Coordinated expression of the renin–angiotensin genes in the lumbar spinal cord: Lateralization and effects of unilateral brain injury

Georgy Bakalkin | Anika Kahle | Daniil Sarkisyan | Hiroyuki Watanabe | Nikolay Lukoyanov | Liliana S. Carvalho | Vladimir Galatenko | Mathias Hallberg | Olga Nosova

1Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden
2Departamento de Biomedicina, Faculdade de Medicina; Instituto de Investigação e Inovação em Saúde; Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal
3Faculty of Mechanics and Mathematics, Lomonosov Moscow State University, Moscow, Russia

Correspondence
Olga Nosova, Department of Pharmaceutical Biosciences, Uppsala University, 751 24 Uppsala, Sweden. Email: olga.kononenko@farmbio.uu.se

Present address
Vladimir Galatenko, Evotec International GmbH, Göttingen, Germany

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Abstract
In spite of its apparent symmetry, the spinal cord is asymmetric in its reflexes and gene expression patterns including leftward expression bias of the opioid and glutamate genes. To examine whether this is a general phenomenon for neurotransmitter and neurohormonal genes, we here characterized expression and co-expression (transcriptionally coordinated) patterns of genes of the renin–angiotensin system (RAS) that is involved in neuroprotection and pathological neuroplasticity in the left and right lumbar spinal cord. We also tested whether the RAS expression patterns were affected by unilateral brain injury (UBI) that rewired lumbar spinal neurocircuits. The left and right halves of the lumbar spinal cord were analysed in intact rats, and rats with left- or right-sided unilateral cortical injury, and left- or right-sided sham surgery. The findings were (i) lateralized expression of the RAS genes Ace, Agtr2 and Ren with higher levels on the left side; (ii) the asymmetry in coordination of the RAS gene expression that was stronger on the right side; (iii) the decay in coordination of co-expression of the RAS and neuroplasticity-related genes induced by the right-side but not left-side sham surgery and UBI; and (iv) the UBI-induced shift to negative regulatory interactions between RAS and neuroplasticity-related genes on the contralesional spinal side. Thus, the RAS genes may be a part of lateralized gene co-expression networks and have a role in a side-specific regulation of spinal neurocircuits.

Keywords
brain injury, co-expression patterns, gene expression, neuroplasticity, renin-angiotensin system, spinal cord

Abbreviations:
Ace, gene coding for angiotensin-converting enzymes; Ace2, gene coding angiotensin-converting enzymes 2; Agtr1a, Agtr1b, Agtr2 and Mas1 genes coding for angiotensin receptors AT1a, AT1b, AT2 and Mas1; Agtrap, gene coding for angiotensin II receptor-associated protein; Atg, angiotensinogen; BDNF, brain-derived neurotrophic factor; HPDCI, highest posterior density continuous intervals; RAS, renin–angiotensin system; Ren, renin; TBI, traumatic brain injury; UBI, unilateral brain injury.
A general view is that the spinal cord is bilaterally symmetric in its anatomy, functions and molecular features. However, several lines of evidence suggest its functional and molecular asymmetries (Bakalkin et al., 1986; Bakalkin & Kobylansky, 1989; Chazov et al., 1981; de Kovel et al., 2017; Hultborn & Malmsten, 1983a, 1983b; Kononenko et al., 2017, 2018; Lukoyanov et al., 2020; Malmsten, 1983; Nathan et al., 1990; Ocklenburg et al., 2017; Watanabe et al., 2020, 2021; Zhang et al., 2020). Mono- and polysynaptic segmental reflexes in rats and cats (Hultborn & Malmsten, 1983a, 1983b; Malmsten, 1983) and the nociceptive withdrawal reflexes in rats (Zhang et al., 2020) are asymmetric and all displayed greater activity on the right body side. Furthermore, bilateral lumbar deafferentation differently abolished the left and right hindlimb postural reflexes, suggesting a side-specific mechanism of sensorimotor integration (Zhang et al., 2020). Symmetric spinal neural circuits controlling activity of the left and right hindlimbs may be differently activated by opioid agonists on the left or right side (Bakalkin et al., 1986; Bakalkin & Kobylansky, 1989; Chazov et al., 1981; Watanabe et al., 2020, 2021). δ-Opioid agonists induced hindlimb postural asymmetry with left limb flexion, whereas the right hindlimb was flexed after administration of δ-agonist. Consistently, expression of the opioid receptors and also glutamate ion channels was lateralized in the spinal cord, whereas their interregional co-expression patterns were side specific, and intra-segmental (between the dorsal and ventral domains) co-expression profiles were differently affected by the left- and right-side brain injury (Kononenko et al., 2017; Watanabe et al., 2021; Zhang et al., 2020). All lateralized opioid and glutamate genes were expressed at higher levels on the left side both in the cervical and lumbar spinal cord. Asymmetry in hindlimb posture and withdrawal reflexes was induced by a unilateral injury of the hindlimb sensorimotor cortex (UBI) and persisted after complete spinal transection, suggesting that it is encoded in the lumbar spinal circuits (Watanabe et al., 2020, 2021; Zhang et al., 2020). The UBI modified expression of neuroplasticity-related genes, as well as robustly impaired coordination of their expression within and between the ipsi- and contralesional halves of lumbar spinal cord; the effects were more pronounced after the left-side compared with the right-side injury (Lukoyanov et al., 2020; Zhang et al., 2020). The asymmetric changes in gene expression may in part underlie the persisting effects of the brain injury on spinal neurocircuits (Lukoyanov et al., 2020; Zhang et al., 2020). These findings raised the questions whether other neurotransmitter and neurohormonal systems besides the opioid and glutamate genes are asymmetrically expressed in the spinal cord, whether they are lateralized to one side and whether their lateralization is associated with neuroplasticity-related genes when spinal neurocircuits are rewired.

The renin–angiotensin system (RAS) is a hormonal system that regulates blood pressure and electrolyte balance. Angiotensinogen (Agt) is cleaved by renin (Ren) to angiotensin I that is converted by angiotensin-converting enzyme (Ace) to angiotensin II acting as an endocrine, autocrine/paracrine and intracrine hormone through activation of AT1a, AT1b and AT2 receptors. Ang II is degraded by Ace2 to Ang 1–7 that activates the Mas1 receptor (Mas1), a G protein-coupled receptor that is not activated by Ang II itself (Figure 1) (Hallberg, 2015; Santos et al., 2018). The AT1R-associated protein (Agtrap) suppresses Ang II-mediated pathological responses through its interaction with the AT1R (Wakui, 2020) and induces angiotensin independent effects including regulation of sarcoplasmic/endoplasmic reticulum calcium ATPase-2a activation (Uneda et al., 2017). In the brain and spinal cord, the RAS functions as a neurohormonal system that regulates processing of sensory and nociceptive information, neuronal plasticity and survival. The RAS genes are expressed in the spinal cord and dorsal root ganglia where their products are co-localized with substance P and calcitonin gene-related peptide (Patil et al., 2010). The RAS is upregulated under conditions of chronic pain and after axotomy or nerve crush, indicating a role in neuronal plasticity (Bessaguet et al., 2018; Perdomo-Pantoja et al., 2020; Schwengel et al., 2016). Activation of angiotensin receptors promotes regeneration after nerve crush (Lucius et al., 1998) and reinnervation, remyelination and functional outcome after sciatic nerve injury (Reinecke et al., 2003). The RAS components Ace2, the Mas and the AT2 receptors exert protective and regenerative actions after their activation (Iwai & Horiuchi, 2009). In stroke models, activation of RAS leads to neuroprotection driven by the brain-derived neurotrophic factor (BDNF) signalling pathway. Expression of the RAS genes that regulate synaptic transmission and neuroplasticity may be coordinated with expression of neuroplasticity-related genes. These findings suggest that the RAS may be involved in neuroplastic spinal responses, for instance, to brain injury, and as such may undergo rearrangements at the transcriptional level in spinal neural circuits.

Here, we examined whether expression of the RAS genes and their co-expression (transcriptionally coordinated) patterns are lateralized in the lumbar spinal cord and whether the lateralized RAS co-expression patterns are associated with expression of...
the neuroplasticity-related genes and affected by the UBI. Previously identified differences in expression of individual genes between the grossly similar left and right sides of the central nervous system were generally subtle, of the order of 1.3-fold (de Kovel et al., 2017, 2018; Karlebach & Francks, 2015; Kononenko et al., 2017; Messina et al., 2021; Watanabe et al., 2015, 2021; Zhang et al., 2020). Therefore, in this study, the emphasis was on the intra- and inter-area coordination of gene expression that demonstrated robust, several-fold left–right differences (Kononenko et al., 2017; Watanabe et al., 2021; Zhang et al., 2020). Co-expression analysis generally provides a wealth of information on the power and structure of gene regulatory networks (Dobrin et al., 2009; Long et al., 2016; Lukoyanov et al., 2020). Strength and direction of pairwise gene–gene correlations were analysed to characterize strength and structure of co-expression patterns of the RAS and neuroplasticity-related genes.

Human and animal studies showed that the effects of brain and spinal cord injury on locomotor behavior and neurotransmitter systems were dependent on the injury side (Hussain et al., 2012; Kononenko et al., 2018; Pearlson & Robinson, 1981; Perennou et al., 2008; Robinson, 1979; Sainburg et al., 2016; Spinazzola et al., 2003; Stancher et al., 2018). Right but not left somatosensory lesions produced behavioral hyperactivity and bilaterally decreased cerebral norepinephrine and dynorphin levels. Because of sensorimotor lateralization and its differential responses to the left- and right-side injuries, the effects of the left- and right-side lesions on the expression levels and co-regulation patterns of RAS genes were compared. The brain lesion was restricted to the hindlimb sensorimotor cortex with the aim to examine molecular changes in the lumbar spinal circuits controlling hindlimb motor functions. Unilateral sham surgery was performed to control for UBI-induced brain injury. The sham surgery that by itself caused tissue injury (Cole et al., 2011) was controlled by analysis of intact rats. The RAS genes analysed were the angiotensinogen gene (Agt) that gives rise to angiotensin, renin (Ren), angiotensin II receptor-associated protein (Agtr1a), angiotensin-converting enzymes (Ace and Ace2) and Agtr1a, Agtr1b, Agtr2 and Mas1 genes coding for angiotensin receptors AT1a, AT1b, AT2 and Mas1 (Figure 1). Neuroplasticity-related genes selected for analysis were those coding for transcriptional regulators of synaptic plasticity (cFos, Egr1 and Nfkbia) and regulators of axonal sprouting, synapse formation, neuronal survival and neuroinflammation (Arc, Bdnf, Dlg4, Homer-1, Gap43, Syt4 and Tgfb1) and for essential components of the glutamate system critical for neuroplastic responses (GluR1, Grin2a and Grin2b).

2 MATERIALS AND METHODS

Male Wistar Hannover rats (Charles River Laboratories, Spain), weighing 150–200 g, were used in the study. The animals received food and water ad libitum and were kept in a 12-h day–night cycle at a constant environmental temperature of 21°C (humidity: 65%). Approval for animal experiments was obtained from the Malmö/Lund ethical committee on animal experiments (No. M7-16) and the ethical committee of the Faculty of Medicine of
analysed that were the intact group, left and right sham (\textit{ tex. The average lesion volume was 6.05/mm}^3, without accounting for tissue shrinkage due to fixation. The lesion volumes in rats with the UBI were confined to the hindlimb sensorimotor area. The expression stability of candidate reference genes was computed for five sets of samples that were the left and right halves of the lumbar spinal cord obtained from intact rats, the left- and right-sided sham surgery groups and the left- and right-sided UBI groups and was as follows (from high to low): \textit{Actb}, \textit{Gapdh}, \textit{Gusb}, \textit{Hprt}, \textit{Pgk}, \textit{Ppia}, \textit{Ppia13a}, \textit{Tbp} and \textit{Tfrc}.

2.3 | RNA purification, quality evaluation and cDNA synthesis

Total RNA was purified by using RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA concentrations were measured with NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). RNA (500 ng) was reverse-transcribed to cDNA with the cDNA \\textit{iScript} kit (Bio-Rad Laboratories, CA, USA) according to manufacturer’s protocol. cDNA samples were aliquoted and stored at -20\degree C.

2.4 | Quantitative RT-PCR

All procedures were conducted strictly in accordance with the established guidelines for the qRCR-based analysis of gene expression, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines (MIQE) (Bustin et al., 2009; Taylor et al., 2019). The raw qRT-qPCR data were obtained by the CFX Maestro Software for CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA). mRNA levels of genes of interest were normalized to geometric mean of expression levels of two reference genes \textit{Actb} and \textit{Gapdh} selected out of 10 genes (\textit{Actb}, \textit{B2m}, \textit{Gapdh}, \textit{Gusb}, \textit{Hprt}, \textit{Pgk}, \textit{Ppia}, \textit{Ppia13a}, \textit{Tbp} and \textit{Tfrc}) using the geNorm program (https://genorm.cmgg.be/; Kononenko et al., 2017, 2018; Vandesompele et al., 2002).

The expression stability of candidate reference genes was computed for five sets of samples that were the left and right halves of the lumbar spinal cord obtained from intact rats, the left- and right-sided sham surgery groups and the left- and right-sided UBI groups and was as follows (from high to low): \textit{Actb}, \textit{Gapdh}, \textit{Gusb}, \textit{Hprt}, \textit{Pgk}, \textit{Ppia}, \textit{Ppia13a}, \textit{Tbp} and \textit{Gusb}.

In each experiment, internal reference gene stability measure M did not exceed 0.5 value at the 1.5 threshold value imposed by the MIQE (Bustin et al., 2009; Taylor et al., 2019). The number of reference genes was optimized using the pairwise stability measure (V value) calculated by the geNorm program. The V value for \textit{Actb} and \textit{Gapdh}, the top reference genes, was 0.12 that did not exceed the 0.15 threshold, demonstrating that analysis of these two genes is sufficient for normalization.

TaqMan assay in 384-well format was applied. cDNAs were mixed with PrimePCR Probe assay (Table S1) and iTaq Universal Probes supermix (Bio-Rad Laboratories, CA, USA) for qPCR with a CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA) according to manufacturer’s instructions. The following conditions were applied for the two-step TaqMan probe-based reaction: 3 min at 95\degree C, then 40 cycles for 5 s at
95°C, followed by incubation for 30 s at 60°C. No template control reactions were run in parallel.

The number of RNA samples in each individual group and combined groups used in analysis of expression levels of the RAS genes is shown in Table S2. The differences in the number of rats/samples analysed were due to the technical errors in manipulation with RNA samples (occasional RNA loss).

2.5 Neuroplasticity-related genes

Neuronal activity dependent AP-1 constituent cFos; Gap-43 coding for growth-associated protein regulating axonal growth and neural network formation; brain-derived neurotrophic factor Bdnf; activity-regulated cytoskeletal Arc gene implicated in neuroplasticity; Egr1 regulating transcription of growth factors; Nfkbia regulating activity-dependent neuronal functions; GluR1 and Grin2b coding for the glutamate ionotropic receptor AMPA Type Subunit 1 and NMDA receptor subunit, respectively, both involved in glutamate signalling and synaptic plasticity; Homer-1 giving rise to Homer Scaffold Protein 1 involved in glutamate signalling-mediated nociceptive plasticity; and Syt4 (Synaptotagmin 4) playing a role in dendrite formation and synaptic plasticity (Adkins et al., 2006; Dolan et al., 2011; Epstein & Finkbeiner, 2018; Grasselli & Strata, 2013; Harris et al., 2016; Hayashi et al., 2000; Larsson & Broman, 2008; O’Mahony et al., 2006; Tappe et al., 2006; Vavrek et al., 2006) were analysed as neuroplasticity-related genes. RNA was prepared from all rats of five groups that were intact rats, the left and right sham groups and left and right UBI groups (n = 10 rats/group) and used in the analysis.

2.6 Statistical analysis

We analysed (i) expression levels of RAS genes (Agt, Agtrap, Ace, Agtr2, Ren, Agtr1a, Agtr1b, Ace2 and Mas1) in the left and right halves of the lumbar spinal cord; (ii) their laterализition; and (iii) correlations of expression levels within left and right spinal cord. Normality of data distribution was tested using the Kolmogorov–Smirnov test, and homogeneity of variances by Levene’s test. Expression levels for each gene in each the left and right spinal domain were analysed with nine Kruskal–Wallis tests (data with non-normal distribution) with group (intact control, left-side sham, right-side sham, left-side UBI and right-side UBI) as a factor.

In the absence of significant differences between the given animal groups in Kruskal–Wallis test, the groups were combined. The pooled data set was used for analysis of lateralization of RAS gene expression using Wilcoxon matched-pairs signed-rank test (data with non-normal distribution). The significance level was set to $P \leq 0.05$. The $P$-value was adjusted to compensate for multiple comparisons using Bonferroni correction.

Spearman’s rank correlations were computed to assess interactions between gene expressions within spinal domains. The sum of correlation squares was used to characterize the aggregated level of intra-area co-regulation of expression, or, in other words, the coordination strength. Comparison of correlation sets was performed by Wilcoxon signed-rank test (left vs. right domain) and Kruskal–Wallis test. The latter was used to compare (i) three groups of animals that were intact rats, the combined group with left impact (left sham and left UBI together) and the combined group with right impact (right sham and right UBI together) and (ii) five groups of animals that were intact rats, the left and right sham rats and the left and right UBI rats.

Because the comparison of gene–gene coordination strength ignored correlation signs, a separate analysis was performed to assess differences in the proportion of positive and negative correlations between animal groups. Generalized linear model (GLM) with binomial response distribution and logit link modelled the probability of Spearman correlation between gene expressions to be positive (p+). Bayesian GLM was fitted by calling Stan 2.19.3 (Carpenter et al., 2017) from R 4.0 (R Core Team, 2018) using brms 2.13 (Bürkner, 2017) interface. Models had no intercepts with indexing approach to predictors (McElreath, 2019). Default weakly informative normal(0, 1) priors were provided by brms according to Stan recommendations (Stan Development Team, 2019). Three Markov chain Monte Carlo (MCMC) chains of 40,000 iterations were simulated, with a warm-up of 20,000 runs to ensure that effective sample size for each estimated parameter well exceeded 10,000 (Kruschke, 2015), producing stable estimates of 95% highest posterior density continuous intervals (HPDCI). MCMC diagnostics were performed according to Stan manual.

Median of the posterior distribution, 95% HPDCI and adjusted $P$-values are reported as computed by R package emmeans 1.4.7 (Searle et al., 2012). Adjusted $P$-values were produced by frequentist summary in emmeans using the multivariate $t$-distribution with the same covariance structure as the estimates. The differences between groups were defined as significant if the corresponding 95% HPDCI interval did not include zero and adjusted $P$-value was $\leq 0.05$. R scripts are available upon request.
3 RESULTS

Expression levels were analysed in the left and right halves of the lumbar spinal cord of intact rats, and rats exposed to the left- and right-side sham surgery, or left- and right-side UBI. Significant differences in the same direction between the intact control group and each the UBI group and respective sham surgery group were considered as effects of body injury induced by surgical procedures including a craniotomy, whereas those between the UBI group and respective sham surgery group as the effects of brain injury.

3.1 Expression levels of RAS genes: UBI effects and left–right side differences

The RAS genes Agt, Ren, Ace, Ace2, Agtrap, Agtr1a, Agtr1b, Agtr2 and Mas1 were analysed. Expression levels of Agtr1a, Agtr1b and Ace2 RAS genes were below the detection level in the lumbar spinal cord and were not further analysed. The Kolmogorov–Smirnov and Levene’s tests revealed substantial deviations from normality and difference in the variances between the rat groups, respectively, for several genes, and therefore, Kruskal–Wallis test was used for further analysis. No significant differences in expression levels of six RAS genes including Agt, Ren, Ace, Agtrap, Agtr2 and Mas1 between the intact control, left- and right-side sham surgery and left- and right-side UBI groups were revealed both for the left- and right-side spinal samples, which were analysed separately.

In the absence of significant differences, the data for five animal groups were combined into the left and right spinal cord samples, and the expression levels were compared between the samples. Wilcoxon matched-pairs test revealed significantly higher expression levels of the Ace (Z = 3.6, adjusted P = 0.002), Agtr2 (Z = 2.6, adjusted P = 0.05) and Ren (Z = 4.9, adjusted P = 1.0 × 10⁻⁶) genes in the left spinal half compared with the right spinal half (Figure 2). The expression levels of the six genes for each of the five animal groups are shown in Figure 1S.

3.2 Co-expression patterns of RAS genes

We next compared the intra- and inter-area coordination of gene expression between the left and right spinal cord. We examined whether expression of the RAS genes correlated within the left and right halves of the lumbar spinal cord and between these halves and whether UBI affects RAS gene–gene co-expression patterns. The aggregated level of co-regulation of expression, or, in other words, the gene–gene coordination strength was characterized by analysis of the sum of correlation squares. Note that analysis of the coordination strength disregarded both correlation signs and changes in the proportion of positive and negative correlations; the proportion was then analysed separately in the following sections.

Spearman’s rank correlations between expression levels of six RAS genes (15 correlations corresponding to 15 gene pairs) were calculated for each of five animal group separately, and in each group separately for the left and right halves. No significant differences between the groups in the gene–gene coordination strength (Kruskal–Wallis test) were revealed. In the absence of significant intra-areas differences, the groups were pooled together and the data were analysed separately for the left and right spinal halves. Analysis of Spearman’s rank correlations in the pooled sample identified seven and two nominally significant pairwise correlations in the right (Ace-Agt, Ace-Agtrap, Ace-Ren, Agt-Agtrap, Agtrap-Ren, Ace-Agtrap and Agtrap-Agtr2) and left (Agt-Agtr2 and Agt-Agtrap) spinal halves, respectively (Figure 3a). Four of the right side (Ace-Agtrap, Agt-Agtrap, Agtrap-Ren and Agtrap-Agtr2) and none of the left side correlations remained significant after Bonferroni corrections (Figure 3b). Correlation between Agt-Agtrap was significant in both left and right spinal cord.

The strength of coordination between gene expressions, or, in other words, the aggregated level of intra-area co-regulation of expression, was assessed by analysis of the sum of correlation squares. The level of coordination was 5.5 times higher in the right half compared with the left half (Wilcoxon test, P = 0.0018; Figure 3b), suggesting that expression of the RAS genes is much stronger coordinated in the right spinal cord.

Analysis of interregional (left side vs. right side) Spearman’s rank correlations between expression levels of RAS genes revealed no significant differences between the five animal groups in the gene–gene coordination strength (Kruskal–Wallis test).

3.3 Co-expression of the RAS and neuroplasticity-related genes: Analysis of the coordination strength

Assuming that functions and regulations of the neuro-hormonal RAS and neuroplastic processes in the lumbar spinal cord may be interdependent, we assessed if the left- and right-side injuries produced effects on co-expression patterns of the RAS–neuroplasticity-related genes. Intra-regional Spearman’s rank correlations between expression levels of the six RAS genes and 13 plasticity-related genes (78 correlations corresponding
to 78 gene pairs) were calculated for the left and right spinal halves. The gene–gene coordination strength was compared among (i) the intact control group, (ii) the left-side impact group consisted of the left UBI and left sham surgery rats, and (iii) the right-side impact group consisted of rats with the right UBI and right sham surgery (Figure 4). We presumed that differences between the intact control group and the UBI group, and between the intact control group and respective sham surgery group, if they occurred in the same direction, were due to the effects of body injury (e.g. by tissue incision or inflammation).

No significant differences between the intact control rats and the left-side impact group in both left and right spinal halves were evident. In contrast, the right-side impact substantially reduced the gene–gene coordination strength compared with the intact control group in both left (multiple comparison test, \( P_{\text{adj}} = 6.2 \times 10^{-6} \); 3.7-fold) and right (multiple comparison test, \( P_{\text{adj}} = 2.1 \times 10^{-4} \); 2.6-fold) spinal cord (Figure 4a). Furthermore, the coordination strength was significantly lower in the right-side impact group compared with the left-side impact in both the left (\( P_{\text{adj}} = 2.1 \times 10^{-3} \); 2.5-fold) and right (\( P_{\text{adj}} = 0.016 \); 2.0-fold) spinal cord (Figure 4a). The intra-regional coordination strength further analysed in the five rat groups did not reveal the UBI effects and did not lead to meaningful biological patterns.

Thus, the coordination pattern between the RAS and neuroplasticity-related genes was similar in the left and right spinal cord in intact control animals, and it was similarly impaired on both spinal sides in rats with the right sham surgery or right UBI, but not in rats with the left-side impact (the left sham surgery and left UBI rats). This pattern is visualized on the heatmap for intra-area Spearman’s rank correlations in the three rat groups (Figure 4b).
3.4 The coordination of RAS genes with genes involved in neuroplasticity: Analysis of the proportion of positive and negative correlations

Analysis of the coordination strength did not take into account changes in the signs of the correlations. We next examined if UBI or sham surgery induces changes in the proportion of positive and negative intra-area correlations between the six RAS genes and 13 neuroplasticity-related genes for three group of rats. The number of rats and RNA samples used in analysis of the RAS gene expression in the left and right spinal cord tissue is shown in Table S1. Neuroplasticity-related genes were analysed in RNA preparations from the left and right spinal cord tissue of all rats of five groups (intact group, left and right sham groups and left and right UBI groups; n = 10 rats/group).

The positive intra-area correlations dominated in all five rat groups, suggesting that RAS and plasticity genes are positively co-regulated (Figure 5a). The proportion of positive intra-area correlations was reduced after UBI on the contralesional side in comparison with intact control group (median of the posterior distribution as estimate [median] = −0.178, 95% HPDCI = [−0.278, −0.073], P = 0.006) and sham surgery (median = −0.187, 95% HPDCI = [−0.289, −0.084], P = 0.003) groups and on the ipsilesional side in comparison with intact control group (median = −0.18, 95% HPDCI = [−0.283, −0.078], P = 0.005) but not sham injury group (Figure 5b). Thus, the UBI significantly decreased the proportion of positive correlations between the RAS and neuroplasticity-related genes in the contralesional lumbar spinal cord.

4 DISCUSSION

This study uncovers the lateralized expression and co-expression patterns for the RAS genes and their coordination with neuroplasticity-related genes in the lumbar spinal cord. The principal findings include (i) an asymmetric expression of the RAS genes Ace, Agtr2 and Ren with higher levels on the left side (Figure 6a); (ii) the coordination of expression patterns of RAS genes that is higher on the right side (Figure 6b); (iii) the decay in coordination of RAS and neuroplasticity-related gene expression induced by both the right-side sham surgery and right-side brain injury (Figure 6c); and (iv) the UBI-induced downward shift in the proportion of positive correlations between the RAS and neuroplasticity-related genes on the contralesional side (Figure 6c).

The asymmetric expression of the RAS genes in the spinal cord fits to the pattern identified for the genes of the glutamate and opioid systems (Kononenko et al., 2017, 2018; Zhang et al., 2020). Expression of the Grin2b
and Oprd1 genes that code for the NMDA receptor subunit and δ-opioid receptor, respectively, in the lumbar spinal cord and expression of the opioid receptors and opioid peptide precursors in the cervical spinal cord were lateralized to the left. Thus, three RAS genes along with all these lateralized genes demonstrated higher left-side vs. right-side expression.

The RAS genes, whose individual expression levels were not significantly affected by brain lesion, were dysregulated by both UBI and unilateral sham surgery in coordination of their co-expression with neuroplasticity-related genes. Co-expression of RAS–neuroplasticity-related genes was affected by the right-side but not left-side impact by sham surgery or UBI; the coordination strength was declined in both the left and right lumbar spinal halves (Figure 6). The differences between the intact rats and rats with either UBI or sham surgery were likely due to tissue incision and inflammation induced by these procedures. Both sham surgery and UBI included a craniotomy that damaged tissues and produced inflammatory response (Cole et al., 2011) that likely affected gene expression patterns. These data replicated the decline in the coordination of expression of the opioid genes in the cervical spinal cord that was induced by the right-side unilateral traumatic brain injury or right-side unilateral sham surgery, although no significant effects of the left-side injuries were evident (Kononenko et al., 2017).

The UBI group compared with both intact rats and sham surgery rats exhibited the decreased proportion of positive correlations between the RAS and neuroplasticity-related genes on the contralesional spinal side. The pre-existing positive interactions between the expression patterns of the RAS and neuroplasticity-related genes were likely transformed to more negative interactions between these gene groups that may be characteristics of the pathological state.

Changes in coordination strength and proportion of positive correlations is an integral picture reflecting alterations in cells of the same type or different types in a tissue or brain area (Dobrin et al., 2009; Kononenko et al., 2017; Long et al., 2016; Zhang et al., 2020). The RAS and neuroplasticity-related genes may be expressed in the same or different cell types and subtypes. Angiotensin AT1a, AT1b and AT2 Type 1a receptors were detected in neurons (Lenkei et al., 1996; Premer et al., 2013; Rodriguez-Pallares et al., 2008; Saavedra, 1999), whereas AT1b also in glia (Premer et al., 2013). Expression of Mas1 receptor was localized to both neurons (Freund et al., 2012) and glial cells (Regenhardt et al., 2013). Renin was detected in neurons in the brainstem and glia in the hypothalamus and cortex (Morimoto et al., 2002). Angiotensinogen is mostly produced by glia (Paul et al., 2006; Stornetta et al., 1988). Ace is expressed in neurons but not in astrocytes and microglia cells in the superficial dorsal horn (Ogata et al., 2016). Interactions between the cells and cell assemblies that are mediated by extracellular signalling molecules may be a prerequisite for coherent regulation of the RAS and neuroplasticity-related genes in the spinal cord. Angiotensins as neurohormones have a role in modulation of neural circuits; they are produced within the circuits and activate transcription of Bdnf and Gap43, resulting in different behavioural phenomena (Bernstein...
et al., 2014; Hobara et al., 2007; Schwengel et al., 2016; Yang et al., 2001). Coherent regulation of neural circuits by paracrine and autocrine angiotensin signalling is an intriguing possibility considering lateralization of the RAS along with the RAS lateralized responses to unilateral body and brain injuries.

Co-regulation by the same transcription program(s) may underlie co-expression patterns of the RAS genes and the RAS and neuroplasticity-associated genes that may be transcribed in the same or different cells. Further in silico analysis of cis-elements aimed to identify binding sites for common transcription factors in promoters of these genes, followed by experimental verification of the predictions is required to understand the co-expression patterns described in the study.

The left and right sides of the central nervous system including the lumbar spinal cord are grossly anatomically similar. Consistently, identified left–right molecular differences were mostly subtle, of the order of 1.3-fold (de Kovel et al., 2018; Karlebach & Francks, 2015; Kononenko et al., 2017; Marlin et al., 2015; Messina et al., 2021; Watanabe et al., 2015, 2021; Wu et al., 2005; Zhang et al., 2020). In contrast, the left–right differences revealed by the gene-set and gene co-expression analyses were much greater, reproducible and highly significant compared with those in expression of individual genes (Abdolmaleky et al., 2019; de Kovel et al., 2017, 2018; Karlebach & Francks, 2015; Kononenko et al., 2017; Ocklenburg et al., 2017; Watanabe et al., 2021; Zhang et al., 2020). Having revealed lateralization of individual RAS genes that was between 1.3- and 2.0-fold, we put the emphasis in this study on co-expression patterns as a more sensitive approach to detect the left–right differences. This approach was based on analysis of correlations among RAS genes and among these RAS genes and plasticity-related genes. This analysis identified strong 2.0–5.5-fold left–right differences in all (i) the coordination of expression among RAS genes and among RAS and neuroplasticity-related genes and (ii) the structure of RAS–neuroplasticity-related gene interactions (Figure 6). To analyse gene–gene correlations, gene expression levels were measured in RNA preparations from the bulk tissue samples by RT-PCR that is a quantitative molecular technique. This approach allowed to correlate levels of expression of multiple genes that may be expressed in the same or different cell types in the area of interest. A limitation was that this approach does not have anatomical specificity and does not identify lateralized neural circuits. On the other hand, methods with anatomical resolution including in situ hybridization and immunohistochemistry do not allow analysis of multiple gene–gene correlations and gene sets and therefore could not be applied for analysis of lateralized gene–gene co-expression patterns and gene networks. Furthermore, single-gene targeted in situ hybridization or other single-gene in situ methods are not considered as a promising avenue to follow-up analyses in the lateralization studies (de Kovel et al., 2018). Canonical two-dimensional snapshot variants of these methods and even construction of three-dimensional gene expression maps may be misleading in quantitative analysis of lateralized expression due to problems with the background and normalization and due to imperfect brain symmetry, left–right differences in cell proportions and technical challenges in mounting on slides perfectly anatomically symmetrical slices (de Kovel et al., 2018; Morandi-Raikova et al., 2021).

5 | CONCLUSIONS

We demonstrated that expression and co-expression patterns of the RAS genes are side specific in the lumbar spinal cord. Furthermore, the expression patterns of the
RAS genes are tightly coordinated with expression of the neuroplasticity-related genes. This coordination undergoes robust changes upon transition to a pathological condition induced by a remote unilateral sham surgery or brain lesion, and these alterations are polarized in the left-right or ipsi-/contralesional directions. In bilaterally symmetric animals, most genes in the left- and right-side tissues are expressed at the same levels and may be regulated by the same mechanisms (Sladitschek et al., 2020). However, as revealed in this and previous studies, expression and co-expression patterns for the RAS, opioid and glutamate genes are different between the left and right hemispheres and/or symmetric spinal cords (Kononenko et al., 2017; Lukoyanov et al., 2020; Watanabe et al., 2020; Zhang et al., 2020). This lateraled neurohormonal and neurotransmitter mechanism may control the balance in activity of the left- and right-sided processes and may mediate the effects of the unilateral brain injury on spinal sensorimotor functions (Lukoyanov et al., 2020; Watanabe et al., 2020; Zhang et al., 2020).

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ETHICAL STATEMENT
All the experimental procedures were carried out in accordance with the European Union guidelines 86/609/EEC for the use of experimental animals and local legislation for ethics of experiments on animals. Approval for animal experiments was obtained from the Malmö/Lund ethical committee on animal experiments (No. M7-16) and the ethical committee of the Faculty of Medicine of Porto University and Portuguese Direcção-Geral de Alimentação e Veterinária (No. 0421/000/000/2018).

AUTHOR CONTRIBUTIONS
GB, MH and ON designed research; GB, ON, AK and NL analysed data; AK, HW, NL, LSC and ON performed research; GB, MH and ON wrote the paper; DS and VG performed statistical treatment.

PEER REVIEW
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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.14401193.v1.

ORCID
Olga Nosova https://orcid.org/0000-0002-1332-7067

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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