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Mineralocorticoid and glucocorticoid receptors in sciatic nerve function and regeneration

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ABSTRACT

The contribution of the two corticosteroid (mineralocorticoid and glucocorticoid) receptor (MR and GR) pathways to the function and regeneration of the sciatic nerve was investigated. We found that the corticosterone-inactivating enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD2) was up-regulated 7 days after lesion in freeze-injured nerve.

The maintenance of a low intracellular level of corticosterone by HSD2 activity in the regenerating nerve is concordant with the improvement of nervous function in injured animals (as measured by walking ability) after treatment by the GR antagonist mifepristone and with the reduction in GR participation in accumulation of the mRNA for numerous endogenous genes (from the renin–angiotensin system and other classical mineralocorticoid-responsive genes), in the same animals. Furthermore, using the MR antagonist spironolactone, we demonstrated that MR plays an active role in the function of the intact sciatic nerve: MR is required for walking ability and participates in the control of the accumulation of the mRNA for several endogenous genes. However, after injury, changes in gene expression cannot be fully explained by changes in MR/GR activity, due to an HSD2 effect, and other signalling pathway(s) induced by the lesion likely combine with the effect of the corticosteroid receptors.

1. Introduction

In a previous work, we demonstrated that the glucocorticoid signalling pathway is fully active in cultured Schwann cells (SC), and that the glucocorticoid inactivating enzyme 11β-hydroxysteroid dehydrogenase type 2 (HSD2) is significantly expressed in quiescent SC and is strongly down-regulated by forskolin (Fk) [1], which increases the intracellular cAMP concentration. The type 2 enzyme oxidizes the 11β-hydroxyl to an oxo group and converts corticosterone to 11-dehydrocorticosterone. This result suggested that SC are able to regulate their own intracellular levels of glucocorticoids *via* cAMP-regulated HSD2 expression. Variations in cAMP during development and in response to nerve injury could thus physiologically modulate the anti-inflammatory, immunosuppressive and developmental control exerted by glucocorticoids and possibly allow aldosterone (or corticosterone) to execute a specific gene expression program upon formation of high affinity complexes with the mineralocorticoid receptor (MR). Therefore, to specify GR action and to determine if and how MR can intervene, we decided to investigate the expression and regulation of endogenous genes in SC cultures and *in vivo* in peripheral nerves. We chose to examine genes known to be mineralocorticoid-responsive in specialized epithelia, and genes of the renin–angiotensin system (RAS). Most of these genes could be also glucocorticoid-responsive, depending on the tissue concerned.

The existence of components of the RAS in cultured SC (local synthesis of angiotensinogen, ATG, and renin) and in the injured nerve (increased expression of the angiotensin II receptors, ATR1a and ATR2) has been already described [2,3]. In addition, applied near an injury, angiotensin II has been reported to accelerate functional recovery *via* the ATR2 receptor [4]. A relationship between production of angiotensin II and aldosterone has been reported: several extra-adrenal tissues displaying local RAS activity have been suspected to locally produce aldosterone [5] and, reciprocally, in different tissues as in the hypothalamus, aldosterone up-regulates the RAS [6]. Moreover, angiotensin II has been shown to act *via* ATR1 and MR in human coronary smooth muscle cells [7].

Polarized cells of kidney and colon and myelinating SC of peripheral nerves possess interesting analogies. SC exhibit precise patterns of ion channel distribution in the nodal and paranodal regions and form specialized junctions [8,9]. They express Na/K-ATPase, a membrane protein complex regulating elec-

Abbreviations: HSD2, 11β-hydroxysteroid dehydrogenase type 2; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; Fk, forskolin; HSD1, 11β-hydroxysteroid dehydrogenase type 1; SC, Schwann cell(s); RAS, renin–angiotensin system; ATG, angiotensinogen; ATR1a, angiotensin receptor type 1a; ATR2, angiotensin receptor type 2; ENaC, epithelial sodium channel; PR, progesterone receptor; p.i., post-injury.

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trolyte homeostasis [10]. In the nephron, Na/K-ATPase allows Na+ extrusion from the basolateral membrane. It participates in the formation of tight junctions and induction of polarity in epithelial cells [11–13]. In the nerve, Na/K-ATPase is involved in the recovery of electric potential after propagation of the action potential but also in signal transduction, monitoring important cellular events such as proliferation, apoptosis, cellular interactions and migration [14]. The specific role of several of the ATPase subunits in the nerve regeneration process was underlined by the altered composition of the Na/K-ATPase in the injured sciatic nerve. However, data on the subunit composition diverge according to reports [15,16]. In the nephron, the epithelial partner for driving Na+/K+ homeostasis is the α subunit of the epithelium sodium channel (ENaCα), which mediates Na+ entry at the apical membrane of the cell. In mineralocorticoid-target epithelia, aldosterone could at least partly act on the transcription/mRNA accumulation of ENaCα. In retinal glial cells (Müller cells), ENaCα and MR expression have been reported. In addition, ENaCα mRNA accumulation was stimulated by aldosterone, consistent with a role for mineralocorticoids in the regulation of the extracellular Na+ [17].

In order to define the genomic role of MR and GR in the course of regeneration, the following parameters have been successively examined. First, HSD2 activity and MR and GR expression in the intact and regenerating nerve were estimated, and correlations between HSD2 level and global concentrations of corticosterone and cAMP were investigated. Second, regulation by corticosteroids was studied by following the mRNA accumulation from the two groups of genes (i.e. genes of the RAS and of polarized epithelia). Direct regulation by corticosteroids was analyzed in SC after administration of aldosterone or RU28362 (agonists of MR and GR, respectively) in SC cultures. Moreover, the *in vivo* effects of mifepristone (RU38486, RU486 thereafter) and spironolactone (transcriptional inhibitors of GR and MR, respectively) were evaluated on mRNA accumulation and walking ability in intact and 7-day lesioned rats. Results provide new important information on the functional modalities of the two corticosteroid receptors and physiological importance of these two groups of genes.

2. Materials and methods

2.1. Surgical procedures

Male Sprague–Dawley rats (7–8-week old) were purchased from CERJ (Le Genest St Isle, France). Animal procedures were performed in accordance with the guidelines of the IASP Committee for Research and Ethical Issues. Prior to surgery, rats were anaesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). The right sciatic nerve was cryolesioned as previously described [18]. Rats were sacrificed 3, 7, 15 or 21 days after surgery. The proximal part (next to dorsal root ganglia), and the distal part of the injured nerve (including the lesion site) or the whole nerve were aseptically collected.

2.2. Schwann cell (SC) cultures

SC from 2- or 3-day-old Sprague–Dawley rat sciatic nerves were purified and propagated as previously described [1,19]. Different preparations were frozen and used after thawing and 3-fold divisions. Cells were incubated 48 h in charcoal/dextran-coated extracted foetal calf serum containing insulin (5 μg/ml) with or without Fk (10 μM) before addition of steroids (10 nM aldosterone or pure GR agonist RU28362 (17α-1-propynyl-11β, 17β-dihydroxy-6-methyl-androsta-1,4,6-triene-3-one) in ethanol) or ethanol alone. Aldosterone was from Sigma and RU28362 was donated by Hoechst-Marion-Roussel (now Aventis). Dose-response curves for mRNA accumulation were performed after a 4h30 incubation period with increasing aldosterone concentrations, giving EC50 values between 0.1 and 1 nM for several studied genes (renin B, ATR2 and Na/K-ATPase α3-subunit). In this work, a concentration of 10 nM aldosterone was used, above the Kd value ~0.5 nM for aldosterone binding to MR [20], but in the range of the rat plasma concentration when aldosterone synthesis is stimulated. Plasma concentrations of 0.47–1.04 nM in normal rats [21,22] can reach 4.4–14.4 nM in rats under a low-salt diet [22,23]. The 10 nM aldosterone concentration remains two low to activate GR [20].

2.3. Measure of cAMP concentrations

Each nerve was powdered under liquid nitrogen and homogenized in 5% trichloroacetic acid (TCA) in a teflon/glass Potter-Elverjehm homogenizer (1–2 ml/10 mg). After 15 min centrifugation at 6500 × *g*, supernatants were taken. TCA was eliminated by 5 extractions with diethyl ether saturated with 1N HCl (4 vol) and the final aqueous phase was dried at 60 °C under an air stream. After addition of 200 μl buffer (Tris 20 mM, EDTA 4 mM, pH7), the cAMP content was determined using the [^{3}H]cAMP Biotrak assay system (TRK432) from GE Healthcare. The protein content of the pellet was determined with a BCA kit (Perbio).

Immunohistological analysis of cellular cAMP content was performed using 1/1000 rabbit anti-cyclic AMP polyclonal antiserum (Chemicon) in phosphate buffer saline-tween (PBST), followed by 1/200 goat anti-rabbit Alexa Fluor 488 antibody (Invitrogen, Life technologies, Paisley, UK).

2.4. Antagonist treatments and the walking ability test

Control and injured rats were treated or not by spironolactone (17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone acetate: 20 mg/kg/day) or mifepristone (RU486, 11β-[*p*-(dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one: 100 mg/kg/day). Suspensions in 0.5% methylcellulose were administrated by daily gavage (0.35 ml) from the day following sciatic nerve cryolesion until 1 day before sacrifice (day 2 to day 6). On days 6 and 7, walking ability was tested using the "locotronic" device (Intellibio, Nancy, France) that automatically counted the hind footfalls (errors) during rat walking along an horizontal scale. Previous work has established an inverse relationship between the number of errors and time after injury provided regeneration occurs [18].

2.5. Measure of corticosterone concentration

Corticosterone was extracted from nerves or plasmas and fractionated by HPLC and then derivatized with heptafluorobutyric anhydride before analysis by GC/MS (Trace GC gas chromatograph Carlo Erba, Milan, Italy coupled to an Automass Solo mass spectrophotometer, Thermo Electron, Les Ulis, France) as previously described [24].

2.6. Quantitative RT-PCR

Total RNA preparation and coding strand cDNA synthesis was performed as previously described using 250 ng nerve total RNA [18]. PCR experiments were carried out using a real time PCR apparatus ABI PRISM 7000 (Applied Biosystems). Assays were performed in 20 μl according to Applied Biosystems' recommendations with the cDNA from 10–20 ng total RNA for the studied mRNAs and 10–20 pg total RNA for standard 18S RNA. We used Taqman methodology for rat Na/K-ATPase-α1, α2, α3, β1, β2 subunits and γ subunits FXYD4 and FXYD7 (Rn00560766, Rn00560789, Rn00560813, Rn00565405,

Rn00560819, Rn00574355 and Rn00469666, respectively), ENaCα, β and γ subunits (Rn00580652, Rn005561892, Rn00566891), HSD1 (Rn00567167) and HSD2 (Rn00492539), Renin A and B (Rn00561847 and Rn01490672), ATG (Rn00593114), ATR1a and ATR2 (Rn00578456 and Rn00560677), CYP11B1 and CYP11B2 (Rn02607234 and Rn02396730), 18S (Hs99999901) and SYBR-Green technology for the 3 house-keeping genes: glyceraldehyde 3-phosphate dehydrogenase, GAPDH (forward primer: TGATTCTACCCACGGCAAGT; reverse primer: AGCATCACCCCATTTGATGT), actin (forward primer: TATGAGCTGCCTGACGGTC; reverse primer: AGTTTCATGGATGCCACAGG) and hypoxanthine-guanine phosphoribosyltransferase, HPRT (forward primer: GCACGAGGGACTTACCTCAC; reverse primer: CTAATCACGACGCTGGGACT). Quantification of mRNA was performed by the ΔCt method (normalization by subtracting the geometric mean of cycle thresholds (Ct) of actin, GAPDH and HPRT for kinetic studies or Ct of standard 18S for the antagonist study, from the Ct of the gene of interest). The two normalization procedures gave very close ΔCt values. Estimation of the corresponding mRNA content was made by calculation of $2^{-\Delta Ct}$. Since the same efficiency was expected ($E \sim 1$) for the different genes (User Bulletin 2, Abi Prism 7700 Sequence Detection System) and the same threshold value was used for Ct determination of all genes, a rough comparison in contents of the different mRNA can be made [25]. For convenience, all $2^{-\Delta Ct}$ values were multiplied by 1000, giving arbitrary units. For SC, one arbitrary unit corresponds to 523 mRNA sequences/ng total RNA or 1.3 sequence/cell.

2.7. HSD2 activity in nerve explants

A piece of nerve (1 cm) was carefully removed and relieved of epineurium before being minced in a drop of physiological serum. Nerve fragments were incubated 6 h at 5% CO_2 and 37 °C in 1.5 ml of DMEM-glutamax containing 2% foetal calf serum, 6.67 nM ^{3}H-corticosterone and 100 μM NAD. Supernatants were removed and extracted with 15 ml methanol containing 1.5 mg 3-ter-butyl-4-hydroxy-anisol and 50 μM corticosterone and 11-dehydrocorticosterone as carriers. After centrifugation at 4500 rpm for 10 min, the supernatant and pellet wash were concentrated to ~100 μl and layered on a thin layer fluorescent chromatography plate for migration with chloroform/methanol/water (100:5:0.1). The radioactivity co migrating with the dehydrocorticosterone peak in the profile obtained with a Tracemaster 20 apparatus (Berthold, Thoiry, France) was expressed as percentage of total slot radioactivity and normalized to the initial corticosterone concentration.

2.8. Statistical analysis

Values reported are means ± SEM. Two-way variance analyses (ANOVA, Prism software, Graphpad) were performed, followed by Bonferroni post-tests. For comparison of two unpaired groups, the Mann–Whitney test or *t*-test with the Welch correction were used as indicated. *P* values of 0.05 or less were considered as significant.

3. Results

3.1. Maximal accumulation of 11β-hydroxysteroid dehydrogenase type 2 (HSD2) mRNA and increased HSD2 activity in the regenerating nerve, 7 days after cryolesion

We have already described the expression of HSD2 mRNA and the lack of detection of 11β-hydroxysteroid dehydrogenase type 1 (HSD1) mRNA in pure SC cultures using the SYBR-Green technology [2]. These results were reproduced with Taqman probes and the study extended to the whole nerve. In the intact nerve, HSD2 mRNA was significantly expressed at a level corresponding to that observed in SC cultured without Fk. HSD1 mRNA was also expressed in the nerve, an expression likely related to synthesis in cells other than SC. In fact, fibroblasts and macrophages contain significant levels of HSD1 mRNA [26,27], as do co-cultures of SC and fibroblasts (F.C. unpublished).

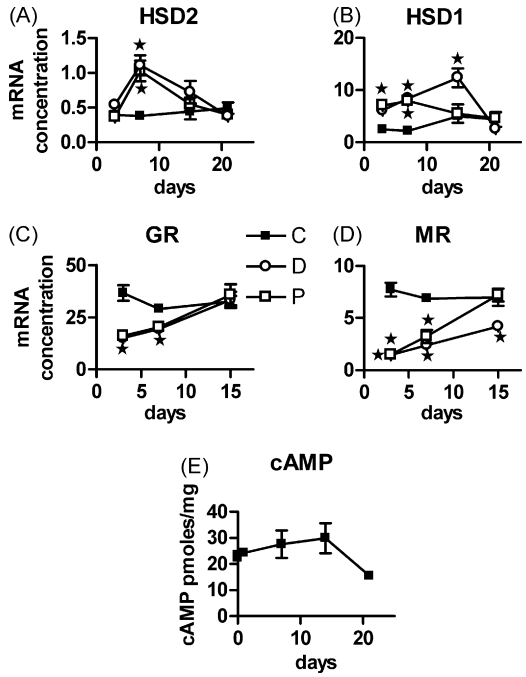

Fig. 1. Levels of 11β-hydroxysteroid dehydrogenase type 1 and 2 (HSD1 and HSD2) mRNA, cAMP and glucocorticoid and mineralocorticoid receptor (GR and MR) mRNAs in the nerve as a function of time (days) after cryolesion. (A) HSD2; (B) HSD1; (C) GR; (D) MR and (E) cAMP. Control (C): ■, proximal segment (P): □, distal segment (D): ○. cAMP in intact (time = 0) and in injured nerve: ■. Values are means ± SEM, $n = 7$–8 nerves except for cAMP $n = 3$–4 nerves. *Significant difference from control, $p < 0.05$, variance analysis (ANOVA, two factors) and Bonferroni post-test.

Between 3 and 7 days after cryolesion, HSD2 mRNA levels significantly increased (~3-fold) in proximal and distal parts of the nerve, followed by a progressive decrease at 15- and 21-day post-injury (p.i.) (Fig. 1A). The equivalent HSD2 mRNA accumulation in both parts of the nerve agrees with a global response of SC to cryolesion. The stimulation of HSD1 differed, with a main late increase in the distal part (Fig. 1B). HSD2 activity was also 2-fold higher in 7- and 15-day lesioned nerve explants (5.04 pmoles/nerve/6 h and 4.95 pmoles/nerve/6 h at 7 and 15 days, respectively) than in the intact nerve explants (2.5 pmoles/nerve/6 h and 2.67 pmoles/nerve/6 h at 7 and 15 days, respectively). Since the number of SC was similar in the intact and lesioned nerve 7 days after lesion (M. Sineus and G.G., unpublished data), the global increase in HSD2 activity was due to an increased activity in each SC.

3.2. Fall then recovery of corticosteroid receptor mRNA concentrations after cryolesion

Since HSD2 levels clearly increased after cryolesion, we wondered if the local inactivation of glucocorticoids was associated with changes in the expression of corticosteroid receptors.

Measurements of GR and MR mRNAs in the intact nerve showed a higher expression (30.5 and 6.7 arbitrary units) than previously reported for cultured SC (corresponding to 15.3 and 0.86 arbitrary units; ~8000 and 450 sequences/ng RNA [1]). Thus, the concentration in the nerve was doubled for GR and was ~8-fold higher for MR and the relative concentration of MR mRNA reached ~22% that of

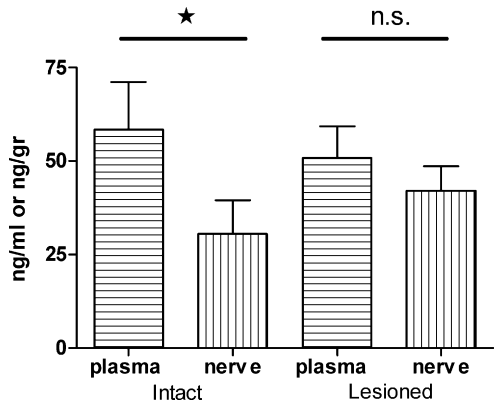

Fig. 2. Corticosterone concentration in plasma and nerve of intact and injured nerves. Independent plasma samples from 16 control animals and 16 lesioned animals and pools of 8 nerves (8 pools for intact nerves and 2 for lesioned nerves) were extracted and prepared for gas-chromatography/mass spectroscopy (GC/MS) analysis. Values represent means ± SEM. * indicates significant difference using the *t*-test with the Welch correction, $p < 0.05$; n.s.: non-significant.

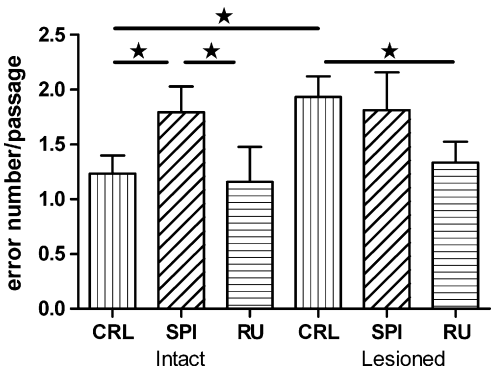

Fig. 3. Walking ability of intact and injured rats after treatment with receptor antagonists (spironolactone and mifepristone). Rats were treated or not by daily gavage (day 2 to 6) with 100 mg/kg/day mifepristone (RU486) or 20 mg/kg/day spironolactone. On day 6 and 7, 4 animals per group (8 for control) were allowed to walk 4–5 times successively in a locotronic device which recorded errors in hind leg position. Values represent means ± SEM. * indicates significant difference using the Mann–Whitney test, $p < 0.05$.

GR mRNA in the intact nerve. Such an expression level substantiates a critical role for this receptor in peripheral nerve physiology.

Three days after cryolesion, both GR and MR mRNA levels dropped respectively to 40% and 19% of control (Fig. 1C and D). At 7 days p.i., mRNA levels were partially restored in both parts of the nerve. At 15 days p.i., control levels were recovered except for MR in the distal part (Fig. 1D). These data showed that the expression of GR and MR followed the course of Wallerian degeneration. The recovery of GR occurred simultaneously in the two nerve fragments, while the delayed recovery of MR expression at 15 days in the distal part suggests a relationship between MR synthesis and progressive axonal regeneration along the nerve.

These data show that the content of GR mRNA was high enough, as early as 7 days p.i., to promote efficient gene expression (equivalent to the GR content in SC) and suggest an activity for MR.

3.3. No decrease in intracellular cAMP and small change in nerve corticosterone concentration following cryolesion

To determine whether the increase of HSD2 mRNA in the regenerating nerve could be related to changes in cAMP concentration, as previously demonstrated in SC cultures, we measured the cAMP concentration at different times after nerve cryolesion. Control (10-week old) rat nerves contained ~24 pmoles cAMP/mg protein, a value close to that estimated for SC cultured without Fk (1.9 pmoles/10^6 SC corresponding to 13.6 pmoles/mg). After cryolesion, the mean concentration of cAMP did not change significantly, although an increase was observed in some animals, resulting in a low mean increase to ~30 pmoles/mg protein. Later, at 21 days, a decrease to 15.4 pmoles cAMP per mg protein was recorded (Fig. 1E). From 7 days after cryolesion, we observed a moderate increase by immunohistological analysis of longitudinal nerve slices (20 μm) using a specific antibody against cAMP (not shown). Thus, in the nerve, the increase in HSD2 activity is not correlated with a global decrease in cAMP.

The *in vivo* effect of HSD2 in oxidizing corticosterone was investigated by measuring the corticosterone concentrations in the plasma and in the nerve of intact and 7-day injured animals. They were significantly higher in the plasma (58.4 ± 12.9 ng/ml) than in the nerve (30.5 ± 9 ng/g) of intact animals, (Fig. 2). At 7 days after cryolesion, the nerve concentration was closer to that of plasma and the difference between plasma and nerve became non-significant (50.9 ± 8.5 ng/ml and 42 ± 6.7 ng/g). The increase in HSD2 activity observed at 7 days in injured animals may contribute to counteract the nerve corticosterone increase induced by lesion. Such a mechanism may reduce, at the intracellular SC level, the unfavourable effect of corticosterone on events such as dedifferentiation occurring during regeneration.

Moreover, the lack of detection of the mRNAs for CYP11B1 and B2, the terminal enzymes involved in the synthesis of corticosterone and aldosterone, respectively, ruled out any local synthesis from 11-deoxycorticosterone (F.C., not shown).

Since at 7 days after cryolesion, expression of GR and MR mRNA were increasing, the role of HSD2 could be to reduce the efficiency of the GR signalling pathway in favour of the MR pathway. To test this hypothesis, we chose to analyze the expression of selected endogenous genes in different groups of rats: intact and lesioned, treated or not with receptor antagonists. In the same rats, we first evaluated the functional walking ability using the locotronic device.

3.4. Effect of mineralocorticoid and glucocorticoid receptor (MR and GR) inhibitors on walking ability

Some steroid analogues display antagonist properties by inhibiting the agonist-induced transcription *via* binding to receptors. Mifepristone (RU486) antagonizes both mammalian GR and progesterone receptor (PR). The PR mRNA content in the intact and lesioned nerve (0.13 arbitrary units) is still lower than in SC (0.24 arbitrary units corresponding to 125 mRNA sequences/ng total RNA or 0.3 sequence/cell). It is ~235-fold lower than the nerve GR mRNA content and thus confirms a minor participation of PR, if any, in the mifepristone effect. Similarly, spironolactone inhibits the mineralocorticoid effect *via* MR. Although some side and indirect effects cannot be eliminated, this approach has already permitted the demonstration of involvement of steroid receptors in various biological events [28].

We analyzed the effect of antagonists on walking ability in intact and lesioned nerve. As expected, sciatic nerve cryolesion was followed by an increase in error number 7 days p.i. (Fig. 3). In intact rats, the inhibition of the MR signalling pathway by spironolactone triggered an increased number of errors, consistent with a functional role of MR in the coordination of walking. However, an effect of MR on upstream motor circuitry (corticospinal and intraspinal tracts) in intact animals cannot be ruled out. The negative effect of spironolactone was not reproduced by the GR antagonist mifepristone (Fig. 3).

In the lesioned rat, spironolactone treatment did not change the number of errors, which remained close to that obtained in untreated lesioned animals. Inhibition of the GR signalling pathway by mifepristone was accompanied by an improvement in walking

Table 1
Maximal mRNA accumulation (between 1 and 15 h) of components of the renin–angiotensin system, of Na/K-ATPase and ENaC subunits in Schwann cells (SC) cultured without forskolin and treated or not with 10 nM corticosteroid and comparison with mRNA accumulation in intact and 7-day lesioned nerve. GC: pure glucocorticoid, RU28362 or MC: mineralocorticoid, aldosterone; values in columns a–c are means from 2 to 3 different SC preparations. Values in column e (lesioned nerve) are means of distal and proximal segment values except for ENaCα where only the distal value was taken; values in columns d and e are means of 5–10 nerves. ↑: Stimulation >1.5; ↓: Inhibition <0.7. *: Values differ significantly from control values (untreated SC or animals), $p < 0.05$, according to the Mann–Whitney test. $^{\Delta}$: There was stimulation or increased stimulation at day 15 after lesion. nd: not detected.

	Schwann cells			Sciatic nerve		Nerve amplification
Treatment	Control (arbitrary units)	GC (fold)	MC (fold)	Intact (arbitrary units)	Lesioned nerve (arbitrary units)	(fold)
mRNAs	a	b	c	d	e	d/a
ATG	0.075	↑1.7*	1.4	12	↓4$^{\Delta}$*	160
Renin A	nd	nd	nd	0.005	0.005	–
Renin B	0.002	1.4	↑5.5*	0.006	↑0.04$^{\Delta}$*	3
ATR1a	0.007	↑13*	↑10*	2	↑4*	285
ATR2	0.003	↑3*	↑4.3*	0.2	↑0.8*	67
Na/K-ATPase						
α3	0.002	1	↑5*	0.45	↑3.5*	225
α2	0.025	↑2.5*	1	30	↓10*	1200
β1	0.25	↑3.2*	↑2.4*	5	↑37$^{\Delta}$*	20
FXYD7	0.4	↑1.8*	1.4	16	↓2*	40
β2	2.5	1	0.8	26	↓3*	10
α1	25	1.2	1.2	100	↓50*	4
ENaC α	0.25	1	1	0.3	↑0.6*	1.2
ENaC β	nd	nd	nd	2.7	↓1.3*	–
ENaC γ	nd	nd	nd	0.9	↓0.4*	–

coordination. This indicates an unfavourable effect of glucocorticoids on walking ability in the course of regeneration.

Gene expression was further investigated in corticosteroid-treated SC cultures and in intact and 7-day lesioned sciatic nerves of rats treated or not with receptor antagonists. Two classes of gene mRNAs were studied: mRNAs coding for proteins and enzymes of the RAS and those from other classical mineralocorticoid target genes displaying both epithelial and neural functions.

3.5. Components of the renin–angiotensin system (RAS) are regulated by GR and MR

Angiotensinogen (ATG) is the precursor of angiotensin II and other related peptides. The expression of ATG mRNA was detected in SC as well as in total nerve. Glucocorticoid treatment produced a weak stimulation of expression in SC (Table 1). In the intact nerve, the ATG level of expression was ~160-fold higher than in SC (Table 1). A 3-fold reduction was observed at 7 days p.i., before an increase in the distal and proximal parts of the lesioned nerve at 15 days (Fig. 4A). The administration of mifepristone induced an increase in ATG mRNA content (+36% and +125% in the intact and lesioned nerve, respectively) (Table 2) which may correspond to the release of a repressive action of glucocorticoids *via* GR.

Renin and then angiotensin converting enzyme successively cleave ATG. SC only expressed the B form of renin (Table 1). In the nerve, both the A and B forms were expressed (Fig. 4B and C). Renin B is a non-secreted intracellular form, mainly found in the brain and able to allow local synthesis of angiotensin peptides from ATG [29]. In SC, the most prominent effect was a 5.5-fold stimulation of the B form expression by aldosterone (Table 1). The basal mRNA level in the nerve was ~3-fold higher than that of SC (Table 1). It was increased by lesion as soon as 3 days p.i. and was very high at 15 days in the distal part (Fig. 4C). The partial inhibition observed by spironolactone (−34%) and to a lesser extent by RU486 (−24%) in the intact nerve and at 7 days after lesion showed that MR and GR exert a stimulatory effect on the synthesis of renin B mRNA (Table 2). Involvement of the MR signalling pathway in renin B mRNA accumulation is coherent with the quick stimulatory response observed with aldosterone in SC.

The angiotensin II effects are mediated by interaction with angiotensin receptors of type 1a and 2 (ATR1a and ATR2). The mRNAs of both receptors were detected in SC, but their levels were considerably lower than in the nerve (285- and 67-fold for ATR1a and ATR2, respectively) (Table 1). A stimulatory effect of both glucocorticoid and mineralocorticoid was observed in SC. In the lesioned nerve, a moderate increase in both angiotensin receptor mRNAs accumulation occurred at 3–7 days (Fig. 4D and E). The level of expression of ATR1 mRNA was 5–10-fold that of ATR2. Use of MR and GR antagonists in intact and injured nerve demonstrated a stimulatory effect of GR in the expression of ATR1a mRNA (51%

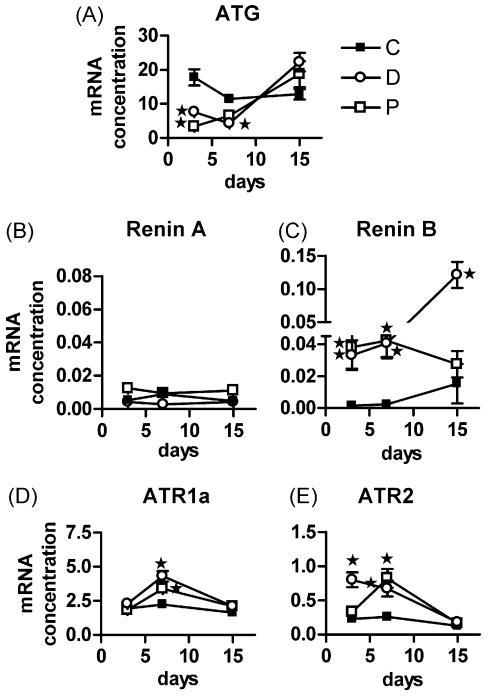

Fig. 4. Variations in accumulation of mRNAs of components of the renin–angiotensin system (RAS) as a function of time (days) after nerve cryolesion. (A) Angiotensinogen (ATG); (B) renin A; (C) renin B; (D) angiotensin receptor type 1a (ATR1a); (E) angiotensin receptor type 2 (ATR2). Control (C): ■; proximal segment (P): □; distal segment (D): ○. Values are means ± SEM, $n = 4–10$ nerves. *Significant difference from control, $p < 0.05$, variance analysis (ANOVA, two factors) and Bonferroni post-test.

Table 2
Effect of *in vivo* administration of antagonists spironolactone (SPIR) and mifepristone (RU486) (day 2 to day 6) on mRNA accumulation in intact and 7-day lesioned sciatic nerve. In columns a–d; numbers preceded by "−": inhibition of mRNA accumulation expressed in % of values from untreated animals; preceded by "+": increased mRNA accumulation corresponding to repression (underlined values). ↑ or ↓: increased or decreased mRNA accumulation after lesion (expressed in fold ≥1.5 and ≤0.7 in column e) or increased and decreased receptor participation (≥5% in the two right columns) in activation or repression (underlined). Values are mean for 4–8 nerves. *: Values differ significantly from control values (untreated animals), $p < 0.05$, according to the Mann–Whitney test. $^{\Delta}$: There was stimulation or increased stimulation at day 15 after lesion.

	Intact nerve		7-day lesioned nerve		Lesion effect at 7 days	Variation (Δ) in the participation of receptor signalling pathway	
	SPIR (%) a	RU486 (%) b	SPIR (%) c	RU486 (%) d	(fold) e	MR (Δ%) c–a	GR (Δ%) d–b
ATG	0	+36*	0	+125*	↓0.33*$^{\Delta}$	0	↑+89
Renin B	−34*	−24	−34*	−24	↑6.7*$^{\Delta}$	0	0
ATR1a	0	−51*	0	−20	↑2*	0	↓+31
ATR2	+79*	0	+85*	+47	↑4*	↑+6	↑+47
Na/K-ATPase							
α3	−11	−39*	+42	+93*	↑7.8*	↑+53	↑+132
β1	−17	−44*	0	−13	↑7.4*$^{\Delta}$	↓+17	↓+31
α1	−30	−18	−31*	−21	↓0.5*	−1	−3
α2	0	−63*	0	0	↓0.33*	0	↓+63
β2	−20	−48*	−36*	−43*	↓0.53*	↑−16	↓+5
ENaCα	0	−22*	−14*	−12	↑2*	↑−14	↓+10
ENaCβ	−18	−58*	−17	−53*	↓0.5*	+1	↓+5
ENaCγ	−35	−66*	−22	−58*	↓0.45*	↓+13	↓+8

and 20% inhibition by RU486, respectively; Table 2), but no effect of MR. In contradiction with the stimulation observed in SC, MR and GR clearly inhibited the expression of ATR2 mRNA (79% and 85% increase by the antagonist spironolactone in intact and lesioned nerves respectively and a smaller increase by RU486 only in the lesioned nerve, 47%). A reduction in repression due to reduced amounts of GR and MR at 3–7 days p.i. relatively to the intact nerve (as shown for their mRNAs in Fig. 1C and D) may account for some increase in ATR2 expression, but does not explain the increase in ATR1a mRNA. Alternatively, a dominant stimulatory mechanism specific to the ATR2 mRNA may occur after lesion.

3.6. Changes in Na/K-ATPase subunit expression and regulation in cultured SC and in the nerve during regeneration

Na/K-ATPase is composed of a catalytic α subunit (4 isoforms) and a β subunit (3 isoforms). A third subunit belonging to the FXYD family has been described as a regulator of the α catalytic subunit [30,31]. The subunit composition differs according to the tissue: α1 is ubiquitous while the other α subunits display a tissue-specific pattern of expression, with α2 expressed in myocytes, skeleton muscle and glial cells and α3 predominating in neurons [32]. The α3 subunit is regulated by aldosterone in both kidney cells and the hippocampus [33]. It binds cardiotonic steroids such as ouabain in different tissues and even progesterone in Xenopus oocytes [34]. The insertion of the α-subunit in the membrane requires the interaction with a β subunit.

In SC cultures, the ubiquitous α1 subunit was expressed at a relatively constant high level (25 arbitrary units) without exhibiting sensitivity to corticosteroids. The β2 subunit appears as the preferential associated subunit in cultured SC (2.5 arbitrary units) since other subunits (α2, α3, β1 and FXYD7) displayed lower levels of expression (<1 arbitrary units). In repeated experiments from several SC preparations, the α3 and β1 subunits displayed mineralocorticoid responsiveness (Table 1) only in the absence of Fk. Glucocorticoid responsiveness occurred in the presence of Fk for the α3 subunit, but both in the presence and absence of Fk for the β1 subunit (not shown). This indicates that the expression of the β1 subunit is maintained whatever the cAMP concentration. A third subunit, containing the FXYD motive, was present as FXYD7 but not FXYD4. FXYD7 was slightly increased by corticosteroids (Table 1) but inhibited by Fk (not shown). Thus, when HSD2 expression was optimal, at low cAMP in SC, the Na/K-ATPase complexes are enriched in α3 and FXYD7 mRNAs.

In the intact nerve, the mRNA levels of the different Na/K-ATPase subunits are higher than in SC at the same order of magnitude (α1) and up to 1–3 orders of magnitude higher (α2, α3, β1, β2 and FXYD7) (Table 1). Deduced from their mRNA contents in the intact nerve, Na/K-ATPase should be essentially constituted of the α1 (or α2) subunit associated with β2 and to a less extent with β1 subunits. FXYD7 specifically associates with α–β1 isozymes and in the brain, possibly only with α1–β1 isozymes [31].

After cryolesion, four Na/K-ATPase subunits underwent an important decrease (α1, α2, β2, FXYD7), while two (α3 and β1) were increased (Fig. 5). Thus, in the injured nerve, it would be expected from the mRNA contents that Na/K-ATPase complexes were enriched in α3 and β1 subunits. The α1 decrease (2-fold) was

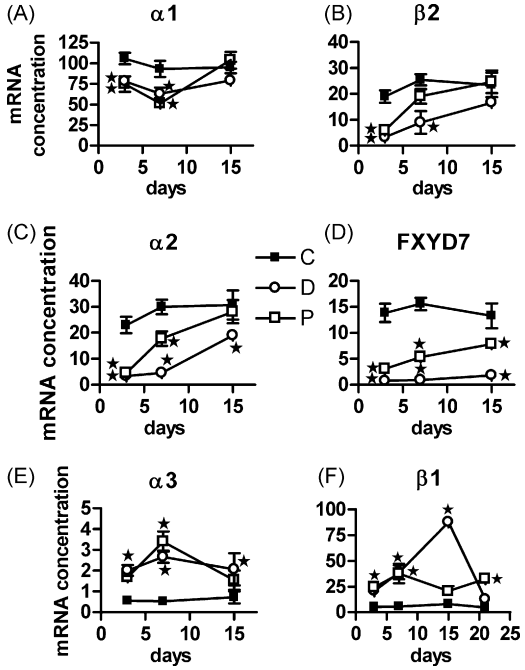

Fig. 5. Variations in accumulation of Na/K-ATPase subunit mRNAs as a function of time (days) after nerve cryolesion. (A) α1; (B) β2; (C) α2; (D) FXYD7; (E) α3 and (F) β1. Control (C): ■; proximal segment (P): □; distal segment (D): ○. Values are means ± SEM, $n = 4–10$ nerves. *Significant difference from control, $p < 0.05$, variance analysis (ANOVA, two factors) and Bonferroni post-test.

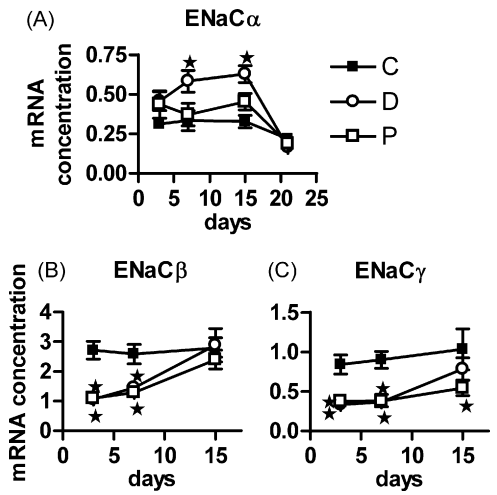

Fig. 6. Variations in accumulation of ENaC subunit mRNAs as a function of time (days) after nerve cryolesion. (A) ENaCα; (B) ENaCβ and (C) ENaCγ. Control (C): ■; proximal segment (P): □; distal segment (D): ○. Values are means ± SEM, $n = 7–9$ nerves. *Significant difference from control, $p < 0.05$, variance analysis (ANOVA, two factors) and Bonferroni post-test.

moderate and progressive with a minimum at 7 days followed by a recovery at 15 days (Table 1 and Fig. 5A). The α2, β2 and FXYD7 subunit mRNAs exhibited acute and rapid falls at 3 days (5–50 times) (Fig. 5). The recovery of the basal levels for these subunit mRNAs occurred first in the proximal part, then in the distal segment, in agreement with the progression of regeneration along the nerve. The mRNA contents of α3 and β1 subunits were increased 8 and 7-fold after cryolesion, but with different kinetics (Fig. 5E and F). The peak of expression was at 7 days in both parts of the nerve for the α3 subunit with a α3 proximal concentration moderately higher than that of the distal one at 7 days. An equivalent concentration of β1 was observed at 7 days in both parts, but the maximum was only reached in the distal part at 15 days. It is remarkable that the two Na/K-ATPase subunits, shown to display aldosterone responsiveness in SC (α3 and β1), are both up-regulated after nerve cryolesion at a time of high HSD2 mRNA content (7 days). This observation prompted us to check for the MR participation in the up-regulation of α3 and β1 subunits.

Use of receptor antagonists confirms the involvement of MR together with GR in the regulation of α3 and β1 subunits of Na/K-ATPase (Table 2). However, they displayed very different regulation patterns. For β1, GR and MR participation decreased after lesion while for α3 there was an inversion from activation to repression. For α1 mRNAs, the participation of GR and MR appeared unchanged by lesion. This contrasted with corticosteroid insensitivity observed in SC cultures and its moderate decrease after lesion. For α2, lesion abolished GR participation and mRNA decreased after lesion. Finally, β2 mRNA decreased after lesion, but MR and GR participation were increased and slightly decreased, respectively.

3.7. Epithelial sodium channel subunit expression in SC and nerves

In SC, Epithelial sodium Channel α (ENaCα) expression was almost constant, without any effect of corticosteroid stimulation (Table 1) and at a level very similar to that of the intact nerve. In the regenerating nerve, ENaCα mRNA underwent rapid and prolonged stimulation (Fig. 6A). A significant increase was recorded from 3 to 15 days in the distal part. The use of corticosteroid inhibitors indicated that ENaCα was regulated by GR in the intact nerve and that lesion induced MR participation while reducing that of GR.

The ENaCβ and γ subunits were undetected in SC but were significantly expressed in the nerve. Their expression was strongly decreased at 3 days post-injury, and then basal levels were slowly and partially restored at 15 days. The recovery was more efficient in the distal part than in the proximal part of the nerve (Fig. 6B and C). Use of corticosteroid inhibitors showed that the decrease in β and γ subunit mRNAs was accompanied with a moderate reduction of MR and GR activities.

4. Discussion

4.1. General conclusion

Our data indicate that a precise equilibrium between MR and GR pathways may exist in peripheral nerve. MR and GR play opposite roles in walking coordination and exhibit both common and distinct functions in gene regulation. In the intact nerve, MR sustains walking coordination and precise genomic activity. After nerve injury, the MR/GR ratio decreases: MR falls more than GR and at 7 days p.i. corticosteroid level in the lesioned nerve was closer to the plasma level than that in the intact nerve. At this time, we found an *in vivo* increase of HSD2 activity in the injured nerve that could help to maintain a low enough intracellular corticosterone level. The decrease in the GR pathway, which may avoid several detrimental effects of GR on walking ability and other regenerative parameters such as SC dedifferentiation, was illustrated by the decrease in the mRNA accumulation of a majority of glucocorticoid-activated genes. Simultaneously, lesion diversely affects MR-regulated genes and reinforces both MR and GR repressive effects. Thus, these results indicate that the modulation of glucocorticosteroid levels by HSD2 activity participates in, but does not fully explain, the changes observed in the expression of the studied genes, suggesting the involvement of (an) additional mechanism(s) triggered by lesion.

In this work, we identified several genes of the RAS system (Renin B, ATR1a, ATR2) and of epithelial target cells (Na/K-ATPase α3 and β1) which were up-regulated by lesion and likely involved in the regenerative program. The importance of ion channel sequestration and the establishment of different types of specialized junctions by the action of RAS and Na/K-ATPase likely constitute one aspect of the nervous requirement for MR function [35,36].

4.2. Corticosterone levels in the nerve

The origin of corticosterone appeared to be purely plasmatic and corticosterone measurements by GC–MS showed a significant difference between the plasma and the nerve of intact animals (plasma > nerve) but not of injured animals. A more efficient penetration of corticosterone into the lesioned nerve than into the intact nerve, resulting from the blood–nerve barrier disruption [37,38] could explain this observation. In addition, the global measurement of corticosterone concentration in the nerve does not take into account variations in the volume of the interstitial fluid between the intact and lesioned nerve (oedema formation) and a possible reduction of the oxidized metabolite to corticosterone mediated by HSD1 in neighbouring fibroblasts or macrophages. Finally, a local intracellular decrease of corticosterone in the MR microenvironment, resulting from co-localisation of MR and HSD2 in the endoplasmic reticulum could be sufficient to ensure MR function [39].

4.3. Opposite roles of MR and GR on walking ability

The opposite effects of MR and GR on walking ability in the intact and in injured animals are an illustration of the interplay between both corticosteroid pathways in the peripheral nerve.

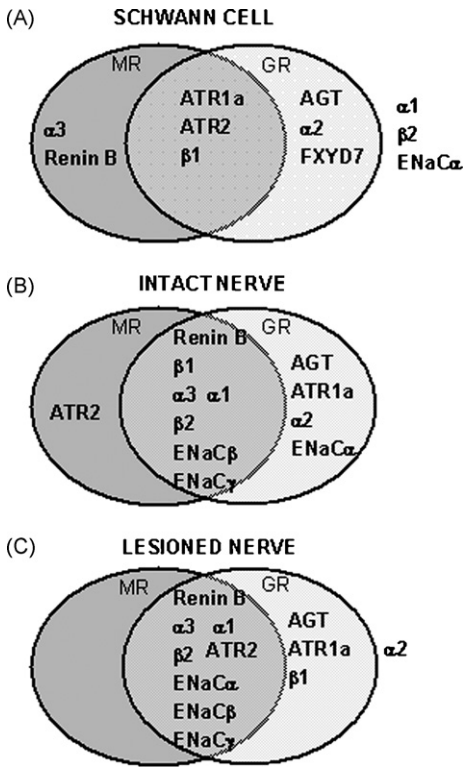

Fig. 7. Gene regulation by MR and GR in SC cultures (A) and in the intact (B) and lesioned (C) nerve. Each ellipse represents a domain of activity (either MR and GR). At their intersection are genes which were regulated by both receptors. Outside, genes which were not regulated.

The favourable effect of MR activity observed in the intact animals was not found in the lesioned animals. This could be due to alteration in the gross motor activity of the sciatic nerve by injury. Reciprocally, lack of GR detrimental effects in intact animals suggests that a pre-existing fragility (developmental or degenerative state) may be required for the glucocorticoid inhibitory effect.

4.4. Genomic MR and GR activity in the intact and regenerating nerve

In the intact nerve, MR or a combination of MR and GR sustains the expression of numerous genes: different subunits of Na/K-ATPase: α1, α3, β1 and β2, ENaCβ and γ and RAS genes (renin B and ATR2) (Fig. 7). Others only depended on GR activity (Na/K-ATPase α2, ENaCα, ATG and ATR1a). All are activated except ATG and ATR2, that are repressed by corticosteroids (Table 2).

At 7 days after cryolesion, several mRNAs were significantly increased: 3 mRNAs of the RAS (Renin B, ATR1a and ATR2), 2 subunits mRNAs of Na/K-ATPase (α3 and β1) and ENaCα mRNA. ATG mRNAs exhibited a late increase. Others were down-regulated (Table 1 and Figs. 4–6).

After lesion, the participation of MR and GR in the transcription of several genes was modified. A majority of RU486-sensitive genes (7/12) showed a reduction in GR stimulation (Table 2) and this agrees with expected reduced corticosterone concentration caused by HSD2 activity. Expression of 3 genes was repressed (ATG, ATR2 and Na/K-ATPase α3) and this repression was enhanced by lesion. The change in MR effect (on 6/12 genes) was complex: amplified repression for 2 genes, increased stimulation for 2 genes, decreased stimulation for the 2 last genes (Table 2).

Systematic correlation between the increase in MR activity/decrease in GR activity and gene expression and, in the absence of MR activity, decrease in GR activity and decrease in gene expression was verified for half of genes. Such a result argues for additive pathway(s) able to induce (β1), amplify (Renin B), inhibit (β2) stimulation or counteract repressive effects (ATR2 and α3) activated by lesion. Variations in mRNA stability can, however, also affect the accumulation of the different mRNAs. Modalities in regulation should be specific to each promoter structure in interaction with a precise cellular context.

4.5. Schwann cells (SC) in culture and in the regenerating nerve

The comparison of the mRNA levels measured in SC cultures and in the intact nerve showed an important amplification of mRNA accumulation in the nerve except for ENaCα (see last column in Table 1). SC cultured in the absence of Fk (in a quiescent state) presented limited analogies (3/10 genes) in gene regulation with the intact nerve, classified as dependent on mineralocorticoid alone, glucocorticoid alone, on both corticosteroids or no dependency (Fig. 7). Such differences can result from loss of intercellular contacts between SC and the axon which induced or maintained further differentiation of SC, a state which may be accompanied by a reinforcement of mRNA accumulation of numerous genes (Table 1). Alternatively, the synthesis of selected genes may take place in other cell types such as neurons, fibroblasts, epithelial cells and macrophages. In fact, axons have been shown to contain different mRNAs available for local protein synthesis [40] and the α1, α3 and β1 subunits of the Na/K-ATPase have been detected in axons of the central nervous system [41].

Another *in vivo* difference consists in the lack of variation in the nerve cAMP concentration after lesion. From a strong and prolonged decrease of cAMP in the endoneurium of crushed nerves reported by Poduslo and Walikonis [42], we expected a fall in cAMP concentration after lesion. Our measurements of cAMP in the whole nerve showed no decrease or a moderate increase following cryolesion, which is coherent with other reports, indicating that injury induces an increase in the cAMP level [43].

To conclude, SC cultures do not appear to reproduce satisfactorily the *in vivo* nerve situation in terms of mRNA contents and regulation and correlation between cAMP and HSD2. On the whole, they underestimated the biological responses and number of genes controlled by corticosteroids.

4.6. Renin–angiotensin system (RAS) in the intact and regenerating nerve

Angiotensin II does not cross the blood–brain barrier [44] and the same may be true for the blood–nerve barrier. Thus, the presence of substrate, metabolizing enzymes and angiotensin receptors in the nerve indicates that a local system replaces the systemic system.

The level of expression of ATR1 mRNA which was dependent on GR in the intact animals was ~10-fold that of ATR2. At 3 and 7 days after cryolesion, an increase in ATR2 mRNA relatively to ATR1 mRNA took place, coherent with previous observations in the sciatic and optic nerves [45]. A role in tissue repair and regeneration has already been ascribed to ATR2, which also displayed ATR1 antagonistic effects in term of growth and blood pressure [46]. Thus, a transient role of ATR2 is perfectly coherent with the nerve regeneration program. A first phase with maximal ATR2 action would be followed by a second phase with prevalent ATR1 activity. ATR1 in the nerve may also act in reconstituting the blood–nerve barrier [47] and intercellular communications [48].

4.7. Expression and function of Na/K-ATPase and ENaC subunits during regeneration

In the intact nerve, the ATPase composition, extrapolated from subunit mRNA content, suggests that the enzyme may be mainly

constituted of α1, α2 and β2 subunits. Protein expression data obtained by Kawai et al. [15] by an immunological approach also showed a high expression of α1 and α2 subunits in the sciatic nerve. Upon cryolesion, we observed a decrease in α1, α2 and β2 and an increase in α3 and β1 subunits mRNAs. These results differ from protein data obtained in the transected or crushed nerve by the same authors, who found a disappearance of the α3 and β1 subunits from distal part and the increase of α2 and β2 subunits. Although we cannot rule out a very important variation in translational efficiency and protein stability after lesion, complete inversion of results was surprising. Bias arising from the use of different antibodies with various strengths, accessibility and specific/non-specific labelling may be responsible for part of discrepancies. An argument in favour of the immunological variability was the results obtained by Arteaga et al. in an axotomy/regeneration model (introduction of nerve stumps into a silicone chamber) [16]. These authors did not detect the β2 but detected the β1 subunit and observed an injury-induced decrease in α1 and α2 subunits and a significant increase in α3 in the regenerating nerve. These last results are concordant with our present study.

Recently, the α3 subunit was detected precisely in the Schmidt-Lanterman incisures [49]. The high affinity of the α3 subunit for ATP allows the Na/K-ATPase to function in conditions where ATP concentration is low [17,50]. We observed its up-regulation after injury, which may favour the functioning of Na/K-ATPase pump when cells are energetically exhausted, as after injury. Furthermore, the α3 subunit could favour regeneration, as observed for the goldfish optic nerve [51]. Increased expression of the β1 subunit is reported during hepatic regeneration [52]. This subunit anchors the Na/K-ATPase at the lateral membrane of adjoining cells in association with adherens junctions [53]. Therefore, its over-expression in the nerve could favour the reconstitution of cell–cell interactions and maturation of cell polarity after injury. Antagonist studies indicate that MR was not essential for the up-regulation of α3 and β1 that requires the intervention of an additional signalling component triggered by lesion.

We have demonstrated, for the first time, the expression of ENaCα in SC and in the nerve. This protein shares with Na/K-ATPase the control of ionic homeostasis. ENaCα in SC may take up excess of Na+ at the axolemma level. In contrast to SC where the β and γ ENaC subunits were undetected, these last subunits were expressed in the nerve. A role of mechanical sensor has also been attributed to this class of ENaC/degenerin proteins especially in the nervous system and the vasculature [54,55].

4.8. Clinical prospect

From a clinical point of view, our study points to the possibility of using mineralocorticoids and antiglucocorticoids to improve nervous function in peripheral nerve. After injury, we directly demonstrate the importance of decreasing the activity of the glucocorticoid pathway by use of the antiglucocorticosteroid RU486. In fact, it reinforces a spontaneous physiological response to injury that is to lower the activity of the glucocorticoid pathway. We also document a positive effect of mineralocorticoids on motor coordination of the hind limbs of the intact rat and gene regulation. Interestingly, loss of coordination after spironolactone administration is reported as unfavourable side effect (http://forums.rxmuscle.com/archive/index.php/t-27739.html). These data constitute an indication to examine motor coordination in the case of mineralocorticoid insufficiency and to test the potential benefit effect of mineralocorticoid administration (fludrocortisone) in the case of deficient motor coordination. The utility of such treatments in various human neuropathies could also be investigated.

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