Semi-quantitative distribution of excitatory amino acid (glutamate) transporters 1–3 (EAAT1-3) and the cystine-glutamate exchanger (xCT) in the adult murine spinal cord

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ARTICLE INFO

Keywords:
Slc1a1
Slc1a2
Slc1a3
Slc7a11
Spinal cord
Glutamate transporter

ABSTRACT

Proper glutamatergic neurotransmission requires a balance between glutamate release and removal. The removal is mainly catalyzed by the glutamate transporters EAAT1-3, while the glutamate-cystine exchanger (system xc– with specific subunit xCT) represents one of the release mechanisms. Previous studies of the spinal cord have focused on the cellular distribution of EAAT1-3 with special reference to the dorsal horn, but have not provided quantitative data and have not systematically compared multiple segments. Here we have studied the distribution of EAAT1-3 and xCT in sections of multiple spinal cord segments using knockout tissue as negative controls. EAAT2 and EAAT3 were evenly expressed in all gray matter areas at all segmental levels, albeit with slightly higher levels in laminae 1–4 (dorsal horn). Somewhat higher levels of EAAT2 were also seen in lamina 9 (ventral horn), while EAAT3 was also detected in the lateral spinal nucleus. EAAT1 was concentrated in laminae 1–3, lamina 10, the intermediolateral nucleus and the sacral parasympathetic nucleus, while xCT was concentrated in laminae 1–3, lamina 10 and the leptomeninges. The levels of these four transporters were low in white matter, which represents 42% of the spinal cord volume. Quantitative immunoblotting revealed that the average level of EAAT1 in the whole spinal cord was 0.6 ± 0.1% of that in the cerebellum, while the levels of EAAT2, EAAT3 and xCT were, respectively, 41.6 ± 12%, 39.8 ± 7.6%, and 30.8 ± 4.3% of the levels in the hippocampus (mean values ± SEM). Conclusions: Because the hippocampal tissue content of EAAT2 protein is two orders of magnitude higher than the content of the EAAT3, it follows that most of the gray matter in the spinal cord depends almost exclusively on EAAT2 for glutamate removal, while the lamina involved in the processing of autonomic and nociceptive information rely on a complex system of transporters.

1. Introduction

It is well established that glutamatergic neurotransmission is fundamental for sensory, motor and autonomic systems of the spinal cord (Tao et al., 2005; Todd, 2010; Abbott et al., 2014), and that a high signal-to-noise ratio requires a careful balance between glutamate removal and release. The removal is catalyzed by the excitatory amino acid transporters EAAT1, EAAT2 and EAAT3 (Lehre et al., 1995; Furuta et al., 1997; Shibata et al., 1997; Yamada et al., 1998; Kanai et al., 1995; Tao et al., 2004, 2005; Queen et al., 2007; Danbolt, 2001; Zhou and Danbolt, 2013), while the release is more complicated in the sense that both vesicular and non-vesicular mechanisms are involved. Both channels and carriers support non-vesicular release (Abudara et al., 2018). A carrier that appears to be of particular importance is the glutamate-cystine exchanger system xc– with xCT (slc7a11) as specific subunit (Baker et al., 2002; Augustin et al., 2007; De Bundel et al., 2011; Massie et al., 2011). Whereas the distribution of xCT in the brain has been studied in detail (Ottestad-Hansen et al., 2018), this information is lacking for the spinal cord. Data are available on the distributions of the EAAT-type of transporters in the spinal cord, but the focus has been at the cellular level and with a particular attention to the dorsal horn.
2. Materials and methods

2.1. Materials

Sodium dodecyl sulfate (SDS) of high purity (>99% C12 alkyl sulfate) was provided by Pierce (Rockford, IL). Electrophoresis equipment was from Hoefer Scientific Instruments (Holliston, MA). Molecular mass markers for SDS-PAGE and nitrocellulose sheets (0.22 m pores; 100% nitrocellulose) were obtained from GE Life Science (Chicago, USA). Paraformaldehyde and glutaraldehyde EM grade were from TAAB Laboratories Inc., Indianapolis, IN, USA) with access to water ad libitum as described previously (Lehre et al., 2011). Biopsies from ear or tail were collected for determining genotype. Mice were housed at the animal facility of the Vrije Universiteit Brussel (Brussels, Belgium). Care was taken to avoid suffering and to minimize the number of mice used.

2.2. Animals

The mice deficient in EAAT2 (GLT-1; slc1a2; Tanaka et al., 1997), EAAT1 (GLAST; slc1a3; Watabe et al., 1998) or EAAT3 (B6N (Cg)-Slc1a1tm1b(KOMP)Wtsi/J; Stock No. 024411, Jackson Laboratory, Bar Harbor, ME; RRID:IMSR_JAX:024411; Hu et al., 2018) were in C57BL/6 background and were generated by a heterozygote breeding scheme. They were kept in individually ventilated (IVC) cages at constant temperature and humidity, and were fed with Harlan Teklad 2018 (Harlan Laboratories Inc., Indianapolis, IN, USA) with access to water ad libitum as described previously (Lehre et al., 2011). Biopsies from ear or tail were collected for determining genotype. Mice were housed at the animal facility of the Vrije Universiteit Brussel (Brussels, Belgium). Care was taken to avoid suffering and to minimize the number of mice used.

2.3. Antibodies

Affinity-purified anti-peptide antibodies to rat glutamate transporters (EAAT1; EAAT2; EAAT3; xCT) were from the same batches as described previously (listed in Table 1). Because antibody batches may differ from each other, we identify antibody batches by the unique identification number (“Ab#”). They are given by our electronic laboratory information system (software provided by Science Linker AS; Oslo, Norway), and by their Research Resource Identifiers (RRID; www.rdrds.org). The EAAT1 (GLAST) antibody Ab#314 (RRID: AB_2314561; Li et al., 2012) was directed to the C-terminal residues 522-541 of rat EAAT1 (GLAST; slc1a3; Watase et al., 1998) or EAAT3 (B6N (Cg)-Slc1a1tm1b(KOMP)Wtsi/J; Stock No. 024411, Jackson Laboratory, Bar Harbor, ME; RRID:IMSR_JAX:024411; Hu et al., 2018) were in C57BL/6 background and were generated by a heterozygote breeding scheme. They were kept in individually ventilated (IVC) cages at constant temperature and humidity, and were fed with Harlan Teklad 2018 (Harlan Laboratories Inc., Indianapolis, IN, USA) with access to water ad libitum as described previously (Lehre et al., 2011). Biopsies from ear or tail were collected for determining genotype. Mice were housed at the animal facility of the Vrije Universiteit Brussel (Brussels, Belgium). Care was taken to avoid suffering and to minimize the number of mice used.

### Table 1

| Name     | id       | RRID               | Ref.             |
|----------|----------|--------------------|------------------|
| EAAT1    | Ab#314   | AB_2314561         | Li et al. (2012) |
| EAAT2    | Ab#360   | AB_2714081         | Holmseth et al. (2012a) |
| EAAT3    | Ab#340   | AB_2714090         | Li et al. (2012) |
| xCT      | Ab#618   | AB_2714118         | Van Liefferinge et al. (2016) |
EAAT1. The EAAT2 (GLT-1) antibody Ab#360 (RRID: AB_2714081; Holmseth et al., 2012a) and Ab#8 (RRID: AB_2714090; Li et al., 2012) were directed to rat EAAT2 residues 12–26 and 493–508 respectively (Pines et al., 1992). The EAAT3 antibodies Ab#340 (RRID: AB_2714057; Holmseth et al., 2012b) and Ab#371 (RRID: AB_2714048; Holmseth et al., 2005) were directed to the C-terminus of rat EAAT3 residues 510–524 and 479–498 respectively (Bjorjas et al., 1996). The xCT antibody Ab#618 (RRID: AB_2714118; Van Liefferinge et al., 2016) was raised in rabbit against a peptide corresponding to residues 1–37 of rat xCT and was shown to be specific when tested both by immuno blotting and by immunohistochemistry (Van Liefferinge et al., 2016; Ottes- tad-Hansen et al., 2018). Biotinylated donkey anti-rabbit IgG (Cat. No. RPN1004, lot. no. 399303), biotinylated donkey anti-sheep IgG (Cat. No. RPN1025, lot. no. 399303) and streptavidin-biotinylated horseradish peroxidase complex (Cat. No. RPN1051) were from GE Life Sciences. Briefly, an average pixel intensity of an unlabeled region in the blot image was defined as the background. The signal was calculated extracted using Image Studio Lite Ver5.2 provided by LI-COR Biosciences. Briefly, they were deeply anesthetized by intraperitoneal injection with ZRF cocktail (at least 0.1 ml per 10 g body weight) or pentobarbital (Dolethal®, at least 200 mg/kg; xCT mice). ZRF is a mixture of Zola-zepam (3.3 mg/ml; CAS 31352-82-6), Tiletamine (3.3 mg/ml; CAS 14176-49-9), Xylazine (0.5 mg/ml; CAS 7361-61-7) and Fentanyl (2.6 μg/ml; CAS 437-38-7). After cessation of all reflexes, the mice were perfused transcardially first with 0.1 M NaPi pH 7.4 to wash out blood and then immediately afterwards with 4% formaldehyde in 0.1 M NaPi pH 7.4 with or without 0.05% (v/v) gluta raudehydrogenase for 5 min. The segments of spinal cord were carefully dissected with blades and scissors, collected and immersed in fixative for about 2–3 h at room temperature. Sections (40 μm thick) were cut from the fixed tissue using either a Vibratome 1000 plus® (Vibratome, Bannockburn, UK) or a Microtome HM450 (Thermo Scientific™, Waltham, USA). For microsectioning, the tissues were cryo-protected with graded sucrose solutions (10%, 20% and 30% (w/v)). Diaminobenzidine-peroxidase labeling was done as previously described (Lebre et al., 1995). The sections were treated (5 min) with 1% hydrogen peroxide in 0.1 M NaPi pH 7.4 to inactivate endogenous peroxidase and then treated (30 min) with 1 M ethanolamine in 0.1 M NaPi pH 7.4. After being washed in PBS, the sections were incubated for 1 h in Tris buffered saline (TBS: 300 mM NaCl in 100 mM Tris-HCl buffer, pH 7.4) containing 10% (v/v) newborn calf serum (NCS) and 0.05% (v/v) Triton X100. Then they were incubated with primary antibodies overnight, followed by biotinylated donkey anti-rabbit/sheep IgG (1: 300; 1 h; room temperature) and streptavidin-biotinylated horseradish peroxidase complex (1: 300; 1 h; room temperature). The sections were examined and photographed on Zeiss Axioskop 2 plus equipped with AxioCam MRc r1.2 camera (Zeiss, Jena, Germany). Immunofluorescent labeling was done as previously described (Zhou et al., 2012). Briefly, the sections were rinsed (3 × 5 min) in TBST (TBS with 0.5% Triton X-100), treated with 1 M ethanolamine in 0.1 M NaPi pH 7.4 (30 min), washed in TBST, and blocked (1 h) in TBST containing 10% NCS and 3% bovine serum albumin (BSA) followed by incubation overnight (room temperature) with primary antibodies (as stated in the figure legends) diluted in 3% NCS and 1% BSA in TBST. Then, sections were washed with TBST before incubation (1 h, room temperature) with secondary antibodies (1:1000). The sections were washed again with TBST, then mounted with ProLong Gold antiFade mountant with 4′, 6-diamidino-2-phenylindole (DAPI; cat. no. P36935; Thermo Fisher Scientific™) and examined either using a Zeiss Axioplan 2 microscope equipped with a Zeiss LSM 510 meta confocal scanner head (Zeiss; Jena, Germany) or infrared scanner LI-COR odyssey scanner (LI-COR Biosciences, Lincoln, USA).

2.6. Histological staining

Spinal cord segments were first identified based on vertebral landmarks (Harrison et al., 2013). Different spinal cord sections representing cervical (C), thoracic (T), lumbar (L) and sacral (S) segments were used. To further verify spinal cord segments and to delineate spinal cord laminae, we performed Nissl staining and compared Nissl stained sections with the detailed descriptions of the mouse spinal cord atlas provided by Watson and co-workers (Watson et al., 2009). Sections were mounted onto SuperFrost Plus slides (ThermoFisher, Waltham, MA, USA), air dried and incubated (3 min) in Walter’s cresyl violet solution on a heating plate. After the removal of excess staining solution, the sections were rinsed in tap water (3 min). Finally the sections were dehydrated and differentiated in graded ethanol (70%, 90% and 100%), cleared in xylene and mounted with Permout (Cat. no: 12377369, Fisher Scientific).
2.7. Quantification of the gray matter area and certain lamina in spinal cord

To estimate the percentage of the gray matter area and of certain spinal laminae, a series of images (one from each segment) of the delineated transsections from Altas of the Mouse Spinal Cord (Watson et al., 2009) were obtained and analyzed using ImageJ. Using the polygon selection tool, a closed line was manually drawn around the regions of interest (ROIs). For each image, all the square pixels representing spinal cord tissue, all the pixels representing total gray matter, and all the pixels only representing a given ROI were determined. Spinal arteries, dorsal and ventral root, and dorsal root ganglion were not included.

For the present study it was assumed that all segments have the same thickness. This approximation was considered acceptable in relation to the scope of the study. Given this, the sum of all the pixels representing spinal cord tissue from all the images was taken as a measure of the total spinal cord volume. Thus, the pixels were treated as though they were voxels with a thickness of a segment. The sums of the pixels from all images containing a given ROI were then taken as measures of the total volumes of each ROI, and the volumes of each ROI was subsequently expressed as percentage of the volume of the entire spinal cord.

3. Results

3.1. Specific labeling was obtained for the glutamate transporter proteins EAAT1-3 and the xCT protein in adult mouse spinal cord

We first tested whether we could obtain immunolabeling in sections from wildtype mice without obtaining labeling in sections from knockout mice (for discussion of the importance of this control see: Holmseth et al., 2012a; Danbolt et al., 2016b). As shown in Figs. 1A, 2A and 4A and 5A, specific labeling was obtained for all the four transporter proteins. The results of the laminar distribution of the four transporter proteins are semi-quantitatively summarized in Table 2.

![Fig. 1. Distribution of EAAT1 in various segments (as indicated) of the adult mouse spinal cord. Panel A: Sections of wildtype (+/+ adult spinal cord were incubated with antibodies to EAAT1 (Ab#314; 0.1 μg/ml). The lack of labelling in the knockout (−/−) tissue processed in parallel, attests to the specificity of the antibodies. Panel B: Delineation of higher magnification images from C1, C5, T10 and S2 segments. Abbreviations: cc, central canal; DH, dorsal horn; df, dorsal funiculus; IML, intermediolateral nucleus; ICI, Intercalated nucleus; LatC, lateral cervical nucleus; If, lateral funiculus; 1Sp, lateral spinal nucleus; SPSy, sacral parasympathetic nucleus; SDCom, sacral dorsal commissural nucleus. vf, ventral funiculus; VH, ventrol horn; 2Sp, lamina 2; 3Sp, lamina 3; 4Sp, lamina 4; 5Sp, lamina 5; 10Sp, lamina 10. Scale bar: 100 μm.](image-url)
3.2. Distribution of EAAT1

As Fig. 1 shows, the highest EAAT1 densities were found in the laminae 1–3 of the dorsal horn, and in lamina 10 surrounding the central canal (cc). This was observed at all segmental levels. Dense staining was also seen in the lateral horn in thoracic segments (the intermediolateral nucleus; IML) where sympathetic preganglionic neurons reside (Figs. 1B and 3C). In the sacral segments, high intensity of EAAT1 was found in the parasympathetic nucleus (SPSy; Fig. 1B). In the intercalated nucleus (ICl), sacral dorsal commissural nucleus (SDCom), lateral spinal nucleus (LSp) and lateral cervical nucleus (LatC) of C1–C3 segments (Fig. 1B). Weak EAAT1 labeling was detected in the ventral (vf) and lateral funiculus (lf) of white matter, but hardly any in the dorsal funiculus (df). Laminae 4, 5, 7, 8 and 9 had little immunolabeling.

3.3. Distribution of EAAT2

EAAT2 staining was dense throughout the entire gray matter in all spinal cord segments. The highest densities were detected in laminae 1–4 of the dorsal horn and in lamina 9 of the ventral horn (Fig. 2). This intense labeling of lamina 9 was particularly evident in cervical and lumbar segments. Moderate labeling was seen in laminae 5–8 and 10, LatC at C1–C3 segment, LSp and lateral horn including IML and SPSy (Figs. 2 and 3BD). Double labeling (using immunofluorescence) confirmed co-localization of EAAT1 and EAAT2 in the IML (Fig. 3EF). Similar to EAAT1 in white matter, weak EAAT2 immunoreactivity was detected in the ventral and lateral funiculi, but not in the dorsal
Table 2
Summary of the semi-quantitative laminar distribution of EAAT1-3 and xCT in adult mouse spinal cord.

|          | EAAT1 | EAAT2 | EAAT3 | xCT |
|----------|-------|-------|-------|-----|
| Dorsal horn |       |       |       |     |
| lamina 1-3 | ++++  | ++++  | ++++  | ++++|
| lamina 4   | 0     | ++++  | ++++  | 0   |
| lamina 5-6 | 0     | +++   | +++   | 0   |
| lamina 10  | +++   | +++   | +++   | +++ |
| Ventral horn |       |       |       |     |
| lamina 7-8 | 0     | ++    | ++    | 0   |
| lamina 9   | (+)   | ++++  | ++++  | 0   |
| Lateral horn |     |       |       |     |
| ICI        | +     | ++    | ++    | (+) |
| IML        | ++++  | ++++  | ++++  | 0   |
| SPSy       | ++++  | ++++  | ++++  | 0   |
| White matter |     |       |       |     |
| LatC       | +     | +     | +     | (+) |
| LSp        | +     | +     | +     | (+) |
| vf         | (+)   | (+)   | (+)   | 0   |
| df         | 0     | 0     | 0     | 0   |

0, not detectable; (+), weakly detectable; +, detectable; ++, quite abundant; ++++, very abundant.

Abbreviations: df, dorsal funiculus; IML, intermediolateral nucleus; ICI, Inter-calcated nucleus; LatC, lateral cervical nucleus; If, lateral funiculus; LSp, lateral spinal nucleus; SPSy, sacral parasympathetic nucleus; Vf, ventral funiculus.

funiculus.

3.4. Distribution of EAAT3

Similar to EAAT2, EAAT3 was present throughout the gray matter in all segments. The highest labeling intensities were in laminae 1-4 of the dorsal horn (Fig. 4AB). Moderate staining intensities were seen in laminae 5-10 and in the lateral spinal nucleus (LSN), which is located in the dorsolateral funiculus (Fig. 4C).

3.5. Distribution of xCT

Similar to EAAT1, xCT was detected in the superficial layer of the dorsal horn (laminae 1–3) and lamina 10 around the central canal at all segmental levels (Fig. 5AB). In addition, xCT immunostaining was detectable in the leptomeninges covering the spinal cord (indicated by arrowheads in Fig. 5C). Because the weak neuronal labeling (indicated by arrows in Fig. 5C) was observed in both sections from wildtype and xCT-deficient mice, this labeling was interpreted as cross-reactivity with a non-xCT molecular species.

3.6. Quantification of relative expression of glutamate transporters EAAT1-3 and xCT protein in mouse spinal cord

The immunohistochemical staining shows the distribution of these proteins within the spinal cord and allows to compare relative expression levels of the respective proteins within the different regions of the spinal cord (as described above), but do not give information on the amounts of transporter protein per unit volume of tissue in absolute terms (mg/g tissue).

First we compared the EAAT1-3 and xCT protein levels in whole mouse spinal cord to the levels in whole mouse hippocampus (EAAT2, EAAT3 and xCT) and cerebellum (EAAT1) by means of immunoblotting. As shown in Fig. 6, the level of EAAT1 in the spinal cord was 0.6 ± 0.1% of that in the cerebellum, while the levels of EAAT2, EAAT3 and xCT in the spinal cord were, respectively, 41.6 ± 12%, 39.8 ± 7.6%, and 30.8 ± 4.3% of the levels in the hippocampus (mean values ± SEM).

Second, because these four transporter proteins have been quantified fairly accurately in young adult Wistar rat hippocampus and cerebellum (Lehre and Danbolt, 1998; Dehnes et al., 1998; Holmseth et al., 2012b), we wanted to find out if numbers obtained from rat can be used to interpret mouse data. Therefore, we tested if the concentrations of these four transporters are similar in young adult mouse hippocampus (C57Bl6) and in young adult Wistar rat hippocampus and cerebellum: no major differences in the tissue concentration of these four transporters were detected between the two species (data not shown).

3.7. Relative volumes of spinal cord components

The immunoblotting above revealed the average transporter concentrations relative to the hippocampus. But because the expression of these transporter proteins is almost negligible in the white matter (see above), we determined the percentages of gray matter relative to white matter and found that to be 42%. This is in good agreement with published assessments both by MRI analysis and histological analysis (Kim et al., 2009; Hashimoto et al., 2007). Similarly, because EAAT1 and xCT are highly concentrated in certain laminae (laminae 1–3 and lamina 10, and in the case of EAAT1 also in the intermediolateral nucleus and the sacral parasympathetic nucleus, we estimated the relative volumes of these parts of gray matter and found that laminae 1, 2, 3 and 10 together account for 8.2% of the spinal cord volume, while the intermediolateral nucleus and the sacral parasympathetic nucleus together only account for 0.4%.

4. Discussion

The present work, using tissues from knockout mice as negative controls, confirms earlier brief reports on the distributions of EAAT1-3 in the spinal cord (Lehre et al., 1995; Furuta et al., 1997; Shibata et al., 1997; Shashidharan et al., 1997; Yamada et al., 1998; Kanai et al., 1995; Tao et al., 2004, 2005; Queen et al., 2007). While the cited studies have mostly focused on the dorsal horn at one segment level, we have studied laminar distribution containing multiple spinal nuclei (e.g. IML and SPSy) in multiple segments and added assessments of the protein expression levels. Finally, this is the first report on the distribution of xCT in the spinal cord.

4.1. The average concentration of EAAT1 is low, but is concentrated in some laminae

As explained above, the average concentration of EAAT1 in the spinal cord is only 0.6 ± 0.1% of the level in the cerebellum. Because the concentration in the hippocampus is 6 times lower (Lehre and Danbolt, 1998), this corresponds to only 3.6% of the hippocampal level. However, the EAAT1 protein is concentrated in laminae 1–3, lamina 10, the intermediolateral nucleus and the sacral parasympathetic nucleus which together represent about 8.4% of the spinal cord volume. If these laminae had contained all of the EAAT1 protein, then the concentration would have been 12 times higher than the average value (corresponding to 7% of the cerebellar level). However, as explained above, there is some EAAT1 in other parts of the spinal cord so the concentration in these laminae has to be lower than 7% of the cerebellar level, but clearly higher than the average value (0.6%).

Given these premises, the following calculations are valid: There is 1.8 mg EAAT1 protein per gram cerebellar tissue (Lehre and Danbolt, 1998), and the gray matter areas with the highest EAAT1 expression therefore has an EAAT1 content that is less than 0.13 mg/g, but higher than 0.011 mg/g tissue.

4.2. The average concentrations of EAAT3 and xCT are low, but xCT is concentrated in some laminae

As explained above, the average concentration of EAAT3 and xCT relative to the hippocampus were, respectively, 39.8 ± 7.6%, and 30.8 ± 4.3%. Because the levels of EAAT3 and xCT in the white matter are very low and because the white matter constitutes 42% of the spinal
cord, it follows that the average concentrations of EAAT3 and xCT in gray matter are, respectively, about 70 and 50% of the hippocampal levels. In contrast to EAAT3, but similar to EAAT1, xCT is concentrated in certain laminae representing 8.2% of the spinal cord volumes. This implies that the concentration in these laminae is higher than the average value, but less than 375% (30.8%/0.082) of the hippocampal value considering that there is some xCT in other parts of the spinal cord and, in particular, in the meninges.

The amounts of EAAT3 (Holmseth et al., 2012b) and xCT (Ottestad-Hansen et al., 2018) in the hippocampus are, respectively, 0.013...
mg/g and 0.01 mg/g. Given these premises, the following calculations are valid: 70% of the hippocampal EAAT3 level corresponds to 0.009 mg/g, while 30.8–375% of the hippocampal xCT level corresponds to 0.003–0.04 mg/g.

4.3. EAAT2 is the major glutamate transporter in the spinal cord and is almost as abundant in spinal cord gray matter as it is in the hippocampus

Because the average concentration of EAAT2 in the spinal cord corresponds to 41.6 ± 12% (see above) and because there is little EAAT1 in white matter, it follows that the average level of EAAT2 in the gray matter (representing 58%) of the spinal cord is about 72% of that in the hippocampus or about 1.3 mg/g (Lehre and Danbolt, 1998). This means that EAAT2 is quantitatively even more dominant in the spinal cord than it is in the hippocampus. In fact, in the ventral horn, where the motor neurons reside, there is hardly any EAAT1 and the EAAT3 level is two orders of magnitude lower than that of EAAT2.

In agreement, selective deletion of EAAT2 in the murine spinal cord has more severe consequences than deletion of EAAT1 or EAAT3 (Sugiyama and Tanaka, 2018; Watase et al., 1998; Peghini et al., 1997; Sato et al., 2005). Pharmacological blockage or genetic deletion of EAAT2 in the spinal cord results in increased vulnerability to traumatic injury (Lepore et al., 2011), loss of about 20% of the ChAT-positive motor neurons and impairment of fine motor skills (Sugiyama and Tanaka, 2018).

The most interesting aspect here may not be that there is some loss of neurons in EAAT2-deficient mice, but that the loss is not much greater considering the dominance of EAAT2 in the ventral horn, and that the...
mice lacking spinal EAAT2 do not develop paralyses (Sugiyama and Tanaka, 2018) like in amyotrophic lateral sclerosis (ALS).

4.4. The contribution of EAAT1 and xCT in glutamate handling may be functionally relevant in the substantia gelatinosa

As explained above, EAAT2 is quantitatively dominant also in the laminae in which EAAT1 and xCT are expressed at the highest levels, possibly by 10 and 30 times, respectively. Nevertheless, both probably have physiological implications: (a) In agreement with this, both EAAT1 and EAAT2 currents were electrophysiologically detectable in the astrocytes of substantia gelatinosa (Zhang et al., 2009). Whether the role of EAAT1 is to contribute to the control of extracellular glutamate or something else (e.g. adjustment of intracellular chloride concentrations; Untiet et al., 2017) remains to be determined. (b) System xc is considered to be a significant supplier of extracellular glutamate as genetic deletion of its specific subunit xCT reduced extracellular glutamate in hippocampus and striatum (Baker et al., 2002; Augustin et al., 2007; De Bundel et al., 2011; Massie et al., 2011) where xCT is expressed at the highest levels (Ottestad-Hansen et al., 2018). According to the estimations presented here, the concentration of xCT protein in the substantia gelatinosa may be higher than in the hippocampus and is thereby likely to be functionally important. In agreement, lamina 2 neurons display slow inward currents (SICs) in addition to excitatory postsynaptic currents (EPSCs). These SICs are possibly triggered by astrocyte-derived glutamate, and the frequency of these spontaneous SICs is positively associated with peripheral inflammation (Bardoni et al., 2010). Thus, this restricted distribution of xCT we describe here in superficial laminae of the dorsal horn fits well with the reported SICs, possibly suggesting that system xc may play roles in sensory processing and chronic pain.

4.5. EAAT1 expression in the intermediolateral nucleus

Available evidence points to glutamate being an important activator of preganglionic neurons of the autonomic nervous system. Firstly, both the sympathetic preganglionic neurons in the intermediolateral nucleus (Morrison et al., 1989, 1991) and the lumbar sacral parasympathetic preganglionic neurons (Miura et al., 2001, 2003) receive glutamatergic...
input from other parts of the CNS. Secondly, microinjections of L-glutamate into the intermediolateral nucleus elicit typical tachycardic responses and increased heart rates (Murugaian et al., 1990; Zhou and Gilbey, 1992). Thirdly, deficits in the supply of neurotransmitter glutamate due to deletion of VGLUT2 in the rostral ventrolateral medulla neurons, impaired frequency-dependent respiratory activation (Abbott et al., 2014). Together, this suggests that glutamate uptake is important for the normal functioning of the autonomic nervous system.

The present report represents the first description of EAAT1 and EAAT2 in the intermediolateral nucleus and in the sacral parasympathetic nucleus. We find that EAAT2 is quantitatively dominant expressed at levels an order of magnitude higher than EAAT1. This probably implies that EAAT1 is of less importance for extracellular glutamate levels. It is therefore legitimate to consider other possible functions. In this context it is interesting to note that the glutamate transporter-associated anion conductance is larger in EAAT1 than in EAAT2 (Wadiche et al., 1995) and may be of importance for adjusting the chloride concentrations in astrocytes (Untiet et al., 2017).

4.6. Future directions

The present report concerns the adult mouse spinal cord. It would be interesting to determine how the distributions and expression levels of these transporters change during development of the spinal cord, as well as how this affects ambient glutamate. For instance, EAAT1 may be more important before postnatal week 3 as it is expressed throughout the gray matter (Shibata et al., 1997), and EAAT2 is transiently expressed in growing axons of spinal cord before astrocytic expression become

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**Fig. 6.** Quantification of relative protein expression of EAAT2 (Panel A: Ab#8, 0.1 μg/ml), EAAT1 (Panel B: Ab#314, 0.1 μg/ml), EAAT3 (Panel C: Ab#340; 0.5 μg/ml) and xCT (Panel D; Ab#618, 0.5 μg/ml) in adult mouse spinal cord. Hippocampus was used as a reference (100%) for EAAT2, EAAT3 and xCT, while cerebellum was defined as a reference for EAAT1. Note that immunoblots for developing with anti-EAAT1 and anti-EAAT2 antibodies were loaded with 6 μg total protein per lane, while the others were loaded with 20 μg total protein per lane. The data represent mean ± SEM of three adult mice aged between 88 and 106 days.
dominant (Yamada et al., 1998) in agreement with cell culture studies (Plachez et al., 2000). Further, it would be interesting to determine the cellular distribution, in particular, whether EAAT2 is also present in axon terminals in the spinal cord. This might be done with a procedure similar to the one we used before (Petri et al., 2015; Zhou et al., 2019), but preferentially using a Cre-line that has a higher neuronal penetrance.

Declaration of competing interest

The authors declare no competing financial interests.

CRediT authorship contribution statement

Qiu-Xiang Hu: Investigation, Validation, Formal analysis. Gesa M. Klatt: Investigation, Validation, Formal analysis. Ruben Gudmundsd-rud: Investigation, Validation, Formal analysis. Sigrid Ottestad-Han sen: Investigation, Supervision. Lise Verbruggen: Resources. Ann Massie: Resources. Niels Christian Danbolt: Data curation, Writing - review & editing. Yun Zhou: Conceptualization, Methodology, Visualization, Supervision, Project administration, Writing - review & editing.

Acknowledgement

This work was supported by the Norwegian Research Council (240844), and from the University of Oslo (SERTA; UNIFOR- FRIMED, Johanne og Einar Eilertsen forskningsfond, Halvor Høie Fond, Anders Jahres fond and Futura fond; UiO: Life Science summer fellowship to HQX and GMK), as well as by the Vrije Universiteit Brussel FRIMED, Johanne og Einar Eilertsen forskningsfond, Halvor Høie Fond, Anders Jahres fond and Futura fond; UiO: Life Science summer fellowship to HQX and GMK), as well as by the Vrije Universiteit Brussel (SRP49). We thank technical staff at animal facility of FHI for technical assistance and Kohichi Tanaka and Hideo Sato for EAAT-2 and xct conventional knockouts.

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