

# Membrane glucocorticoid receptors are localised in the extracellular matrix and signal through the MAPK pathway in mammalian skeletal muscle fibres

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## Key points

- Many studies have previously suggested the existence of stress hormone receptors on the cell membrane of many cell types, including skeletal muscle fibres; however, the exact localisation of these receptors and how they signal to the rest of the cell is poorly understood.
- In this study, we investigated the localisation and the mechanism(s) underlying the physiological functions of these receptors in mouse skeletal muscle cells.
- We found that the receptors were present throughout muscle development and that, in adult muscle fibres, they were localised in the extracellular matrix, satellite cells (muscle stem cells) and close to mitochondria.
- We also found that they signalled to the rest of the cell by activating enzymes called mitogen-activated protein kinases.
- From these results we suggest that, at physiological concentrations, stress hormones may be important in skeletal muscle differentiation, repair and regeneration.

**Abstract** A number of studies have previously proposed the existence of glucocorticoid receptors on the plasma membrane of many cell types, including skeletal muscle fibres. However, their exact localisation and the cellular signalling pathway(s) they utilise to communicate with the rest of the cell are still poorly understood. In this study, we investigated the localisation and the mechanism(s) underlying the non-genomic physiological functions of these receptors in mouse skeletal muscle cells. The results show that the receptors were localised in the cytoplasm in myoblasts, in the nucleus in myotubes, in the extracellular matrix, in satellite cells and in the proximity of mitochondria in adult muscle fibres. Also, they bound laminin in a glucocorticoid-dependent manner. Treating small skeletal muscle fibre bundles with the synthetic glucocorticoid beclomethasone dipropionate increased the phosphorylation (= activation) of extracellular signal-regulated kinases 1 and 2, c-Jun N-terminal kinase and p38 mitogen-activated protein kinase. This occurred within 5 min and depended on the fibre type and the duration of the treatment. It was also abolished by the glucocorticoid receptor inhibitor, mifepristone, and a monoclonal antibody against the receptor. From these results we conclude that the non-genomic/non-canonical physiological functions of glucocorticoids, in adult skeletal muscle fibres, are mediated by a glucocorticoid receptor localised in the extracellular matrix, in satellite cells and close to mitochondria, and involve activation of the mitogen-activated protein kinase pathway.

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**Abbreviations** BDP, beclomethasone dipropionate; EDL, extensor digitorum longus; ERK1&2, extracellular signal-regulated kinases 1 and 2; FAK, focal adhesion kinase; FAKi-14, focal adhesion kinase inhibitor 14; GC, glucocorticoid; GR, glucocorticoid receptor; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; RYR1, type-1 ryanodine receptor.

## Introduction

Glucocorticoids (GCs, steroids) are the main stress hormones in the body. They are synthesised from cholesterol and released from cells in the zona fasciculata of the adrenal cortex. In healthy individuals, they are released in short irregular bursts that peak ~2 h before the start of the day and their plasma concentration rarely exceeds 250 nM except during stress when it can be as high as 700 nM (Krieger *et al.* 1971). GCs are essential for life and affect most tissues in the body including skeletal muscle. Indeed, skeletal muscle plays a key role in the stress response because it enables individuals to evade or to fight the stressor. Despite this, little is known about the physiological functions of GCs in skeletal muscle especially at the low concentrations normally found in plasma. Instead, what has been extensively studied and reported are the effects of treating laboratory animals for long periods with high doses of synthetic GCs (especially dexamethasone) on skeletal muscle function (Nava *et al.* 1996; van Balkom *et al.* 1996; Ma *et al.* 2003) and protein metabolism (Goldberg, 1969; Rannels & Jefferson, 1980; Wing & Goldberg, 1993). Much is also known about the effects of lack of (Addison's disease) and excess of (Cushing's syndrome) GCs on skeletal muscle mass and function.

GCs exert most of their physiological and pharmacological effects through an intracellular glucocorticoid receptor (GR, GCR, NR3C1). The GR belongs to the super family of ligand-activated nuclear transcription factors that include the steroid, thyroid and retinoic acid receptors (Kadmiel & Cidlowski, 2013). It is coded for by a single gene (the *NR3C1* gene) that is spliced into two main isoforms, GR $\alpha$  (which is the active form) and GR $\beta$  (which is inactive and resides in the nucleus where it acts as a dominant negative inhibitor of GR $\alpha$ ) (Kadmiel & Cidlowski, 2013). GR $\alpha$  (henceforth referred to as GR) is expressed in most cells, including muscle fibres (= cells), where the inactive receptor is thought to reside in the cytosol (hence cytosolic GR). In this state, the receptor is maintained in its high affinity conformation and is protected from inactivation by chaperone molecules such as heat shock proteins (HSPs), e.g. HSP 90, and immunophilins, e.g. FK506 binding protein (FKBP) 51 (Pratt & Toft, 2003). However, in a recent study we showed that, in adult mammalian skeletal muscle fibres, GRs were

localised close to the surface of muscle fibres where they co-localised with laminin. We were also able to inhibit their effects on force using a monoclonal antibody against the receptor suggesting that they were easily accessible from the cell surface (Pérez *et al.* 2013). Although a number of studies have previously alluded to the existence of membrane glucocorticoid receptors in other cell types, including human lymphoma cells (Gametchu, 1993), peripheral blood mononuclear cells (Bartholome *et al.* 2004) and neuronal cells (Orchinik *et al.* 1991), their precise location is still unknown. It is also uncertain whether in skeletal muscle the GRs are localised close to the cell surface throughout myogenesis or just in adult muscle fibres.

In addition to their genomic actions, GCs also exert actions that occur within minutes and are relatively insensitive to inhibitors of transcription and translation (Stahn & Buttgerit, 2008; Pérez *et al.* 2013). These non-canonical/non-genomic actions have been described in many cell types including HeLa cells (Lasa *et al.* 2002; Bruna *et al.* 2003), guinea pig neurones (Hua & Chen, 1989), human endometrial cells (Hafezi-Moghadam *et al.* 2002), human adenocarcinoma cells (Croxtall *et al.* 2000), rat thymocytes (Buttgerit *et al.* 1997), airway vasculature (Alangari, 2010) and skeletal muscle (Pérez *et al.* 2013). However, the cellular signal transduction events mediating them are still poorly understood. The receptor/mechanism underlying their actions is also controversial.

The primary aims of this study were twofold: (1) to investigate the localisation and physiological functions of the GR in mouse skeletal muscle cells and (2) to determine the cellular signal transduction events mediating their non-genomic physiological actions in adult mammalian skeletal muscle fibres.

## Methods

### Small intact skeletal muscle fibre bundles

The experiments reported here were performed at room temperature (~20°C) using small skeletal muscle fibre bundles (~540 ± 26  $\mu$ m in diameter) isolated from the extensor digitorum longus (EDL; a fast-twitch) or the soleus (slow-twitch) muscles of adult male C57BL/6 mice. The mice were killed by cervical dislocation according to UK legislation (for a summary of the legislation see Drummond, 2009) and all the experiments conformed

to the University of East Anglia animal welfare committee guidelines. Throughout the experiments, the muscles and muscle fibre bundles were bathed in mammalian Ringer solution with the following composition (in mM): 109 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 4 CaCl<sub>2</sub>, 24 NaHCO<sub>3</sub>, 1 NaHPO<sub>4</sub>, 10 sodium pyruvate plus 400 mg l<sup>-1</sup> fetal bovine serum (FBS). The pH of the Ringer solution was maintained at ~7.42 by continuously bubbling it with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. To avoid any confounding influence of the GCs present in the FBS, serum from the same batch was used in all the experiments.

### Cell culture

C2C12 cells (an immortalised mouse skeletal muscle cell line) were grown on coverslips in growth media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% L-glutamine plus 10% FBS and 1% penicillin and streptomycin (all purchased from Sigma-Aldrich, Poole, UK) for 3–4 days or until 90% confluence. Once 90% confluent, they were induced to differentiate by replacing the growth media with differentiation media, i.e. growth media containing 2% horse serum instead of FBS. At the end of the experiment the cells were washed in phosphate-buffered saline (PBS), air dried and permeabilised for 15 min using Tris-buffered saline with Tween 20 (TBST). They were blocked for non-specific antibody binding for 10 min using 1% bovine serum albumin (BSA) in PBS. Finally, they were immunoblotted for the expression of the GR, laminin and double stranded DNA as described below.

### Determination of the effects of beclomethasone dipropionate on the MAPK pathway

This experiment was performed as previously described in Hamdi & Mutungi (2010, 2011). Briefly, the muscle fibre bundles were mounted horizontally between two stainless steel hooks in a Perspex muscle chamber (total volume ~40 ml) with a glass bottom. Half of the bundles (controls) were treated for 5, 10, 30 and 60 min with the standard Ringer solution plus 34 nM ethanol (the vehicle used to dissolve beclomethasone (beclometasone) dipropionate (BDP)). The other half (experimental) was treated for the same period of time with the Ringer solution containing 250 nM BDP. At the end of the experiment, the fibre bundles were snap-frozen in liquid nitrogen and cytosolic proteins were extracted using NP40 lysis buffer. The proteins were immunoblotted for the activation/phosphorylation of c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase (SAPK)), extracellular signal-regulated kinases 1 and 2 (ERK1&2) and p38 mitogen-activated protein kinase (MAPK) as described below.

### Determination of the receptor/mechanism mediating the effects of BDP in mammalian skeletal muscle fibres

To determine whether the effects of BDP on the MAPK pathway were mediated through the GR and whether the receptor was localised close to the fibre surface, another set of fast-twitch and slow-twitch bundles was divided into three groups. Group 1 (controls) was treated with the standard Ringer solution plus the vehicle used to dissolve the GC (ethanol) and mifepristone (DMSO). Group 2 was pre-incubated for 10 min in Ringer solution containing 10 μM mifepristone (RU486; a GR inhibitor) (Sigma-Aldrich, Gillingham, UK), whereas Group 3 was pre-incubated for the same period in a 1:5000 dilution of a monoclonal anti-GR (ab109022; Abcam, Cambridge, UK). The fibres in Group 2 were then treated for a further 30 min with the Ringer solution containing 10 μM mifepristone alone or mifepristone plus 250 nM BDP, whereas those in Group 3 were treated for 5 min with the Ringer solution plus 1:5000 dilution of the anti-GR alone or 1:5000 dilution of the anti-GR plus 250 nM BDP. To determine whether the effects of BDP were mediated via focal adhesion kinase (FAK), another set of fibres was pre-treated with 10 μM FAK inhibitor (FAKi) 14 (Abcam). They were then incubated for a further 10 min in Ringer solution containing the inhibitor alone or the inhibitor plus 250 nM BDP. At the end of these experiments, the fibre bundles were frozen and cytosolic proteins were extracted using NP40 lysis buffer. They were then immunoblotted for the activation of ERK1&2, JNK and p38 MAPK as described below.

### Immunoblotting

To determine the effects of BDP on the activation/phosphorylation of ERK1&2, JNK and p38 MAPK, equal amounts of the proteins were immunoblotted as previously described in Pérez *et al.* (2013). Briefly, 10 μg of the proteins were separated by standard gel-electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked for non-specific antibody binding using 5% milk. They were then immunoblotted for the expression of phosphorylated (p) ERK1&2, pJNK and pp38 MAPK using a 1:500 dilution of rabbit monoclonal antibodies (4377, 4671 and 9215, respectively) from Cell Signaling Technology (New England Biolabs, Hitchin, UK) and the expression of the GR using a 1:500 dilution of the monoclonal anti-GR from Abcam used in the experiment above (ab109022; Cambridge, UK). Finally, they were visualised using SuperSignal WestPico chemiluminescence substrate (Perbio Science UK Ltd, Cramlington, UK) and exposure to film.

The following day, the membranes were stripped and re-probed with a pan-actin antibody (ab3280,

Abcam, Cambridge, UK) to check for loading. In some experiments, the fibre bundles were fibre typed using monoclonal antibodies against fast and slow MyHCs (Sigma-Aldrich, Gillingham, UK). All the blots were run in duplicate and each experiment was repeated at least twice.

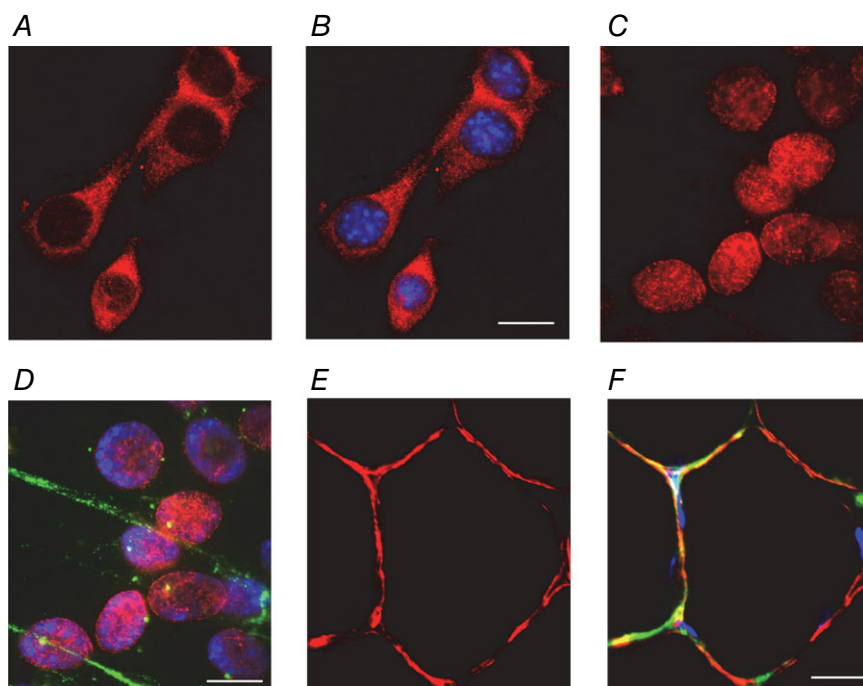
### Immunocytochemistry

The expression and localisation of the GR in myoblasts, myotubes and adult mammalian skeletal muscles fibre bundles was determined as previously described in Pérez *et al.* (2013). Briefly, the EDL and soleus muscles isolated from adult male C57BL/6 mice were mounted perpendicularly on cryostat chucks using tissue Tek OCT compound (Sakura Finetek UK Ltd, Thatcham, UK). They were snap frozen in liquid nitrogen-cooled isopentane and 10  $\mu\text{m}$  thick serial sections were cut. Two of the sections and the two coverslips of myoblasts and myotubes (grown as described above) were blocked for non-specific antibody labelling using 1% BSA dissolved in PBS. They were incubated at room temperature, for at least 1 h, with a 1:200 dilution of a monoclonal rat anti-laminin (MAB1905; Millipore Corporation, Billerica, MA, USA) and a 1:100 dilution of the monoclonal rabbit anti-GR (ab109022; Abcam, Cambridge, UK). They were then washed 5 times in PBS and incubated in 1:500 dilution of species-specific secondary antibodies conjugated to Alexa fluorophores for 1 h. Finally, Hoechst 33342 (a double stranded DNA-staining dye; Life Technologies, Paisley, UK) was added 5 min before the end of this incubation. Two more

sections and coverslips (controls) were incubated with the primary or the secondary antibodies only. At the end of the incubation period, the sections and coverslips were washed 4 times in TBST, dried and mounted for viewing. Finally, they were visualised and photographed using an Axioplan 2 Imaging microscope and AxioVision Release 4.8 software (Carl Zeiss, Cambridge, UK). Like all the immunoblotting experiments, the immunocytochemistry was performed in duplicate and each experiment was repeated at least twice.

### Pull-down assay

To determine whether the GR was able to bind laminin, a modification of the pull-down assay described in De Beer *et al.* (1981) was used. Briefly, 250  $\mu\text{g}$  recombinant human merosin (laminin 2; laminin 211; Millipore (UK) Ltd, Watford, UK), the main laminin in skeletal muscle, or 100  $\mu\text{g}$  recombinant mouse integrin  $\alpha 7\beta 1$  (the main integrins in mammalian skeletal muscle; R&D Systems, Abingdon, UK) were coupled to cyanogen bromide (CNBr)-activated sepharose beads (Sigma-Aldrich), according to the manufacturer's instructions. The excess ligand was washed off with coupling buffer and all the unbound active groups were blocked using 0.2 M glycine. A 300  $\mu\text{l}$  volume of slow-twitch muscle lysate was added to the beads alone, the beads plus laminin, or the beads plus integrin, and left overnight at 4°C on a rotator. Slow-twitch muscle lysate was used because slow muscle expresses 4–5 times more GR than fast-twitch muscle (Pérez *et al.* 2013). The following morning the beads were washed



**Figure 1. The localisation of GRs changes with muscle differentiation**  
Photographs showing the expression and localisation of GRs (red), laminin (green) and double stranded DNA (nucleus; blue) in myoblasts (A and B), myotubes (C and D) and muscle fibres (E and F). Note that the GRs are expressed mainly in the cytoplasm in myoblasts, in the nucleus in myotubes, and in a narrow band along the cell surface in adult muscle fibres. Scale bars: A and B, 5  $\mu\text{m}$ ; C and D, 2.5  $\mu\text{m}$ ; E and F, 15  $\mu\text{m}$ .

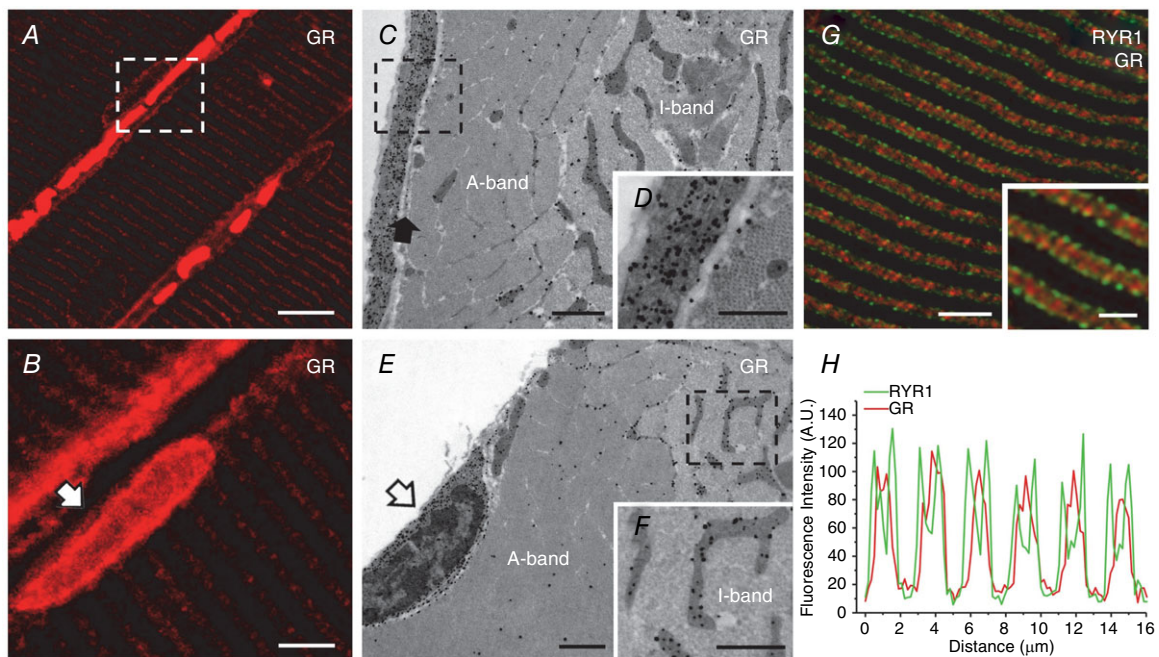


2 times with TBST and once in PBS. They were then mixed with 30–40  $\mu\text{l}$  elution buffer (Laemmli sample loading buffer) and left on a rotator for 1 h. Finally, they were centrifuged at 2000  $g$  for 20 s and the supernatant was collected. A 10  $\mu\text{l}$  volume of each supernatant was separated using standard gel-electrophoresis, transferred onto nitrocellulose membrane and finally immunoblotted for the GR using a 1:500 dilution of the rabbit monoclonal anti-GR antibody from Abcam as described above.

### Immunofluorescence confocal microscopy

Soleus muscles from 6-month-old C57BL/6 mice were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h at room temperature. Small bundles were washed twice with PBS and blocked for 1 h in PBS containing 1% BSA, 10% goat serum and 0.5% Triton

X-100 (added to permeabilise the membrane). They were incubated overnight at 4°C in primary antibody and the following morning they were washed 3 times in PBS. They were incubated with the secondary antibody for 1 h at room temperature before being mounted on coverslips with anti-bleach media (Slowfade Gold antifade reagent; Invitrogen (Molecular Probes) Eugene, Oregon, USA). Primary antibodies were: anti-RYR1 (Ryanodine receptor type 1), 34C (dilution 1:30; Developmental Studies Hybridoma bank, University of Iowa); rabbit monoclonal anti-GR (dilution 1:100; Abcam). Secondary antibodies were: Cy3-labelled goat anti-rabbit IgG (dilution 1:300) for single GR labelling; Cy5-labelled goat anti-mouse IgG (dilution 1:200) and Cy3-labelled goat anti-rabbit IgG for double labelling (dilution 1:300). All secondary antibodies were from Jackson ImmunoResearch Laboratories, Lexington, KY. Specimens were finally viewed using a scanning laser confocal microscope (LS510 META; Carl Zeiss, Jena, Germany).



**Figure 2. GRs are localised in the extracellular matrix and around satellite cells in adult mouse muscle fibres**

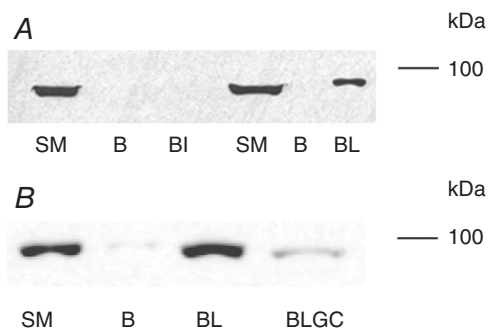
A and B, immunofluorescence confocal microscopy images showing the localisation of GRs in mouse soleus. Note that the GRs are localised either in the proximity of the surface membrane, where they appear to form a narrow and discontinuous band (A; white dashed square) and in satellite cells (B, white arrow) or in the fibre interior where the signal is mostly localised in the I-band. C, D, E and F, immunogold-labelling electron microscopy photographs showing the localisation of GRs in mouse slow-twitch fibres. Note that the GRs (dark dots, i.e. gold particles) are abundant in the extracellular matrix (C and enlargement of the dotted box in D) outside the sarcolemma (C, filled arrow) and in satellite cells (E, open arrow). GRs are also present within the fibre interior almost exclusively at the I-band, and mostly in the proximity of mitochondria (C, E and F). G, a confocal micrograph showing a muscle section immunoblotted for both GRs and RYR1s. Note that the GRs (red) are mostly spread throughout the I-band inside the double green lines generated by the staining of RYR1s, which marks the position of triads. H, graph representing the fluorescence intensity profile calculated from images obtained from samples co-immunostained for RYR1s and GRs. Scale bars: A, 5  $\mu\text{m}$ ; B, 10  $\mu\text{m}$ ; C and E, 5  $\mu\text{m}$ ; D and F, 0.5  $\mu\text{m}$ ; G and inset, 10  $\mu\text{m}$  and 1  $\mu\text{m}$ , respectively.

### Immunogold labelling electron microscopy

Soleus muscles were fixed for 20 min at room temperature in a fixative mixture containing 2% paraformaldehyde and 0.5% glutaraldehyde (in PBS buffer). Small bundles were permeabilised and blocked as described above. After incubation with the rabbit monoclonal anti-GR from Abcam, secondary antibodies conjugated with Nanogold

particles were applied for 2 h at 4°C (dilution 1:100). Samples were then post-fixed with 1% glutaraldehyde (in PBS buffer), at room temperature, and incubated with reagents to enhance the signal (Goldenhance EM Formulation; Nanoprobes, Yaphank, NY, USA) for 5 min. Fibre bundles were finally embedded and sectioned using standard electron microscopy protocols (Boncompagni *et al.* 2009). Ultrathin sections of ~40 nm were cut using a Leica Ultracut R microtome (Leica Microsystems, Vienna, Austria) with a Diatome diamond knife (Diatome Ltd, CH-2501 Biel, Switzerland) and double-stained with uranyl acetate and lead citrate. Sections were viewed using a FP 505 Morgagni Series 268D electron microscope (FEI Company, Brno, Czech Republic), equipped with Megaview III digital camera and Soft Imaging System (Münster, Germany).

As the monoclonal antibody used in the immunocytochemistry, immunofluorescence confocal microscopy and immunogold-labelling electron microscopy has never been tested before for use in these techniques, the findings were confirmed using a polyclonal rabbit anti-GR (Abcam ab35780) that is recommended for use in immunocytochemistry.

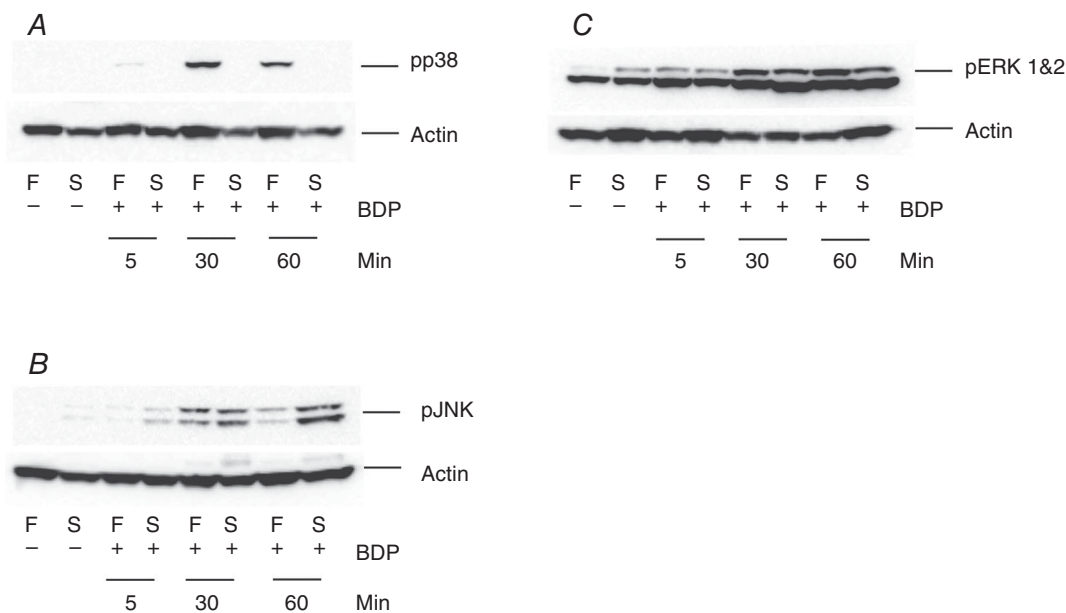


**Figure 3. GRs bind laminin in a GC-dependent manner**

*A* and *B*, Western blots showing the expression of GRs in a slow-twitch muscle (SM; control), in loading buffer used to elute GRs from CNBr-activated sepharose beads alone (B), or with the beads cross-linked with laminin 211 (BL) or with integrin  $\alpha 7 \beta 1$  (BI). In *B* the lysate used in the pull-down assay of the sample labelled (BLGC) was pre-treated with 500 nM BDP for 10 min before it was used in the assay. Note that only the beads coupled with laminin are able to bind and pull down the GR and treating the lysate with GC abolishes this interaction.

### Statistical analysis

As mentioned above, all the immunoblotting experiments were run in duplicate and each experiment was repeated



**Figure 4. The activation of MAPKs by BDP in mammalian skeletal muscle fibres depends on the fibre type and the duration of the treatment**

Western blots showing the effects of treating fast-twitch (F) and slow-twitch (S) muscle fibre bundles with the Ringer solution only (-) or the Ringer solution plus 250 nM BDP (+), for 5, 30 and 60 min, on the phosphorylation of p38 MAPK (*A*), JNK (*B*) and ERK1&2 (*C*). Note that the effects of BDP depend on the fibre type and the duration of the treatment.

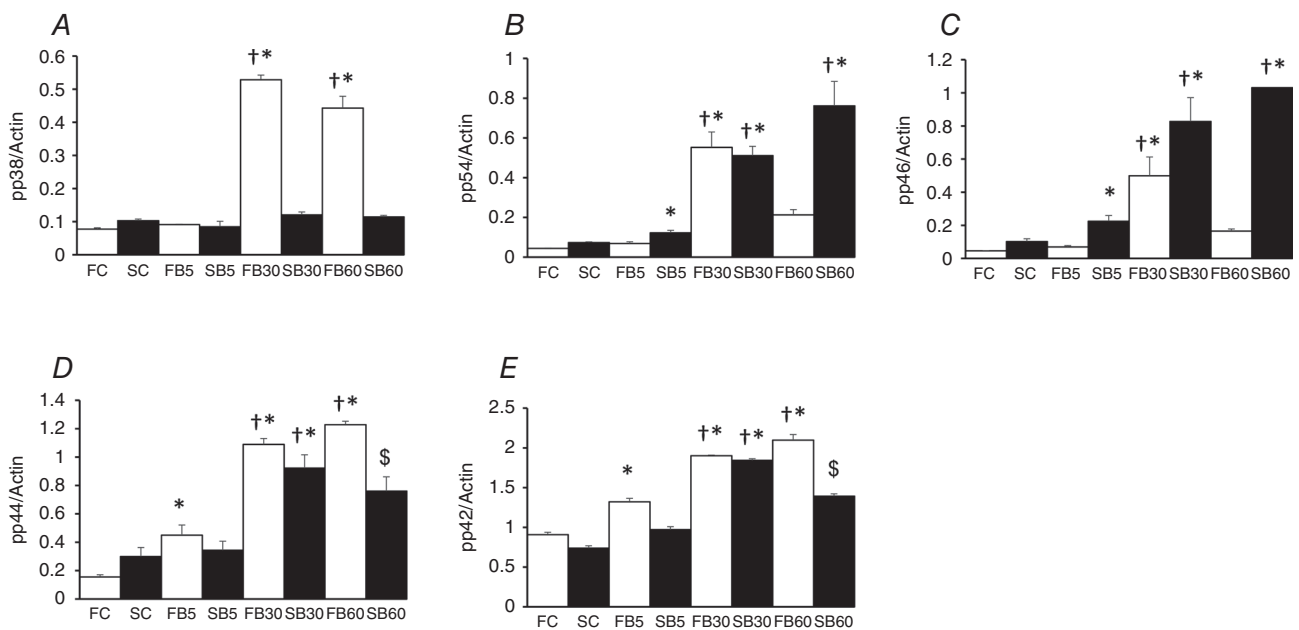
at least twice. To determine the expression of GRs in the various muscles, the Western blots were digitised and the intensity of the various protein bands were analysed using Scion Image from NIH and normalised to that of the loading control (actin). Statistical analysis of the data was performed using SigmaPlot 11.2 (Systat Software Inc., London, UK). The data obtained from each fibre type under control and from the various experimental conditions were compared using a two-way ANOVA with Tukey's *post hoc* test and a  $P < 0.05$  was considered to be statistically significant.

## Results

The results in Fig. 1 show the expression and localisation of GRs in myoblasts (Fig. 1A and B), myotubes (Fig. 1C and D) and adult muscle fibres (Fig. 1E and F). They confirm our previous findings in adult skeletal muscle fibres (Fig. 1E and F) but also show that the receptors were expressed throughout myogenesis and that their localisation, within the cell, changed with differentiation. Thus, they were localised in the cytoplasm in myoblasts (Fig. 1A and B), in the nucleus in myotubes (Fig. 1C and D) and around the periphery of the muscle fibres, where they co-localised with laminin, in adult skeletal muscle fibres

(Fig. 1E and F). Moreover, this shift in the localisation of GRs was accompanied by an increase in the expression of laminin (green staining in Fig. 1D and F).

To visualise the precise localisation of the receptors, in adult mammalian skeletal muscle fibres, a combination of immunofluorescence confocal microscopy (CM) and immunogold-labelling electron microscopy was used. Since we have previously shown that the GR is expressed mainly in oxidative fibres (Pérez *et al.* 2013), the soleus muscle, which contains mostly oxidative fibres (i.e. type I and IIA fibres), was used in this experiment. The immunofluorescence CM confirmed some of our previous findings. For example, it showed that most receptors were localised in clusters on the fibre surface where they generated a narrow, but discontinuous band around the fibres (Fig. 2A; white dashed square). In addition, it provided new evidence showing that the receptors were also localised in large quantities in satellite cells (white arrow, Fig. 2B) and to a lesser extent inside the muscle fibres where they formed a striated pattern. The immunogold labelling confirmed the CM data (Fig. 2C, D and E), and clearly demonstrated that the GR was quite abundant in the extracellular matrix (Fig. 2C) and in satellite cells (Fig. 2E). Gold particles were also found inside the fibres, preferentially, but not exclusively, in



**Figure 5. BDP activates MAPKs in mouse skeletal muscle fibres in a fibre-type and duration-dependent manner**

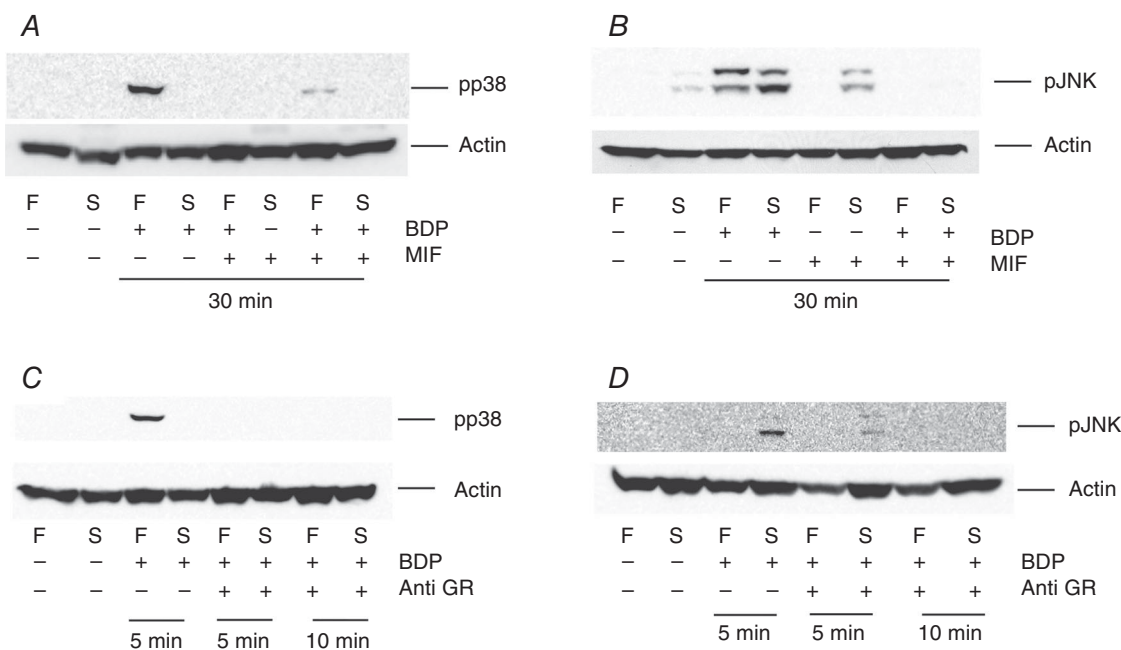
Summary data showing the effects of treating fast-twitch (F) and slow-twitch (S) muscle fibre bundles with the Ringer solution only (FC; SC) or the Ringer solution plus 250 nM BDP for 5 (FB5; SB5), 30 (FB30; SB30) and 60 min (FB60; SB60) on the phosphorylation of p38 MAPK (A), the 54 kDa isoform of JNK (B; p54); the 46 kDa isoform of JNK (C; p46); ERK1 (D; p44) and ERK2 (D; p42). Note that the effects of BDP depend on the fibre type and the duration of the treatment. \* $P < 0.05$  when the data indicated are compared to those of the corresponding untreated (control) fibres. † $P < 0.05$  when the data indicated are compared to those of the corresponding fibres treated with BDP for 5 min. †† $P < 0.05$  when the data indicated are compared to those of the corresponding fibres from the other treatments.

the vicinity of mitochondria (Fig. 2*F*), that we know are specially localised at I-bands (on both sides of Z lines) in adult fibres (Boncompagni *et al.* 2009). To determine the exact location of the GR signal inside the fibres, some of the fibres were double stained with an anti-GR and an antibody (anti-RYR1, 34C) that marks the position of Ca<sup>2+</sup> release units (CRUs), or triads. In adult fibres, CRUs are specifically placed at the transition between A- and I-bands, where they form a very peculiar transverse double-cross striation on either side of the I-band (Boncompagni *et al.* 2009*a*). They also contain the sarcoplasmic reticulum Ca<sup>2+</sup> release channels, type-1 ryanodine receptors (RYR1s). Double labelling for GRs and RYR1s (red and green, respectively, in Fig. 2*G*) indicated that the GR staining inside the fibres was exclusively localised at the I-band, flanked on either side by the two green lines marking the position of the triads. These findings also suggested that some of the gold particles that were not at the I-band may have been non-specific (Fig. 2*C* and *D*). As the I-band region of adult fibres is rich in mitochondria (Boncompagni *et al.* 2009), the results in Fig. 2*G* agree with those in Fig. 2*E* and *F* showing gold particles in the proximity of mitochondria (the dark and branched organelles).

To determine what tethers the GR in the extracellular matrix, laminin 2 (merosin, laminin 211) or integrins of the  $\alpha 7\beta 1$  type coupled to cyanogen bromide-activated

sepharose beads were used to pull down the GR from slow muscle lysates. As the results displayed in Fig. 3*A* show, only laminin-coupled beads were able to bind and pull-down the GR. Moreover, their ability to bind the receptor depended on the presence or absence of the ligand (GC). Thus, adding 500 nM BDP to the muscle lysate significantly reduced the amount of GR pulled down by the beads (Fig. 3*B*) suggesting that the GC was able to dissociate the GR from laminin.

To determine the cellular signalling pathways activated by physiological concentrations of GCs in adult skeletal muscle, small muscle fibre bundles isolated from the EDL (a fast-twitch muscle) and soleus (a slow-twitch muscle) were treated with 250 nM BDP (a synthetic GC widely used in the management of asthma). The phosphorylation (= activation) of JNK, ERK1&2 and p38 MAPK was then determined. As the results displayed in Figs 4 and 5 show, the effects of BDP on the phosphorylation of these kinases was complex and depended on the fibre type and the duration of the treatment. For example, treating the muscle fibre bundles with BDP for 5 min increased the phosphorylation of p38 MAPK and ERK1&2 in the fast-twitch muscle fibres (Figs 4*A* and *C*, and 5*A*, *D* and *E*) and that of JNK in the slow-twitch fibres (Figs 4*B*, and 5*B* and *C*). Increasing the treatment time to 30 min further enhanced the activation of p38 MAPK in the fast-twitch fibres (Figs 4*A* and 5*A*) and that of ERK1&2 (Figs 4*C*, and



**Figure 6. The effects of BDP are mediated through cell surface (membrane) GRs**

Western blots showing the effects of pre-treating fast-twitch (F) and slow-twitch (S) muscle fibre bundles with 10  $\mu$ M mifepristone (MIF, a GR inhibitor; *A* and *B*) or a 1:5000 dilution of an anti-GR (*C* and *D*), for 10 min, on the phosphorylation of p38 MAPK (*A* and *C*) and JNK (*B* and *D*). The muscle fibre bundles were then treated with the compounds for the durations shown below each blot. Note that both mifepristone and the antibody abolish the BDP-induced increase in the phosphorylation of both MAPKs.

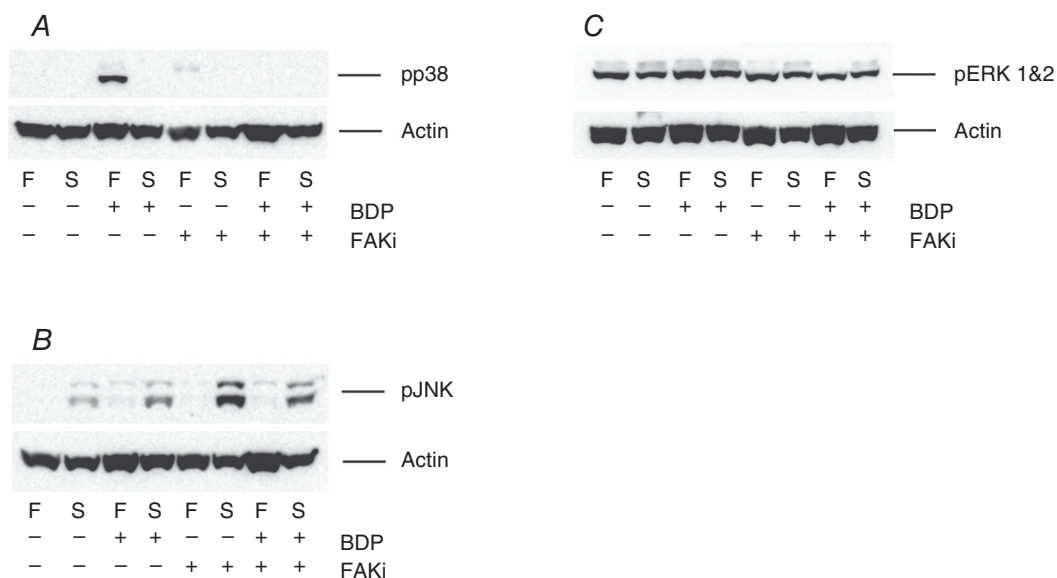


5D and E). However, it led to the activation of JNK in both fibre types (see Figs 4B and C, and 5B and C). In contrast, prolonging the treatment duration to 60 min led to a slight decrease in the phosphorylation of p38 MAPK and a marked decline in that of JNK in the fast-twitch fibres (Fig. 4A and B, and 5A, B and C) but further enhanced that of JNK in the slow-twitch fibres (Fig. 4B, and 5B and C). On the other hand, the phosphorylation of ERK1&2 in the fast-twitch fibres remained elevated and was similar to that seen after the 30 min treatment (Figs 4C, and 5D and E), whereas in the slow-twitch fibres it was slightly reduced (Fig. 5D and E).

The findings in Figs 4 and 5 suggested that the activation of MAPKs by GCs in mammalian skeletal muscles was non-genomic (i.e. it occurred within 5 min). However, they did not tell us the receptor/mechanism underlying this effect. As the mechanisms underlying the non-genomic effects of GCs are still controversial (Lipworth, 2000; Stahn & Buttgerit, 2008; Pérez *et al.* 2013), we investigated whether the activation of the various MAPKs by BDP were mediated through the GR or through another receptor/mechanism. As the results displayed in Fig. 6A and B show, pre-treating the muscle fibre bundles with 10  $\mu$ M mifepristone (RU486, a GR inhibitor), for 10 min, completely abolished or significantly reduced the 30 min BDP-induced activation of p38 MAPK and JNK suggesting that they were mediated by a GR. To determine whether they were mediated by a cytosolic GR or a membrane GR, the experiment

above was repeated using the rabbit monoclonal anti-GR used in the immunocytochemistry and Western blotting experiment. The rationale behind this experiment was that the antibody was too large to cross the cell membrane and that it could only bind the GR and inhibit its effects if the receptor was easily accessible, i.e. located close to the cell surface. As the results presented in Fig. 6C and D show, pre-treating the muscle fibre bundles with the antibody for 10 min completely abolished the BDP-induced activation of JNK and p38 MAPK in both fibre types suggesting that they were mediated by a GR that was easily accessible to the antibody (i.e. a cell surface GR). They also suggested that the antibody bound the GR close to the ligand binding site or caused a conformational change to the receptor that inhibited its activation of the MAPK pathway.

The results presented so far suggested that the effects of BDP on the activation of the MAPK pathway were non-genomic and were mediated by a GR located close to the cell surface. They also suggested that it may be bound to laminin. However, they did not tell us how the GC was able to activate the MAPKs. In mammalian skeletal muscle fibres, laminin is localised in the basal lamina (one of the two layers that form the basement membrane of skeletal muscles) and is thought to communicate with the rest of the cell through integrins (mainly integrin  $\alpha$ 7 $\beta$ 1 in skeletal muscle) and dystroglycan (Gawlik & Durbeej, 2011). This led us to hypothesise that the GR signalled to the MAPK through the activation of focal adhesion kinase (FAK) by integrins (see Fig. 9).



**Figure 7. The BDP-induced activation of p38 MAPK is mediated through focal adhesion kinase (FAK)**

Western blots showing the effects of pre-treating fast-twitch (F) and slow-twitch (S) muscle fibre bundles with 10  $\mu$ M FAK inhibitor (FAKi) 14, for 10 min, on the phosphorylation of p38 MAPK (A), JNK (B) and ERK1&2 (C). The muscle fibre bundles were then treated with the Ringer solution containing BDP alone or BDP plus the inhibitor as shown below each Western blot for a further 10 min. Note that pre-treating the fibre bundles with FAKi 14 abolishes the BDP-induced increase in the phosphorylation of p38 MAPK (A) and ERK1&2 (C) but not that of JNK (B).

To test this hypothesis, we pre-treated another set of muscle fibre bundles with the FAK specific inhibitor FAK inhibitor 14. As the results displayed in Figs 7 and 8 show, pre-treatment of the fibre bundles with the inhibitor completely abolished the GC-induced activation of p38 MAPK (Figs 7A and 8A) and ERK1 (p44; Figs 7C, and 8D and E). However, pre-treatment of the muscle bundles with the inhibitor alone augmented the activation of JNK (especially the 46 kDa isoform) in the slow twitch fibres and returned to the level observed in the BDP-treated fibres when the bundles were treated with both the inhibitor and the GC (Figs 7B, and 8B and C).

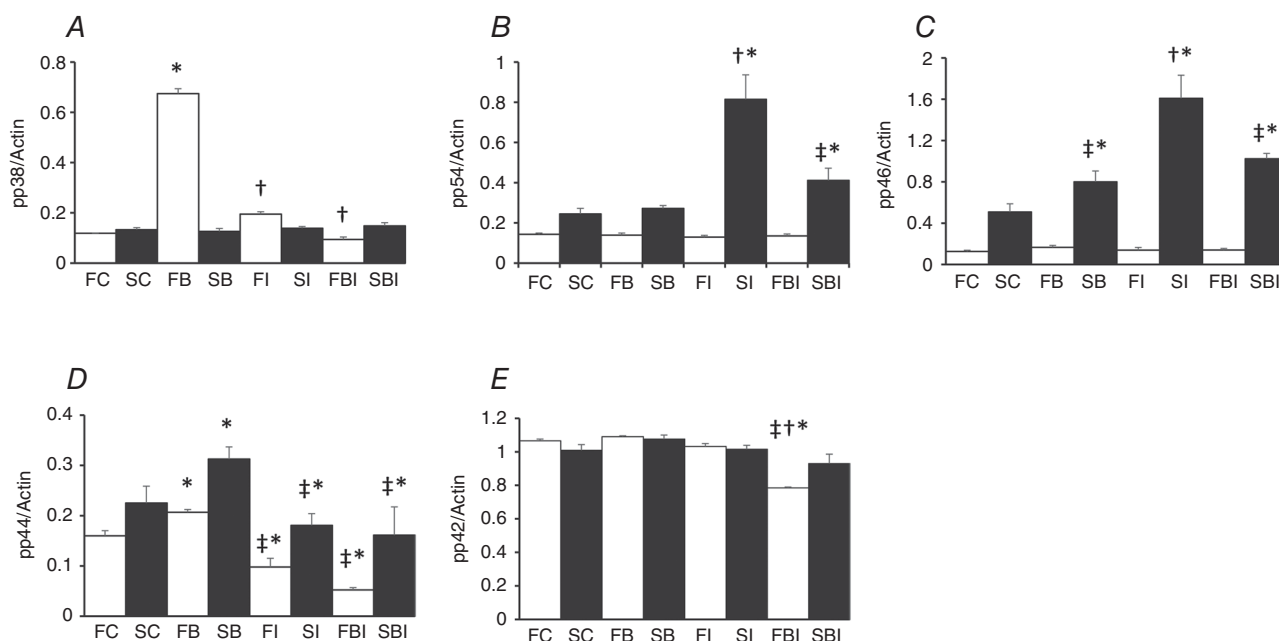
## Discussion

### Localisation of the GR in mouse skeletal muscle cells

The results we report here show for the first time that GRs are expressed at all stages of myogenesis. However, their localisation changes with differentiation (Fig. 1). They also revealed that, in adult mammalian skeletal muscle fibres, they were localised mostly in the extracellular matrix (Fig. 2A and C), in satellite cells (Fig. 2B and D) and in the I-band region where they were closely associated with mitochondria. Although a number of studies have

previously suggested that a membrane glucocorticoid receptor exists in many cell types (Orchinik *et al.* 1991; Gametchu, 1993; Bartholome *et al.* 2004; Pérez *et al.* 2013), this is the first study to show that a GR localised close to the cell membrane exists. However, as our results show, it is localised in the extracellular matrix and in satellite cells and not in the plasma membrane as previous studies have suggested.

Adult mammalian skeletal muscle fibres, unlike most other cell types, are long, cylindrical, multinucleated cells whose nuclei are located close to the fibre surface. Their cytoplasm (= sarcoplasm) is also packed with contractile proteins that are organised into striations consisting of highly regular repeats of thin (actin) and thick (myosin) filaments. Slow-twitch fibres also contain large quantities of mitochondria that are mostly found at the I-band region (Boncompagni *et al.* 2009) and under the sarcolemma (mostly in the proximity of capillaries). Here, we propose that this unique structural organisation of mammalian skeletal muscle fibres is responsible for the distinctive distribution and localisation of GRs in adult mammalian skeletal muscle fibres. We also hypothesise that, as myoblasts differentiate into myotubes, GRs migrate to the nucleus and as contractile proteins are laid down, the nucleus, the GRs and most other cell organelles are



**Figure 8. FAKi 14 inhibits the BDP-induced increase in phosphorylation of p38 MAPK and ERK1 (p44)**

Summary data showing the effects of pre-treating fast-twitch (F) and slow-twitch (S) muscle fibre bundles with the Ringer solution only (FC; SC), the Ringer solution plus 10  $\mu$ M FAKi (FI; SI) or the Ringer solution plus the inhibitor as well as the GC (FBI; SBI) for 10 min, on the phosphorylation of p38 MAPK (A), the 54 kDa isoform of JNK (B; p54); the 46 kDa isoform of JNK (C; p46); ERK1 (D; p44) and ERK2 (E; p42). Note that pre-treatment with FAKi inhibits the BDP-induced increase in the phosphorylation of p38 MAPK and ERK1 but not that of the two JNK isoforms. \* $P < 0.05$  when the data indicated are compared to those of the corresponding untreated (control) fibres. † $P < 0.05$  when the data indicated are compared to those of the corresponding fibres treated with BDP. ‡ $P < 0.05$  when the data indicated are compared to those of the fibres treated with the inhibitor only.

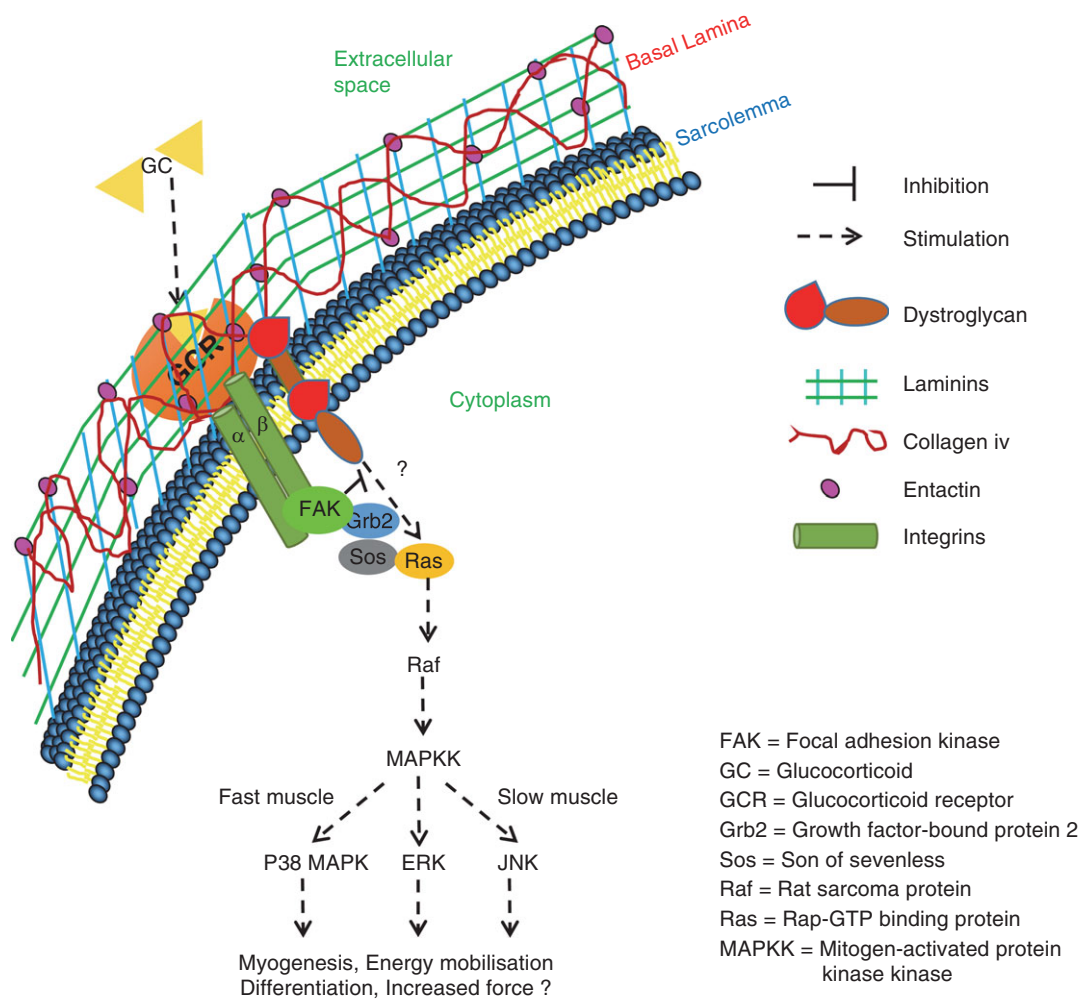
pushed to the periphery of the muscle fibres. However, how the GRs get out of the muscle fibres and localise in the extracellular matrix is uncertain. Also, why and how the receptor localises in the proximity of mitochondria is also uncertain and further studies to investigate these uncertainties are necessary.

Another possibility is that GRs are bound to or are closely associated with cytoskeletal proteins such as dystrophins, dystroglycans, utrophins and laminins. In adult mammalian skeletal muscle fibres, these proteins are important in the maintenance of cell shape, structure and function, and are closely associated with the cell membrane (Gawlik & Durbeej, 2011). Indeed, GCs have been shown to increase the content of dystrophin and utrophin in both normal and dystrophic differentiating human skeletal muscle satellite cells (Sklar *et al.* 1991;

Pasquini *et al.* 1995). In the present study, GRs were also found to be able to bind laminin (Fig. 3). The reason why GC treatment increases the accumulation of these proteins is uncertain. Here we postulate that, in mammalian skeletal muscles, GCs, and hence GRs, may be important for the synthesis, recruitment and/or organisation of these extracellular matrix proteins. Indeed, the present findings show that myoblasts expressed little or no laminin (Fig. 1A and B) and laminin only started to appear in myotubes when the GRs translocated into the nucleus (Fig. 1C and D).

### Effects of GCs on the MAPK pathway

Another novel finding in the present study is the observation that treating mammalian skeletal muscle



**Figure 9. A schematic diagram summarising the mechanism we propose underlies the non-genomic effects of BDP in mammalian muscle fibres**

Our hypothesis is that some of the GRs ('GCR' in the figure) are localised in the basement membrane where they are bound by laminin and that their stimulation by GCs leads to the activation of either integrins (in fast-twitch fibres) or dystroglycan (in slow-twitch fibres). We also think that integrins recruit and activate FAK leading eventually to the phosphorylation of p38 MAPK and ERK1&2, whereas dystroglycans act lower down the pathway and lead to the activation of JNK. We also think that FAK has inhibitory effects on the dystroglycan pathway.

fibre bundles with physiological concentrations of BDP increases the phosphorylation/activation of ERK1&2, JNK and p38 MAPK in a fibre type- and duration-dependent manner (Figs 4 and 5). MAPKs are also activated in brain tissues of rats subjected to short periods of psychological stress (Gutiérrez-Mecinas *et al.* 2011) as well as in hippocampal and PC12 (rat pheochromocytoma) cells treated with low doses of corticosterone (Li *et al.* 2001; Qi *et al.* 2005). However, treatment of most other cell types, including HeLa cells, vascular endothelial cells, macrophages, mast cells and monocytes, with low doses of GCs has been shown to have the opposite effect, i.e. it decreases MAPK activation (Franklin *et al.* 1997; Caelles *et al.* 1997; González *et al.* 2000; Kassel *et al.* 2001; Imasato *et al.* 2002; Lasa *et al.* 2002; Bruna *et al.* 2003). Together these findings suggest that the effects of GCs on the MAPK pathway depend on the tissue that the cells are derived from and the physiological role of stress and MAPKs in that tissue. For example, in tissues such as the brain and skeletal muscle that are directly involved in the stress response and where activation of MAPKs by short term stress is beneficial (Gutiérrez *et al.* 2011; Pérez *et al.* 2013; present study), physiological concentrations of GCs increase the activation of MAPKs. Conversely, in tissues such as the immune system where activation of MAPKs by stress is detrimental to the long term survival of the organism, GCs inhibit their activation (Franklin *et al.* 1997; Caelles *et al.* 1997; González *et al.* 2000; Bruna *et al.* 2003).

### Physiological functions of GCs

In mammalian skeletal muscle cells, all three MAPKs (p38 MAPK, JNK and ERK) have been implicated in the regulation of myogenesis and the expression of skeletal muscle-specific genes (Bennett & Tonks, 1997; Cuenda *et al.* 1999; Weston *et al.* 2003). Additionally, intense exercise (Widegren *et al.* 2000) and marathon running (Yu *et al.* 2001), both of which lead to fibre type switch, have been shown to induce the release of GCs and the activation of p38 and ERK1&2 in the vastus lateralis portion of the quadriceps femoris muscle (note that the vastus lateralis consists mostly of fast-twitch muscle fibres). p38 MAPKs have also been shown to act as molecular switches in the activation of quiescent satellite cells (Jones *et al.* 2005). Myogenesis is important in skeletal muscle development, whereas satellite cell activation is essential for skeletal muscle repair/regeneration. The fact that GC treatment increases the activation of all three MAPKs in mouse skeletal muscle fibres (present study) and strenuous exercise activates p38 and ERK1&2 in fast-twitch fibres suggests that GCs may play an important role in both myogenesis and skeletal muscle repair/regeneration. However, the exact role of GCs in myogenesis and satellite cell

activation is uncertain and further studies to confirm this are necessary.

The GC-induced activation of ERK1&2 we report here may also be responsible for the GC-induced increase in maximum isometric force ( $P_o$ ) in slow-twitch muscle fibres previously reported by Pérez and her colleagues (Pérez *et al.* 2013). Here we speculate that the GC-induced activation of ERK1&2 leads to the phosphorylation of the regulatory myosin light chain (RMLC) and the potentiation of  $P_o$  as previously shown in Hamdi & Mutungi (2010). Fast-twitch and slow-twitch muscle fibres express different isoforms of RMLCs that are phosphorylated at low stimulation frequencies in fast-twitch fibres and at high frequencies in slow-twitch fibres (Moor & Stull, 1984). The phosphorylation of RMLCs in fast-twitch fibres increases sub-maximal force (including twitch contraction), whereas their function in slow-twitch muscles is unknown as their phosphorylation does not seem to affect twitch amplitude (Moore & Stull, 1984). Here we speculate that the phosphorylation of RMLCs in slow-twitch fibres leads to the potentiation of maximum isometric force. However, the exact mechanism underlying the GC-induced increase in force is uncertain and further studies to confirm this are needed.

### Mechanism underlying the effects of GCs in skeletal muscle

As mentioned above, in most cells, GCs inhibit the MAPK pathway. They do this either by up-regulating the synthesis and activation of proteins such as glucocorticoid-induced-leucine zipper, MAPK phosphatase-1 and annexin-1 (González *et al.* 2000; Lasa *et al.* 2002) or by directly interacting with components of the MAPK pathway (Caelles *et al.* 1997; Bruna, 2003). The results reported here suggest that the effects of GCs in mammalian skeletal muscle fibres are mediated through GRs localised close to the cell surface (Fig. 6) and that their stimulation leads to the activation of focal adhesion kinase (FAK, Fig. 9). Here we hypothesise that in mammalian skeletal muscle fibres, membrane GRs are bound to laminin and that their stimulation by GCs leads to a conformational change in laminin and the activation of FAK by integrins (Fig. 9). In mammalian skeletal muscles, laminin binds to and signals through both integrin  $\alpha7\beta1$  and dystroglycan (Gawlik & Durbeek, 2011). Therefore, we think that the conformational change in laminin activates integrin  $\alpha7\beta1$  in fast-twitch muscle fibres and dystroglycan in slow-twitch fibres (Fig. 9). We also think that integrins recruit and activate FAK leading eventually to the phosphorylation of p38 MAPK and ERK1&2, whereas dystroglycans act slightly lower down the pathway and their stimulation leads to the activation of JNK. We also think that FAK has inhibitory effects on



the dystroglycan pathway (Fig. 9). However, the exact mechanism mediating the GC-induced increase in the activation of MAPKs in mammalian skeletal muscle fibres is uncertain and further studies to determine this are necessary.

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## Additional information

### Competing interests

None declared.

### Author contributions

G.M. was responsible for the conception and design of the experiments. He dissected the small muscle fibre bundles and helped with the various treatments. He also did the immunocytochemistry experiments and wrote the manuscript. S.B. and F.P. did all the confocal and electron microscopy experiments including the immunogold labelling. L.A. did most of the Western blotting under the supervision of G.M. He also helped with the treatment of the muscle fibre bundles with the various compounds. E.A. and L.A. did the pull-down assays with the help of D.S. T.P. performed all the C2C12 cell experiments. All authors approved the final version of the manuscript. All the immunocytochemistry and cell signalling experiments were performed in the BioMedical Research Centre, University of East Anglia; whereas, the immunofluorescence confocal microscopy and immunogold labelling electron microscopy experiments were performed in the Department of Neuroscience, Imaging and Clinical Sciences, University G. d' Annunzio.

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