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Glycine is enriched in a neurochemically distinct subset of laminae I-II inhibitory interneurons in the spinal cord of rodents

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Glycine enrichment in a selective subset of laminae I-II inhibitory interneurons

Spinal cord interneurons modulate afferent information transmitted from peripheral receptors. Loss of these modulating interneurons may be involved in the pathophysiology of many diseases. However lack of knowledge about the functionally homogeneous populations from the present heterogeneous groups of interneurons is hindering advancement of possible therapeutic approaches. Immunocytochemistry was used to identify galanin (Gal) and neuronal nitric oxide synthase (nNOS) containing inhibitory interneurons. Gal and nNOS inhibitory interneurons are a known neurochemically distinct population of neurons in laminae I and II of the dorsal horn in rodents. Three different animal strains C57Bl/6 (C57) mice, Sprague Dawley rats and PrP-GFP mice were used. Our aim was to clarify if these neurons are glycine (Gly) enriched and if the three animal strains used have identical distribution of glycine enrichment in nNOS and Gal cells. We report significant differences between the animal strains. A large proportion of Gal-immunoreactive neurons in the C57 mouse (65%) but a low proportion of nNOS cells (28%) are glycine enriched and colocalization of nNOS and Gal occurred in mainly glycine enriched cells. In the rat an inverse relationship can be observed with Gal and nNOS cells being (23%) and (80%) glycine enriched respectively and Gal and nNOS never colocalizing. Furthermore we postulate that neurons expressing GFP may functionally differ from those in which it is not based on significantly higher percentage of glycine enriched neurons in the PrP-GFP mouse in both Gal (78%) and nNOS (78%) neurons than in the C57 mouse.

Contents

1. Acknowledgments

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2. Introduction

The spinal cord was classically regarded as a relay station for signals to be forwarded to higher processing centres in the brain. In recent decades it has however become abundantly clear that spinal cord circuitry plays a fundamental role in modulating afferent information (Melzack and Wall, 1965). C-fibres and Aδ afferents terminating in Rexed laminae I-II and the interneurons that modulate their signalling have been of particular focus. This is partly due to the fact that the complex spinal cord circuitry present in these laminae has been implicated in various pain pathologies (Todd, 2010; Sivilotti and Woolf, 1994). This study focuses on a particular subgroup of inhibitory interneurons in the rat and mouse spinal cord contributing to the rapidly growing literature that attempts to unravel spinal cord interneuron circuitry.

To better understand inhibitory interneurons we must be able to define physiologically homogenous populations from the present heterogeneous group of spinal cord interneurons. Various attempts at this have been made. Interneurons have varying morphology which allows distinction of functional classes depending on shape. A recent study by Yasaka, et al. (2010) concluded that classifying neurons based on morphology allowed distinction of various subgroups some of which have distinct functions. However while allowing some subdivision of distinct populations, there was a substantial population of cells that could not be classified based on morphology, typically about 30% of cells sampled (Todd, 2010).

Furthermore the question has been raised whether these cells actually have specific physiological functions (Todd, 2010). Previous studies in our lab have had success in distinguishing inhibitory interneurons using Immunocytochemistry (ICC) (Todd, 2010), as well as suggesting functional subpopulations (Polgar, et al., 2013). The somatostatin subtype 2A receptor (SST_{2A}) has proven very useful being only expressed on a subset of inhibitory interneurons in laminae I-II labelling around 54% of all inhibitory interneurons. (Iwagaki, et al., 2013). The SST_{2A} positive (SST_{2A}^{+}) neurons can be subdivided further into populations of cells expressing neuronal nitric oxide synthase (nNOS) and/or galanin (Gal) and cells containing neither (figure 1). nNOS positive ($nNOS^{+}$) and Gal positive (Gal^{+}) containing interneurons were of particular interest as various sources of evidence have implicated them in inhibition of itch perception. Evidence for this comes from the fact that nNOS and Gal but not other inhibitory interneurons depend on the Bhlhb5 transcription factor for development. Bhlhb5 Knockout mice are characterized by stereotyped scratching motion. This is hypothesized to be due to the absence of nNOS and Gal-immunoreactive interneurons which form inhibitory synapses either on pruriceptive projection neurons or on excitatory interneurons (Todd, unpublished observations; Ross et al., 2010).

A recently developed transgenic mouse line has been shown to label a subset of the Gal and nNOS population and the question was raised whether this was of functional relevance. The PrP-GFP mouse was derived from the Swiss Webster mouse line and expresses green fluorescent protein (GFP) in Laminae I-II the factors driving GFP expression are not yet fully understood (Hantman and Perl, 2005). However when the expression pattern of GFP in the spinal cord was analyzed it was found that it labels inhibitory nNOS and Gal cells with great exclusivity (Iwagaki, et al., 2013) (figure 1).

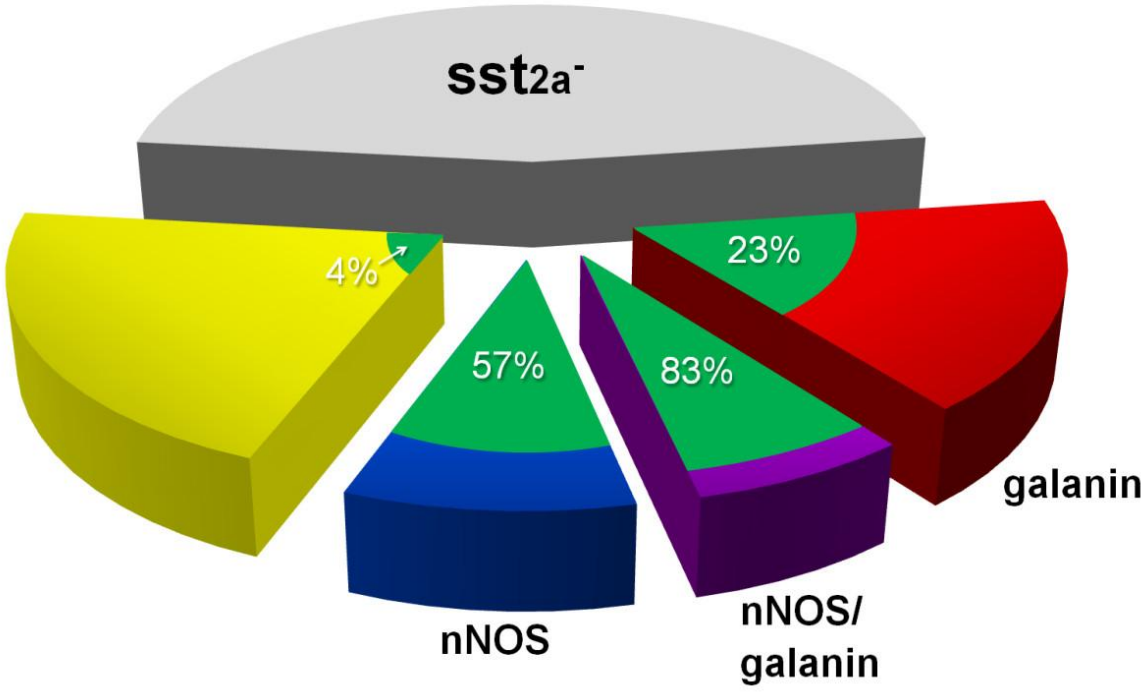

Figure 1. Gal and nNOS expression pattern among SST_{2A}^{+} neurons and their pattern of colocalization with GFP in a subset of these neurons in the dorsal horn of the PrP-GFP mouse. The pie chart shows distribution of types of inhibitory interneurons in laminae I-II. Relative percentages of these inhibitory interneuron populations were estimated in the C57 mouse in which around 54% of inhibitory interneurons are SST_{2A}-immunoreactive. The SST_{2A}^{+} neurons can be subdivided further into nNOS, Gal or nNOS/Gal double labelled cells, these neuronal populations make up 17%, 31%, and 13% respectively. GFP as shown by the green shading is found exclusively in SST_{2A}^{+} cells and is expressed mainly in cells containing Gal and/or nNOS. (Iwagaki, et al., 2013)

The primary neurotransmitters used by $nNOS^{+}$ and Gal^{+} neurons are known in the rat (Spike, Todd and Johnston, 1993; Simmons, Spike and Todd, 1995; Polgar, et al., 2013). However it was unclear whether there were interspecies differences and the same pattern of neurotransmitter expression can be seen in the C57 and PrP-GFP mouse strains. It is known that nearly all inhibitory interneurons in laminae I-II of the dorsal horn contain γ - Aminobutyric acid (GABA) (Polgar, et al., 2013; Keller, et al., 2001) .We therefore looked at glycine the other principal inhibitory neurotransmitter in the spinal cord which nearly always co-localizes with GABA in laminae I-III. Glycine-immunoreactivity is known to differ amongst the SST_{2A}^{+} populations being enriched in some cells. Changes in the number of

glycine enriched neurons within the nNOS and Gal populations can indicate interspecies differences in how these cells function in the different animals.

We used immunocytochemistry (ICC) to label selective populations of inhibitory interneurons with 3 principal aims in mind.

1. Estimate the percentage of Gal and inhibitory nNOS-immunoreactive neurons, in the C57 mouse, Sprague Dawley rat and PrP-GFP mouse laminae I-II, that are glycine enriched and identify differences.
2. Confirm previous studies of inhibitory interneurons in the rat dorsal horn that found that Gal-immunoreactive interneurons contain GABA exclusively (Simmons, Spike and Todd 1995), while in nNOS-immunoreactive inhibitory interneurons there is sometimes colocalization of GABA and glycine (Spike, Todd and Johnston ,1993).
3. Identify whether GFP labelled cells in the PrP-GFP mouse line label a selective subset of Gal^+ and $nNOS^+$ cells, indicated by percentage changes of glycine enriched nNOS and Gal interneurons relative to the C57 mouse line.

While immunocytochemistry is extremely useful for identifying contents of a cell a cautioned approach to these results is urged. While all inhibitory interneurons contain GABA electrophysiological recordings have found synapses that appear to be purely glycinergic in nature (Yasaka, et al., 2007; Keller, et al., 2001). Furthermore glycine has various metabolic roles and is found ubiquitously in all cells. While glycine enrichment may indicate that the neuron is glycinergic, neurons with high metabolic glycine may signal using GABA exclusively (Polgar, et al., 2013). Therefore electrophysiological studies are needed to confirm the results of ICC.

3. Method

3.1. Ethical statement

All experiments were approved by the Ethical Review Process Applications Panel of the University of Glasgow and were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986.

3.2. Animal preparation and tissue processing

Six animals were used in this experiment; two C57 male mice weighing 15-21g, two PrP-GFP female mice weighing 16-23g and two male Sprague Dawley (Harlan) rats weighing 270-290g. The animals were deeply anaesthetized using pentobarbital and transcardially perfused with 4% formaldehyde and 15% picric acid through the left ventricle. The L5 segment of the mice was removed after perfusion and cut into 60 micron thick transverse sections using a vibratome. The same procedure was repeated with the L4 segments from the two rats. The sections were immersed in 50% ethanol for 30 minutes and rinsed repeatedly using phosphate buffered saline (PBS). Primary antibody incubations lasted for 3 days and secondary antibody incubations were overnight (both at 4°C).

3.3. nNOS, Glycine, SST_{2A} and Gal immunoreactivity in the C57Bl6 mice, Sprague Dawley rat and PRP-GFP mouse

Four primary antibodies were applied to the C57 mice and rat sections. The antibodies labelled cells containing glycine (dilution: 1:500; species in which antibody was raised: rat; source: ImmunoSolutions), nNOS (1:2000; guinea pig; PC Emson), Gal (1:1000; rabbit; BaChem), and SST_{2A} (1:2000; rabbit; Gramsch Laboratories). The secondary antibodies had a dilution factor of 1:500 and were conjugated to Rhodamine Red, DyLight 649,

(Jackson Immunoresearch), Pacific Blue and Alexa 488 (Molecular probes). All secondary antibodies originated in donkey and showed species specificity. All sections were mounted in anti fade medium and stored at -20°C. All dilutions were carried out in 5% Normal Donkey Serum in Phosphate buffer for both the primary and secondary antibody.

A slightly different procedure was applied to the PrP-GFP mouse as it is known that all PrP-GFP cells are inhibitory and SST_{2A}^{+} (Iwagaki, et al., 2013; Polgar, et al., 2013). Therefore no SST_{2A} antibody was applied. Single salt phosphate buffer instead of PBS was used in the second PrP-GFP mouse which prevented lysis of GFP containing cells but reduced Gal staining intensity resulting in a lower signal to noise ratio.

Sections were scanned on a Zeiss LSM710 confocal microscope at 40X resolution in oil immersion scanning overlapping images of laminae I-II at 1µm z-seperation. Sections were selected and scanned before quantitative analysis of immunofluorescence took place. Four dorsal horn images were taken from each C57 mouse and each Sprague Dawley rat. 7 dorsal horn sections were scanned from the first PrP-GFP mouse and 6 sections from the second animal. The resulting images were analysed on Neurolucida for confocal software (MBF Bioscience).

The outline of the grey matter and the lamina II/III border was marked on all images based on the ventral border of SST_{2A}-immunoreactive dendrites (Iwagaki, et al., 2013) or determined from known proportional distance of the border (Molander, Xu and Grant, 1984). Cells of the C57 mice and Sprague Dawley rats were identified and marked depending on whether they contained nNOS or GAL or both. Furthermore $nNOS^{+}$ and Gal^{+} interneurons were identified as glycine enriched or glycine negative. Only the top 8µm of the sections were used as glycine staining is unreliable deeper into the section. The SST_{2A} staining assisted in the

identification of Gal cells and allowed distinction between inhibitory and excitatory nNOS cells (Todd, Spike and Polgar, 1998).

In the PrP-GFP mouse cells were identified first as either GFP positive ($GFP^{+)}$ or negative (GFP^{-}), the GFP^{+} cells were then examined for nNOS and Gal-immunoreactivity and finally marked as glycine enriched or negative thus only GFP^{+} cells were included at first. Later on galanin cells which were GFP^{-} were also identified and analysed for glycine enrichment.

3.4. Antibody characterization

All antibodies showed specific binding. The galanin antibody did not bind in Gal KO mice (Makwana, et al., 2010). The nNOS antibody had 155kDa band in a western blot and staining was abolished by pre-incubation with nNOS (Herbison, et al., 1996). SST_{2A} staining was abolished by pre-incubation with the peptide (Polgar, et al., 2013). The glycine antibody was highly specific in dot blot tests (Pow and Crook, 1993).

3.5. Statistical analysis

Statistical calculations were performed using Microsoft Excel 2007. Differences between nNOS and Gal cells were detected using two-sample t-tests. Interspecies differences were analysed using one way ANOVA. Differences were assumed to be significant if $p<0.05$. Two-sample t-tests with Bonferroni corrections were used to test for differences between individual animal strains, significance was reduced to $p<0.0167$. (Schaffer, 1995; Weisstein, 2014).

4. Results

4.1. Immunoreactivity of inhibitory nNOS and Gal neurons in laminae I-II of the Sprague Dawley rat

A total of 153 SST_{2A}^{+} interneurons contained either nNOS or Gal out of a total of 8 dorsal horn sections with a range of 72-81 cells per animal. Out of 77 Gal^{+} cells 18 were glycine enriched making up 23.4% of the population. Out of 76 nNOS-immunoreactive neurons 61 were glycine enriched, making up 80.3% of the population (figure 5a). There is a significant difference ($p<0.05$, two sample t-test) between Gal and nNOS populations with Gal^{+} cells being significantly less glycine enriched than $nNOS^{+}$ populations in the rat. There were no cells that contained both nNOS and Gal (figure 2.)

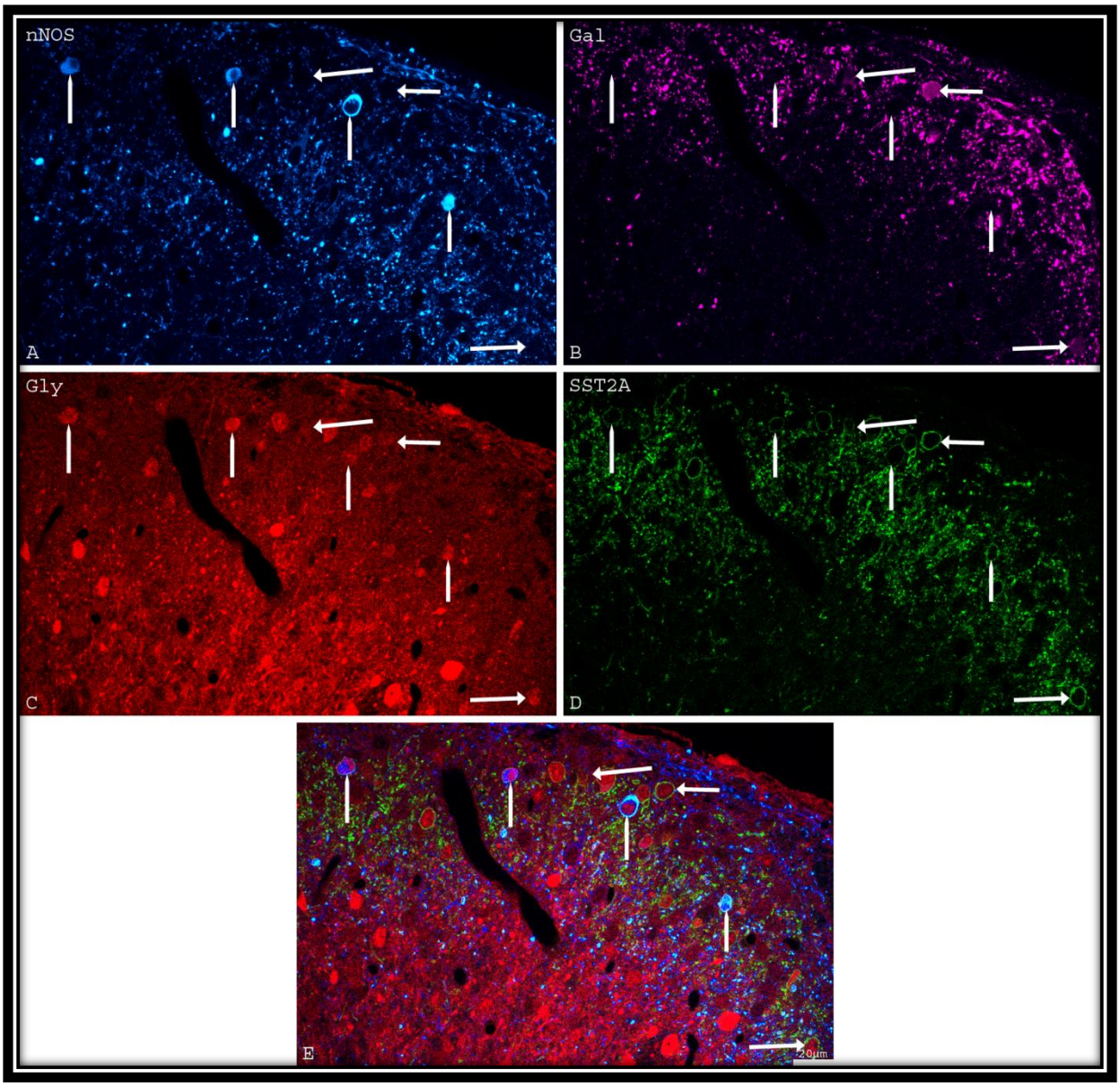

Figure 2. nNOS, Gal, Gly and SST_{2A} in the dorsal horn of a Sprague-Dawley rat.

A single 1µm confocal scan of a section that had been reacted to reveal nNOS (panel A), Gal (panel B), glycine (panel C), SST_{2A} (Panel D) antibodies. In panel A four nNOS-immunoreactive cells are visible indicated by arrows without arrow head. In panel B three Galanin cells are visible indicated by arrows with arrowhead. In panel C it can be seen that 3 of the nNOS cells are glycine enriched and only one Gal cell is glycine enriched. Panel D confirms that both Gal and nNOS cells are SST_{2A}^{+}.

4.2. Immunoreactivity of inhibitory interneurons in laminae I-II of the C57 mouse

A total number of 119 Gal and/or nNOS-immunoreactive cells were counted in a total of 8 dorsal horn sections with 48-71 cells per animal. Out of 67 Gal^{+} neurons 44 were found to be

glycine enriched making up 65.7% of the population. Out of 43 nNOS cells 12 were glycine enriched making up 27.9% of the population (figure 5a). There was a large variation found between the two compared C57 mice with a range of 22.2-37.5% of the $nNOS^+$ neurons being glycine enriched. There is a significant difference between the number of nNOS cells that are glycine enriched and the number of Gal cells that are glycine enriched ($p<0.05$, two sample t-test). In the mouse colocalization of Gal and nNOS has also been found in 9 cells out of these 7 were glycine enriched (77.78%) (figure 3).

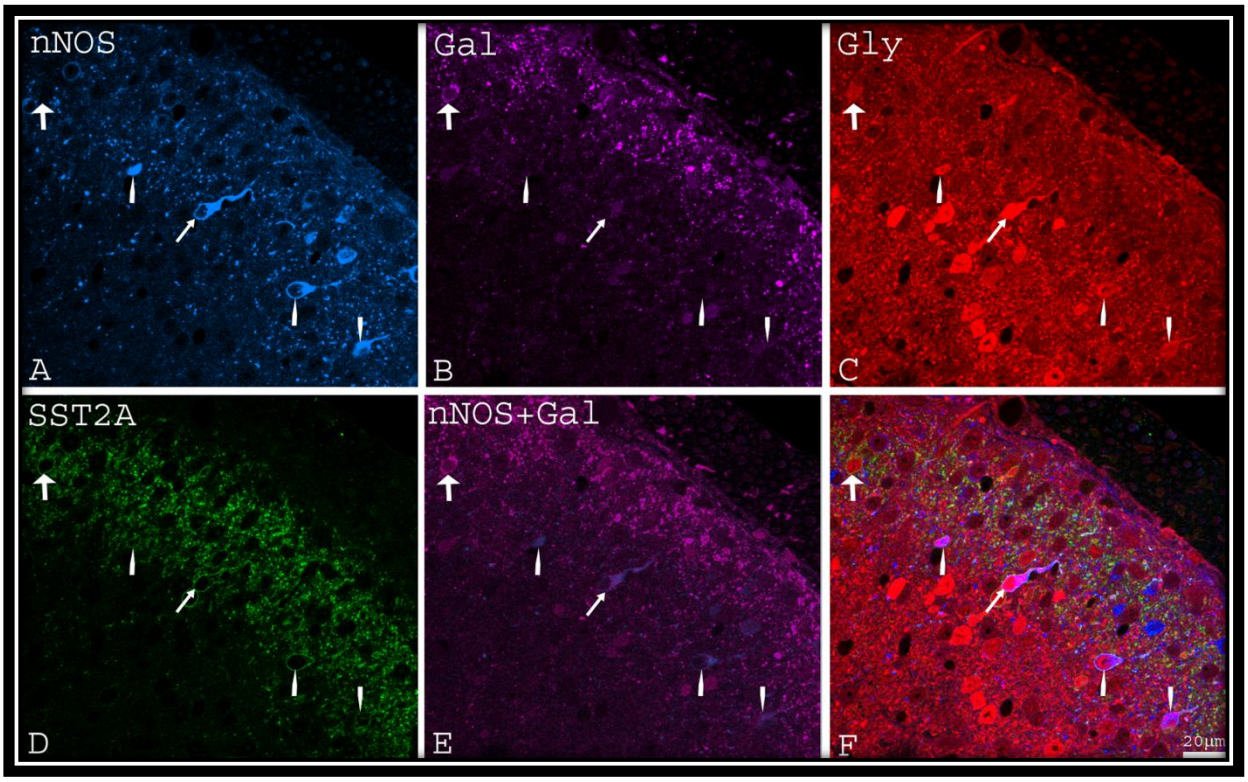

Figure 3. nNOS, Gal, Gly and SST_{2A} in the dorsal horn of a C57 mouse.

A single 1µm confocal scan of a section that has been reacted with nNOS (panel A), Gal (panel B), glycine (panel C), SST_{2A} (Panel D) and co-localization of Gal and nNOS (panel E). In panel A four inhibitory $nNOS^+$ neurons can be seen. In panel B two Gal^+ neurons can be seen. $nNOS^+$ neurons have been indicated with arrows without arrowhead. The Gal^+ cell has been indicated with a very broad arrowhead. Colocalization of Gal and nNOS as can be seen in one cell in Panel E is shown by a medium arrowhead. Panel D shows that all cells labelled were SST_{2A}^+. Lastly in panel C it can be seen that the double labelled cell was highly glycine enriched while only 1 nNOS cell is glycine enriched.

4.3. Immunoreactivity of inhibitory interneurons in laminae I-II of the PRP-GFP mouse

13 dorsal horn sections from 2 animals were analysed with a total of 149 GFP^+ cells. Out of these 58 cells (38.9%) contained Galanin, 67 cells (45.0%) contained nNOS and 17 cells (12.1%) contained both. Finally a small group of GFP^+ cells did not colocalize with either nNOS or Gal with 6 cells making up just about 4.0%. Surprisingly 77.59% of the Gal and 77.61% of the nNOS neurons were glycine enriched (figure 5a). 94.4% of the cells that colocalized Gal and nNOS were glycine enriched (fig. 4). Conversely 0 of the 6 cells that contained neither Gal nor nNOS were glycine enriched.

We also looked at galanin cells in GFP^- cells. In the 13 sections a total of 40 Galanin cells not containing GFP were found. Compared to the GFP^+/Gal^+ cells only a relatively small proportion of 12 cells (30%) of these were glycine enriched. (fig. 5b)

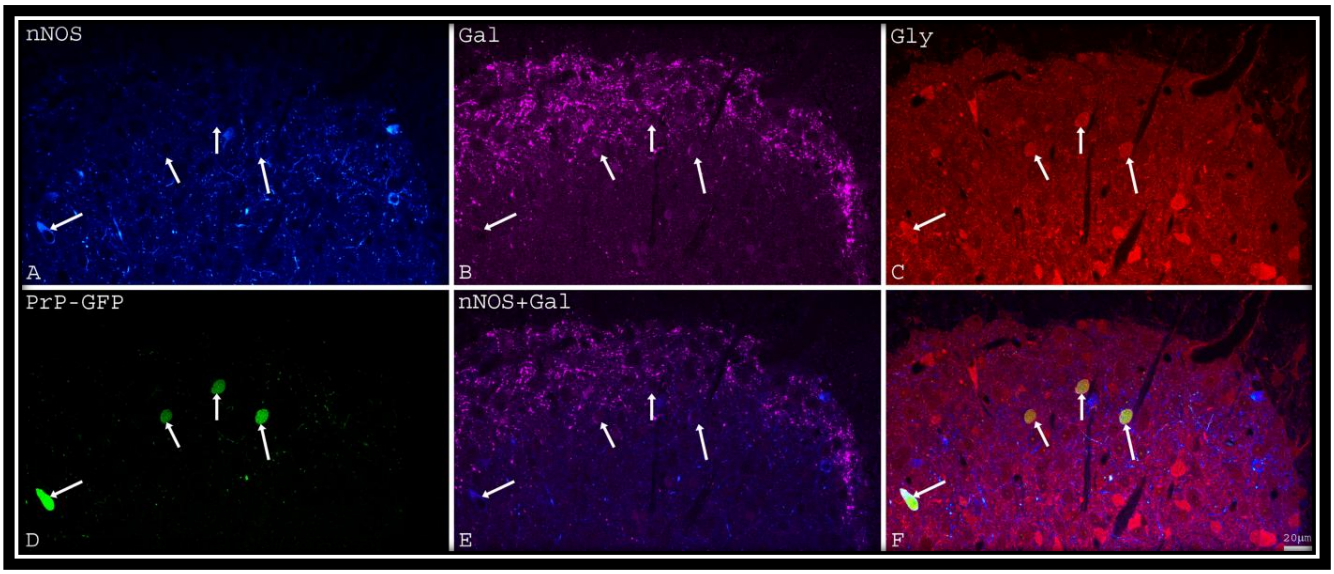

Figure 4. nNOS, Gal, Gly and PrP-GFP in the dorsal horn of a PrP-GFP mouse.

A single 1µm confocal scan of a section that has been reacted with nNOS (panel A), Gal (panel B), glycine (panel C), PrP-GFP (Panel D) and co-localization of Gal and nNOS (panel E). In panel D PrP-GFP cells are indicated by arrows. Panel A and B show GFP cells contain either nNOS and/or Gal. Panel E indicates colocalization of Gal and nNOS in 3 PrP-GFP cells. Panel C shows that all the GFP^+ cells in this image are glycine enriched.

4.4. Variation in glycine enrichment of Gal/nNOS cells

We found that there were significant differences in the percentage of glycine enriched Gal and nNOS cells of the three animals ($p<0.05$, one-way ANOVA). Both F values were greater than the critical f value (f=23.18, f-critical=3.36.) (f=32.29, f-critical=3.12) for Gal^+ and $nNOS^+$ interneurons respectively. Analysis of individual sets revealed that there were significant differences between percentage of glycine enriched cells for both Gal^+ and $nNOS^+$ populations in the C57 mouse and Sprague Dawley rat ($p<0.0167$, two-sample t-test with Bonferroni correction). The Sprague Dawley rat and the PrP-GFP mouse Gal^+ cells were nearly significantly different ($p=0.0169$, two-sample t-test with Bonferroni correction) but $nNOS^+$ cells were not significantly different ($p\approx0.62$, two-sample t-test with Bonferroni correction). There was a significant difference between the percentage of both $nNOS^+$ and

Gal^+ glycine enriched cells in the GFP^+ cells of the PrP-GFP mouse and C57 mouse ($p<0.0167$, two-sample t-test with Bonferroni correction). If however Gal^+ population regardless of colocalization with GFP were analyzed there was no significant difference in percentage of glycine enriched cells between the C57 and PrP-GFP mice ($p \approx 0.60$, two sample t-test.) Differences in GFP negative nNOS cells in respect to glycine content were not tested as we were unable to distinguish excitatory from inhibitory nNOS cells in the PrP-GFP mouse line.

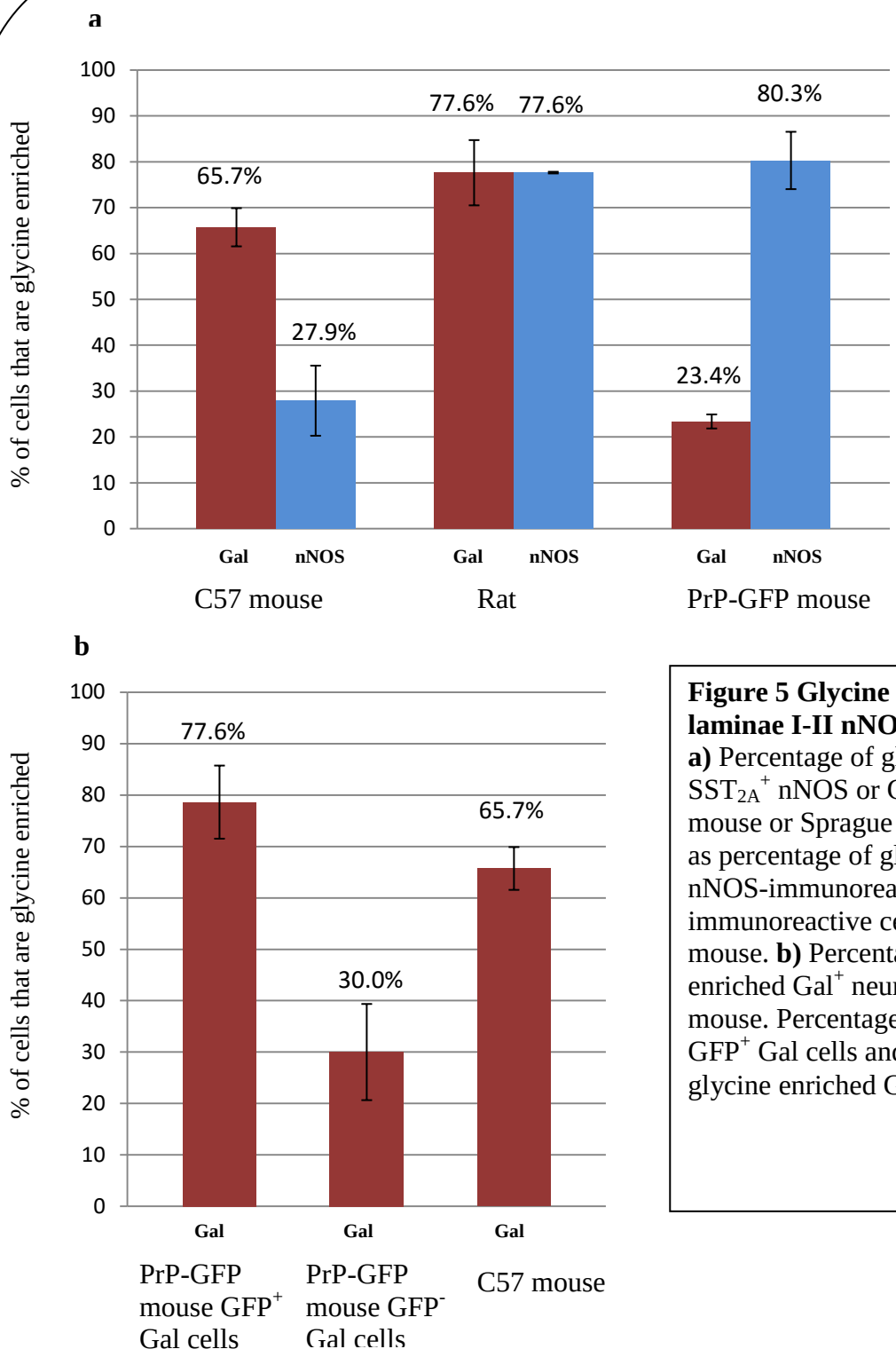

Figure 5 Glycine enrichment in laminae I-II nNOS$^+$ or Gal$^+$ neurons. **a)** Percentage of glycine enriched SST_{2A}^+ nNOS or Gal cells in the C57 mouse or Sprague Dawley rat. As well as percentage of glycine enriched GFP$^+$ nNOS-immunoreactive or GFP$^+$ Gal-immunoreactive cells in the PrP-GFP mouse. **b)** Percentage of glycine enriched Gal$^+$ neurons in the C57 mouse. Percentage of glycine enriched GFP$^+$ Gal cells and percentage of glycine enriched GFP$^-$ Gal cells.

5. Discussion

The main findings of this study are that: (1) There are significant differences between the different animals used in respect to glycine enrichment in Gal and nNOS populations. (2) Some Gal^+ cells are glycine enriched in the rat contradicting previous findings that glycine is never enriched in Gal^+ neurons (Simmons, Spike and Todd, 1995). (3) The cells that express GFP in the PrP-GFP mouse label a specific subset of $nNOS^+$ and Gal^+ cells that are often glycine enriched.

5.1. Variation in glycine enrichment of Gal/nNOS cells

There is a significant difference in percentage of glycine enriched nNOS-immunoreactive neurons and Gal-immunoreactive neurons between the rat and the mouse (Figure 5a). There was a decrease in the percentage of glycine enriched $nNOS^+$ neurons in the C57 mouse compared to the Sprague Dawley rat (27.9% and 80.3% respectively). However there was an increase in percentage of glycine enriched Gal^+ cells in the mouse compared to the rat (65.7% and 23.4% respectively). When looking for significant differences the Bonferroni correction was applied to avoid type I errors, however this technique has been criticised for increasing type II errors. This was not a problem in our study as most of the interspecies differences analyzed were highly significant and a relatively small number of t-tests were performed. Whether the observed change in glycine enrichment is relevant for physiological functioning of these cells is not known. However it has been shown that $nNOS^+$ interneurons and Gal^+ interneurons innervate different targets and respond to slightly different stimuli. Inhibitory nNOS cells target giant lamina I projection neurons which do not receive input from Gal^+ cells (Todd, 2010). $nNOS^+$ cells are thought to originate from Gal^+ cells which at some point undergo a phenotypic switch and start expressing nNOS (Todd, unpublished observations). Therefore the variation in the level of glycine enrichment observed between these two

populations of cells in the two animals could be due to changes in metabolic need for glycine to perform their respective functions which differ slightly in mouse and rat. It could also be due to the Gal^+ cells being glycinergic in the mouse but not in the rat with the opposite being true for the $nNOS^+$ cells in the rat. Another point of interest is that Gal and nNOS colocalize in the mouse but not in the rat. It is not yet clear what function these neurons subserve or what target they innervate. It is clear that they were often glycine enriched (fig. 3, fig. 4). Further investigation of this neurochemically distinct group unique to the mouse is needed.

Knowing that there are interspecies differences between two closely related species raises the issue of whether results found in rodents will be translated to humans. A major problem in clinical research is that potential drug targets are identified and treated in animals however in clinical studies there is a major failure of drugs in phase II clinical trials. The drug is shown to be ineffective which may result due to differences between human and animal models (Arrowsmith, 2011). Therefore any results obtained using these animal strains should be treated cautiously and if possible confirmed in non-human primates or in humans.

5.2. Galanin cells contain glycine in the rat

In previous studies it has been indicated that nNOS inhibitory interneurons in the spinal cord always contain GABA but colocalize with glycine to a high degree (Spike, Todd and Johnston, 1993). Simmons, Spike and Todd, (1995) found that Gal^+ interneurons were exclusively GABAergic and were not glycine enriched. In our study we detected glycine enrichment in Gal-immunoreactive neurons. Various explanations to these contradictory results are plausible. In the study conducted by Simmons, Spike and Todd, (1995) a post embedding stain to detect glycine after having already detected Gal^+ neurons was used. No techniques such as colchicine were used to intensify Gal staining. A bias resulting from only identifying strongly labelled Gal cells in the semi-thin sections on which post embedding

reactions were done may have resulted in the inability to find any glycine enriched Gal^+ neurons. In our experiment we used an immunofluorescent approach having the advantage of being able to identify even weakly labelled Gal cells. However we did not use gluteraldehyde fixative which means that we had reduced glycine retention increasing the signal to noise ratio. Doubts over the primary antibody used to detect glycine in this study have also been raised. Perhaps there is some residual activity with GABA or possibly other amino acids. To ensure antibody specificity immunoprecipitation techniques to check for other possible ligands and non-specific binding should be performed.

5.3. Sampling and antibody critiques

It was known that the glycine antibody showed poor tissue penetration. A variety of pre-emptive measures were taken to avoid falsely labelling cells as not glycine enriched. Picric acid was added during perfusion to enhance antibody penetration. Furthermore only the surface of sections were scanned and analyzed with an average z-depth of 8µm. However no gluteraldehyde fixation could be used in our study due to the antibody used which results in washing out a large proportion of the amino acid transmitter (Pow, Wright and Vaney, 1995; Cotman, et al., 1987). This results in a larger signal to noise ratio which could result in falsely identifying cells as glycine enriched. To avoid observation bias the sections were not analyzed in detail for immunofluorescence before inclusion in the study. Furthermore in future studies SST_{2A} staining in the PrP-GFP mouse should be added as we were unable to identify inhibitory nNOS from excitatory nNOS interneurons if they did no co-localize with GFP. As previously stated glycine enrichment does not necessitate that the neuron is glycinergic. A much more reliable method is staining for glycine transporter 2 (GlyT2). However this does not stain the cell body making colocalization with other stains difficult. A possible solution to this problem would be utilizing a GlyT2-GFP mouse in which cells

expressing the GlyT2 transporter also express GFP in the cell body. It has been shown that in these mice GFP is correctly localized to glycinergic neurons (Zeilhofer, et al., 2005).

5.4. GFP marks a specific subset of nNOS and Gal neurons that are often glycine enriched in the PrP-GFP mouse

In the PrP-GFP mouse and the C57 mouse there was a significant difference between the SST_{2A}^{+}/Gal^{+} population and the GFP^{+}/Gal^{+} population in respect to glycine enrichment (65.7% and 77.6% respectively)(figure 5b). However if all galanin cells in the mouse strains were counted regardless of colocalization with GFP the percentage of glycine enriched Gal^{+} cells was not significantly different (65.7% C57 mouse and 63.8% PrP-GFP mouse). This suggests that the difference is caused by cells expressing GFP being more likely to be glycine enriched and not by a sub-species difference between C57 mice and PrP-GFP mice. nNOS cells in which there was a more drastic rise in glycine enrichment between the mouse strains (27.9% C57 mouse and 77.6% PrP-GFP mouse) most likely contain a significantly higher proportion of glycine enriched cells as well, however without SST_{2A} staining we cannot make definite conclusions as to whether the increase in glycine enrichment was caused by a sub-species difference or by an accumulation of glycine in nNOS cells colocalizing with GFP.

5.5. The role of glycine from Bhlhb5 derived inhibitory interneurons in itch

Various lines of evidence implicate Bhlhb5 derived inhibitory interneurons in suppression of itch perception. Primary evidence comes from the Bhlhb5 KO studies from Ross et al. (2010). Furthermore studies have shown that glycine antagonists (strychnine) are more effective than GABA A (bicuculine) or GABA B (phaclofen) antagonists at suppressing nociceptive mediated relief of itch (Akiyama, et al., 2010). While it is unclear how exactly strychnine inhibits relieve of itch in response to nociceptive stimuli we would like to suggest that it is

caused by inhibiting the action of glycine released by $nNOS^{+}/Gal^{+}$ interneurons on projection and/or excitatory interneurons responsible for pruriception. Furthermore Gal has been shown to co-localize with preprodynorphin (PPD) a precursor to the κ-opioid receptor agonist dynorphin (Sardella, et al., 2011). While it is known that opiates induce itch recent more specific studies have demonstrated that the effects of κ-opioid receptor agonists are anti-pruriceptive and it is μ-opioid receptor agonists that cause increased itch perception. (Togashi, et al., 2002; Kamei and Nagase., 2001). The prion protein is localized in about 50% of GFP expressing cells in Lamina II (Hantman and Perl, 2005) and is central to disease pathogenesis in the transmissible spongiform encephalopathies (TSE). In many TSEs an intense pruritus is observed after onset of the disease (Nisipeanu, and Chapman, 1996) which may be mediated by a loss of nNOS/Gal interneurons. The idea that itch is inhibited by the Gal/nNOS population of cell which are enriched in glycine is a plausible hypothesis and further highlights the importance of especially glycine enriched Gal and nNOS interneurons. Caution however is urged again as itch is a complex phenomenon that is most likely processed at multiple levels throughout the CNS (Schmelz, 2002).

5.6. Concluding remarks

While it is clear that inhibitory interneurons in the spinal cord present an important target for potential anti-nociceptive and anti-itch treatments progress in identifying suitable targets is slow. Unravelling of the intricate interactions between functionally distinct populations of interneurons has also progressed at a conservative pace. The Gal/nNOS neurons may provide an important target for anti-itch therapy. In this paper we have suggested that these neurons contain glycine which may be of functional relevance. The differences we observed between the C57 mouse and Sprague Dawley rat may support significant interspecies differences in how these cells function or may be physiologically irrelevant, a question which needs to be

answered using in vivo electrophysiology determining whether the glycine is used as a neurotransmitter by these neuronal populations. A subset of nNOS/Gal neurons contain GFP and are more likely to colocalize with glycine than those that do not express GFP. The PrP-GFP mouse is important due to being able to select a neurochemically distinct subgroup of cells for electrophysiological recordings hopefully being able to answer how the nNOS/Gal cells signal. In addition the expression of GFP in these cells may indicate that they are affected by TSEs and may be a possible cause of pruritus in these cases. Further investigation is needed on nNOS/Gal inhibitory interneurons to identify their exact functioning and significance in vivo.

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