**Review#1**
**Comments and Suggestions for Authors**

In the manuscript “A System for Assessing Dual Action Modulators of Glycine Transporters and Glycine Receptors” by Sheipouri et al., that was submitted for publication to biomolecules, the authors describe a novel xenopus laevis based coexpression system that allows to analyse the functional interactions of glycine receptors and transporters. The authors demonstrate that upon coexpression of each of the GlyTs together with glycine receptors can limit the local availablility of glycine at the receptor especially when low or intermediate glycine concentrations are applied, and this effect was dependent on the activity of the respective transporter and their expression level. They subsequently used this system to monitor the effects of different glycine/lipid conjugates that have been previously characterized by this group to act specifically on the GlyT2, GlyRs or both. Here they could show, that substances that inhibit GlyT2 and in addition work as a positive allosteric modulator of the GlyR shows strongest effects on the total glycine induced current in this co-expression system.

In general, the study is well conceived, and the experiments support the conclusions reached by the authors. The main findings, how (and at which concentration range) the GlyTs are able to contribute to the regulation of glycinergic currents, are novel and interesting for a broader readership, therefore justifying publication in Biomolecules. There are, however, some points that require clarification before publication.

Specific points:

Stop/flow experiments: The authors demonstrate that in oocytes coexpressing a GlyT1 and GlyR, a significant reduction in the observed glycine induced current occurs after stopping of the superfusion solution. Does this also occur in oocytes only expressing a GlyT?

Furthermore the authors claim, but do not show that the currents observed in their stop flow experiments are almost exclusively mediated by the GlyRs. The data in Fig. S2 however suggest that especially at low concentration (i.e. 10 µM, which is used for some of the experiments 50 % of the current for GlyT1/GlyR coexpressing oocytes were mediated by GlyT1, whereas still 20 % of the current was transporter carried in GlyT2/GlyR expressing oocytes). So, are the currents seen e.g. in Fig. 1 GlyR, GlyT or mixed currents?

- To address both these points, we have updated Figure S2 to include example traces showing the stop-flow reduction of currents in cells expressing GlyT1 and GlyT2 only. Figure S2 and its legend has also been updated to show the percentage of both fast-flow and stop-flow current attributed to GlyTs. Lines 208 – 222 in the main manuscript have been updated to describe Figure S2 and state that Figure 1 shows traces with mixed GlyR and GlyT currents.

How do the authors interprete the difference in the contribution of the individual transporters to the total current? Is this due to differences in expression level of a results e.g. from differences in the transport kinetics?

- The rate at which glycine equilibrium is achieved is dependent on both the number of available transporters and the kinetics (turnover).

- As shown in Figure 3 and Figure S4, different expression levels of GlyTs will change the contribution to the total current. Although the amount of GlyT RNA injected into oocytes was the same for comparable experiments, differences in protein expression levels between GlyT1 and GlyT2 may account for the difference in contribution to total current seen in Figure S2.

- GlyT1 and GlyT2 also differ in their kinetic profiles (turnover ~ 21 $s^{-1}$ for GlyT1 and ~30 $s^{-1}$ for GlyT2) and this is also likely to contribute to these differences.

Figure 3: Here, although I understood the general finding, I found the figure rather confusing:

The authors claim that the more GlyT is present, the stronger the reduction in the current observed after stopping of the superfusion. In A and D: Why is the ratio between the Istop/Iflow is close to 0 at the beginning of the experiment? As far as I understood, the current is expected to be maximal at this point and should decrease from there on? In B and E the ratio of the Istop/Iflow is depicted. This should be, following the authors description be around 1 for oocytes expressing only GlyR, but it is close to 0… so what is actually depicted in this graph?

- The axes in Figure 3 were incorrectly labelled. They have now been corrected to reflect the greatest current when the flow is stopped on cells expressing only GlyR and the least current when the flow is stopped on cells expressing a greater number of GlyTs.

Figure 5: How do the authors interprete the apparent facilitation of the glycine induced current under the stopped flow conditions? Is this really elicited by glycine released be the transporter? Is fo can this be blocked by GlyT1 inhibitors? Why is this not seen in GlyT2 expressing oocytes, since the major driving force is Na+, I would expect the observed effect to be even stronger?

- We interpret facilitation of glycine currents when the flow is stopped on cells expressing $GlyR\alpha_1$/GlyT1 in 1mM $Na^+$ as reverse transport of glycine out of the cell by GlyT1. This is not observed under the same conditions in $GlyR\alpha_1$/GlyT2 expressing cells. This could be due to different rates of reverse transport observed for the different transporters. This explanation has been added to the Results text at lines 344 – 347.

- Furthermore, we address the reviewer's suggestion of showing this experimentally by blocking GlyT1 transport with an inhibitor. Figure S5 has been added to the supplementary materials showing the results of this experiment and these results are described in lines 337-339.

General: The glycine affinity the authors determine for essentially all GlyR subunits is very high (in the area of roughly 10 µM)! on most studiy using a comparable oocyte expression system much lower affinities ( in the range of 100-300 µM) were described. The authors should at least discuss possible reasons for these differences.

- A study by De Saint Jan (2001), shows that glycine $EC_{50}$ at $GlyR\alpha_1$ is highly variable, from 20-280 µM. They find that in cells with low maximal currents (~200 nA), the $EC_{50}$ is greatest. In cells with the greatest maximal currents (~ 10 000 nA) the $EC_{50}$ values are the smallest. Our maximal currents are consistently in the range of 1000's of nA, which could explain why our $EC_{50}$s are also so low. We routinely record from oocytes 3 days after mRNA injection in order to get consistent expression levels.

- This explanation has been added to the Discussion at lines 653 – 660.

The solution exchange system the authors are using is very slow. This has of course a least some impact both on the overall shape of the observed current traces. Additionally, however, slow developing changes in the local concentrations of the ions, especially chloride, cannot be excluded. This might affect both the observed overall current and the activity of the GlyTs. Can the authors at least comment on this possibility?

Choride concentrations are unlikely to change significantly under these conditions. We have not systematically altered the chloride concentration, but with ~100mM Cl- in the external solution it will not be depleted to an extent that is measurable in this system. The glycine concentration where depletion was most readily observed was 10-100 µM (3-4 orders of magnitude lower).

The authors describe nicely that in the coexpression system a marked change in the Hill coefficient was observed. If the only effect of the coexpressed GlyT is as the authors suggest a modulation of the local glycine concentration such a change in the Hill coefficient is not to be expected. So, how do the authors explain this effect?

- The change in Hill co-efficient is expected because the reduction in glycine concentrations at the membrane generated by GlyTs is most marked in the range of 1 – 30 μM, skewing the dose response curve in a non-uniform way and therefore creating an apparent change in Hill co-efficient. This is further explained in lines 471 – 474 and Figure 9.