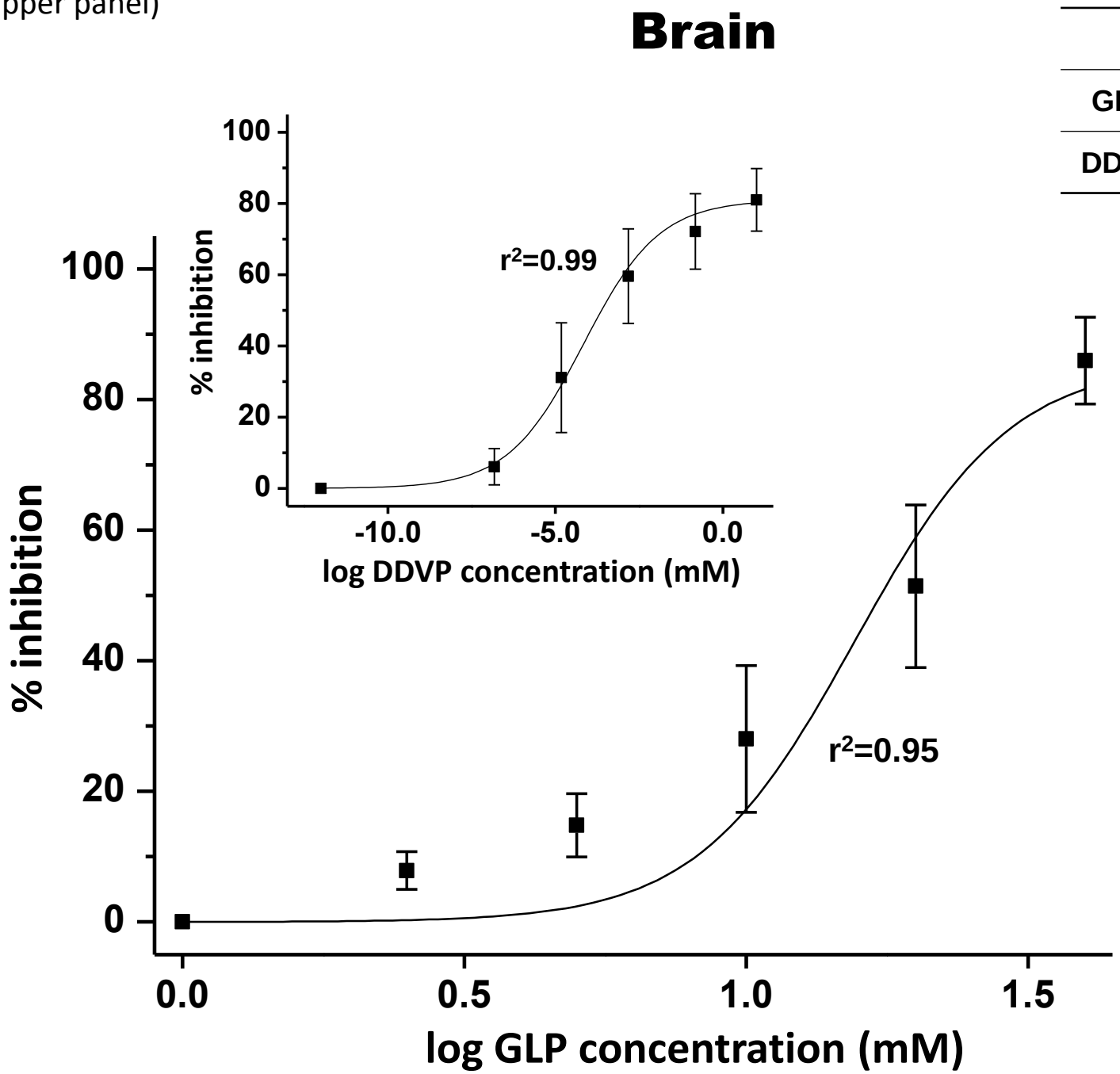
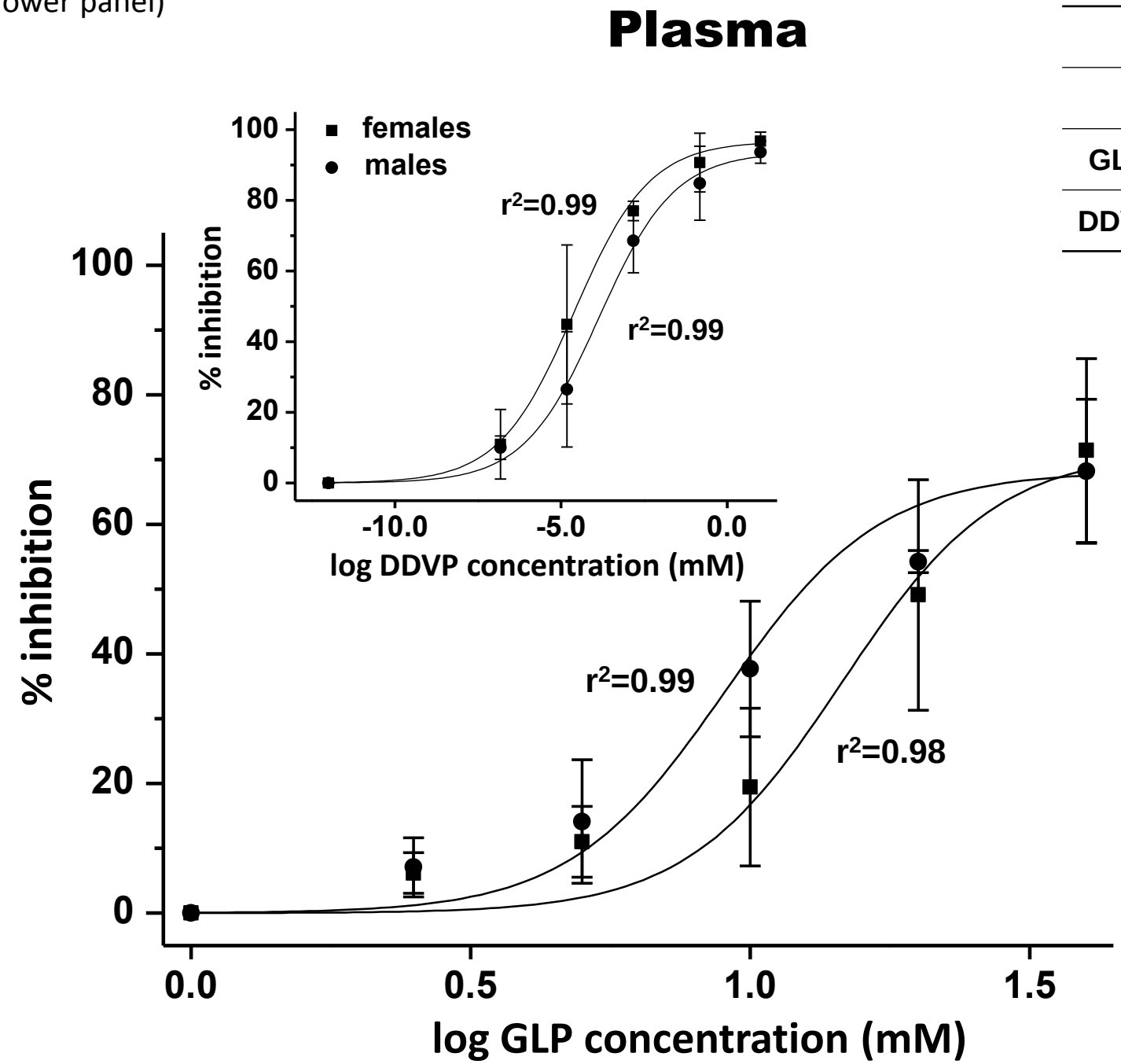

Brain
Basal activity (nmol/min.mg)
Females Males
144±61 a 119±33 a
AChE activity (fold change)
1.5
1.0
0.5
0.0
a
b
c
Control GLP DDVP
Kidney
Basal activity (nmol/min.mg)
Females Males
66.2±10.4 b 61.1±7.2 b
AChE activity (fold change)
1.5
1.0
0.5
0.0
a
b
c
Control GLP DDVP
Liver
Basal activity (nmol/min.mg)
Females Males
24.1±7.2 c 17.1±1.6 c
AChE activity (fold change)
1.5
1.0
0.5
0.0
a
b
c
Control GLP DDVP
Plasma
Basal activity (nmol/min.mg)
Females Males
9.8±1.7 d 4.7±0.8 d§
AChE activity (fold change)
Females
1.5
1.0
0.5
0.0
a
b
c
Control GLP DDVP
Males
1.5
1.0
0.5
0.0
a
b
c
Control GLP DDVP

Fig. 2 (upper panel)



	IC50 (mM)
GLP	17.4
DDVP	2.7×10^{-4}

Fig. 2 (lower panel)



	IC50 (mM)	
	Females	Males
GLP	19.1	12.6
DDVP	2.8×10^{-5}	20×10^{-5}