

## **MEASUREMENT OF GABA AND GLYCINE IN PLANARIANS**

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### **Summary**

We extracted and measured GABA ( $\gamma$ -amino butyric acid) and Gly (glycine), two major inhibitory amino acid neurotransmitters, from planarians. The method used buffer extraction (perchloric acid containing 0.025% of L-cystine and Na<sub>2</sub>EDTA), a simple derivatization, HPLC, and fluorescence detection. The mean retention time ( $\pm$  S.D.) of Gly and GABA was  $2.4 \pm 0.2$  and  $26.6 \pm 0.1$  min, respectively, and the mean ( $\pm$  S.E.M.) amount of GABA was  $108.4 \pm 15.0$  pmole ( $46.2 \pm 3.2$  pmole/mg of planarian). The amount of Gly was only about 1/10<sup>th</sup> the amount of GABA.

Keywords: Method, amino acids, HPLC, Planaria

Previous studies have demonstrated that planarians contain some of the same amino acids found in mammals, including  $\gamma$ -amino butyric acid (GABA), glycine (Gly), glutamate (Glu), and aspartate (Asp) (*e.g.*, (1-5)). A recent study using *in situ* hybridization reported finding a putative homolog of glutamate receptors in planarians (6) and Glu-like immunoreactivity was detected in another flatworm (7). Furthermore, behavioral and pharmacological evidence suggests that there might be a functional role for amino acids in planarians, suggesting that an assay that conveniently measures change in amino acid content in planarians would provide an opportunity to correlate changes in behavior, or other endpoint, with changes in amino acid levels. As a preliminary first step toward this end, we set out to develop a method that efficiently quantifies amino acid levels in planarians. The method utilizes a straightforward extraction procedure, simple derivatization process, high-pressure liquid chromatography (HPLC), and fluorescence detection. To our knowledge, the present results provide the first direct simultaneous quantitative measurement of GABA, Gly, Glu, and Asp in planarians.

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## Methods

### *Animals and drugs.*

Planarians (*Dugesia gonocephala*, s.l.) were purchased from the Carolina Biological Supply Co. (Burlington, N.C.) and were acclimated to the laboratory conditions for at least 24 hours before use. The planarians were used within three days. Glutamate, aspartate, glycine, GABA, sodium borate, potassium cyanide, and naphthalene-2,3-dicarboxaldehyde (NDA) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium citrate and methanol were purchased from Fisher Scientific (Fair Lawn, NJ). Standard solutions of glutamate, aspartate, glycine and GABA were prepared in 0.06 M perchloric acid buffer.

Individual planarians were weighed (approximately 2 mg each), air dried, and then homogenized (Braun-Sonic 2000; B. Braun, Biotech, Allentown, PA, USA) in 125  $\mu$ L of ice-cold perchloric acid buffer (0.15 M) containing 0.025% L-cystine and Na<sub>2</sub>EDTA (5,8). The resulting homogenate was then centrifuged at 14,000  $\times$  *g* for 20 min at 4° C (Refrigerated Multipurpose Centrifuge 5810 R; Eppendorf, Hamburg, Germany) and the supernatant was passed through a 0.2  $\mu$ m filter (Agilent). Stock solutions were prepared by dissolving 6.7 mg of Asp, 7.3 mg of Glu, 4.6 mg Gly, and 5.6 mg GABA into 100 mL of 0.06 M perchloric acid buffer. Serial dilutions were made from the stock solution to yield a working standard of 100 pmole.

### *Derivatization*

For the derivatization, a 10 mM naphthalene-2,3-dicarboxaldehyde (NDA) solution was prepared by dissolving 3.6 mg of NDA into 2 mL of 100% methanol. Potassium cyanide (12 mM) and sodium borate (8 mM, pH 9.5) stock solutions were prepared in double-deionized water (9-12). Planarian filtrate (50  $\mu$ L for the actual sample) of a standard solution (50  $\mu$ L) containing Glu, Asp, GABA, and Gly was reacted with 100  $\mu$ L of sodium borate, 100  $\mu$ L of potassium cyanide, and 40  $\mu$ L of NDA. 0.1 M of NaOH was added to improve peak resolution. The mixture was allowed to react for 10 min and then vortexed for 1 min (10). The ratio of components in the mixture was based on previous work, which measured amino acid levels in microdialysis samples from rat brains (11,12). Reacting primary amines with NDA in the presence of cyanide ion produces a 1-cyano-(f)-isoindole derivative that is highly fluorescent (9,10). 1-Cyano-(f)-isoindole derivatives exhibit minimal time-dependent degradation as compared to o-phthalaldehyde derivatives (9). In contrast to our microdialysis studies, where the low sample volume was the limiting factor for HPLC analysis, the planarian filtrates yielded volumes of at least 100  $\mu$ L, allowing a larger volume of each component to be used, while maintaining the original ratios (11,12).

### *Measurement*

Following derivatization, glutamate and aspartate were separated using a 5  $\mu$ m C<sub>18</sub> reversed-phase column (150  $\times$  4.6 mm; Phenomenex Inc., Torrance, CA, USA). The HPLC-detector system consisted of a vacuum degasser (model #G1322A), quaternary pump (model #G1311A), autosampler (adjustable sample loop, 1-100  $\mu$ L) (model #G1313A) and fluorometric detector (model #G1321A) purchased from Agilent Technologies (Wilmington, DE, USA). The mobile phase consisted of a 5 mM sodium citrate buffer (pH 7.5) used in a linear gradient with elution with 30% methanol. The flow rate was set at 0.75 mL/min. The fluorescence detector wavelength settings were 440 nm for excitation and 480 for emission. The sample injection volume was 50  $\mu$ L.

Chromatographic peaks were identified based on their retention times by comparison to working standard solutions. The amino acid concentrations (pmole) in planarian filtrates were quantified with HPCHEMSTATION software (Agilent) based on peak area by comparison with an external standard calibration curve. Fluorometric data were then converted to pmole of amino acid per mg of planarian (pmole/mg planarian).

### Results

Consistent with prior studies from our laboratory and from others (9-13), a reaction time of at least 5 minutes was required for the derivatization mixture to yield consistent peak areas. We therefore used a reaction time of 10 min for all subsequent work.

GABA and Gly were easily separated in standard solutions. The mean retention time ( $\pm$  S.D.) of Gly was only  $2.4 \pm 0.1$  min; that of GABA was  $26.8 \pm 0.1$  min. The mean  $\pm$  S.D. retention times of GABA, Gly, Glu, and Asp peaks from planarian extractions were  $26.6 \pm 0.1$ ,  $2.4 \pm 0.2$ ,  $4.2 \pm 0.2$ , and  $4.9 \pm 0.1$  min, respectively. The close agreement of the extract peaks with the standard samples is demonstrated in **Table 1**.

**Table 1**

Peak retention times (means  $\pm$  S.D. min) of glycine, glutamate, aspartate, and GABA in working standards and from homogenates of planarians.

<i>Amino acid</i>	<i>Standard</i>	<i>Homogenate</i>
glycine	$2.4 \pm 0.1$	$2.4 \pm 0.2$
glutamate	$4.3 \pm 0.1$	$4.2 \pm 0.2$
aspartate	$5.0 \pm 0.1$	$4.9 \pm 0.1$
GABA	$26.8 \pm 0.1$	$26.6 \pm 0.1$

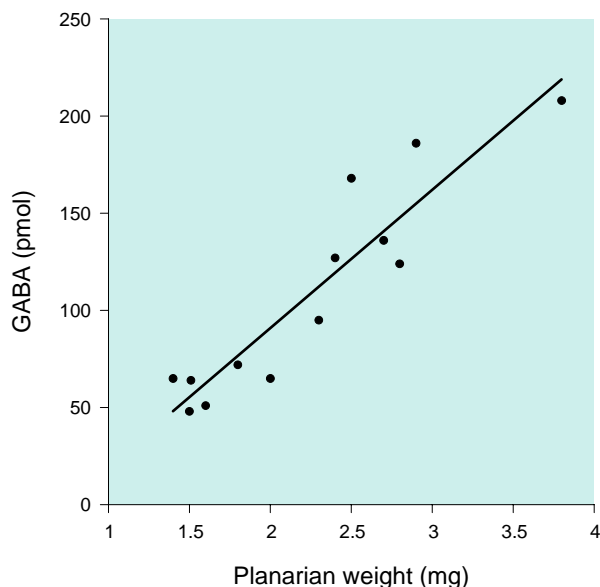
N = 6 each group.

The mean weight  $\pm$  S.E.M. of the 13 planarians was  $2.2 \pm 0.2$  mg (range = 1.4 – 3.8 mg). **Fig. 1** shows the detected amount of GABA plotted against individual planarian weight and the linear fit of the data ( $r = 0.93$ ). The mean  $\pm$  S.E.M. amount of GABA was  $108.4 \pm 15.0$  pmole ( $46.2 \pm 3.2$  pmole/mg planarian). A Gly peak was detected from planarian extracts, but the amount of Gly present in planarians was small, amounting to only about  $1/10^{\text{th}}$  the amount of GABA. It should be noted that because the amino acids in the planarian filtrate are mixed with NDA in order to form their fluorescent CBI derivatives, the values should technically be designated CBI-GABA, CBI-Gly, CBI-Glu, and CBI-Asp. Therefore, based on these and previous data (14), the rank order of amino acids in planarians is Glu > Asp > GABA > Gly.

### Discussion

We are interested in the use of planaria, a type of flatworm having cephalic ganglia and peripheral nerve cords (15), as a model for the correlation of biochemical and behavioral changes (16-25), in particular, the withdrawal following exposure to cocaine and other drugs (5,26-32).

We previously reported the measurement of the excitatory amino acids (Asp and Glu) in planarians (14) and now report the detection and measurement of the excitatory and inhibitory (GABA and Gly) amino acid levels in individual planarians.



**Fig. 1.** Amount of GABA (pmol) in individual planarians (N = 13).

GABA is the major inhibitory neurotransmitter within mammalian brain. It is present at about 10  $\mu\text{mol/g}$  tissue in the nigrostriatal system and at lower concentrations throughout the grey matter (33). In mammals, GABA is formed from glutamate by the action of glutamic acid decarboxylase, an enzyme found only in GABA-synthesizing neurons within the brain, and is metabolized by a transamination reaction (catalyzed by GABA-transaminase (GABA-T)), in which the amino group is transferred to alpha-oxoglutaric acid to yield glutamate and succinic semialdehyde, which then is oxidized to succinic acid. In addition, GABAergic neurons have an active re-uptake system that removes GABA after it has been released. GABA-receptors, particularly the GABA<sub>A</sub> subtype, are the direct or indirect target for several important centrally acting drugs, like benzodiazepines, barbiturates, ethanol, and neurosteroids.

Gly is present in high concentration (approximately 5  $\mu\text{mol/g}$ ) in the grey matter of mammalian spinal cord. The Gly receptor resembles the GABA<sub>A</sub>-receptor in being a multimeric ligand-gated chloride ion channel and several subtypes have been identified. Mutations of the receptor have been associated with neurological disorders involving muscle spasm and hyperexcitability (34).

The functional significance, beyond being constituents of protein, of endogenous amino acids in invertebrates is not known (3,35-37). In mammalian brain the pathway for GABA formation is *via* conversion of Glu to GABA, a reaction enzymatically catalyzed by glutamic acid decarboxylase (GAD) (38). Planarians synthesize GABA by glutamate decarboxylation (39,40), so Glu might also be a precursor to GABA in planarians.

Further information regarding the enzymes and the biochemical pathways that mediate the interactions between Glu and GABA systems in planarians is needed.

Ultimately, we would like to correlate behavioral changes in planarians to changes in amino acid levels. The results reported here represent only a preliminary first step. The present methodology measures the amount of GABA and Gly in whole bodies of planarians. Gly is essential for protein biosynthesis and free amino acids are distributed in the whole body. As a next step, the amounts of GABA and Gly should be measured in more discrete portions of planarians, such as the cephalic ganglia.

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