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Calcium signals and FGF-2 induced neurite growth in cultured parasympathetic neurons: spatial localization and mechanisms of activation

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Abstract

The growth of neuritic processes in developing neurons is tightly controlled by a wide set of extracellular cues, that act by initiating downstream signalling cascades, where calcium signals play a major role. Here we analyze the calcium dependence of the neurite growth promoted by basic Fibroblast Growth Factor (bFGF, or FGF-2) in chick embryonic ciliary ganglion neurons, taking advantage of dissociated, organotypic and compartmentalized cultures. We report that signals at both the growth cone and the soma are involved in the promotion of neurite growth by the factor. Blocking calcium influx through L- and N-type voltage dependent calcium channels and TRPC channels reduces, while release from intracellular stores does not significantly affect, the growth of neuritic processes. Simultaneous recordings of calcium signals elicited by FGF-2 at the soma and at the growth cone show that the factor activates different patterns of responses in the two compartments: steady and sustained responses at the former, oscillations at the latter. At the soma, both voltage dependent channel and TRPC blockers strongly affect steady state levels. At the growth cone, the changes in the oscillatory pattern are more complex; therefore we used a tool based on wavelet analysis to obtain a quantitative evaluation of the effects of the two classes of blockers. We report that the oscillatory behaviour at the growth cone is dramatically affected by all the blockers, pointing to a role for calcium influx through the two classes of channels in the generation of signals at the leading edge of the elongating neurites.

Introduction

In the developing nervous system, the oriented growth of neuritic processes is a crucial step in the establishment of the correct neuronal connections; it is a highly regulated process, under the tight control of a wide array of extracellular cues [12, 29]. Neurotrophic factors play a prominent role in this context: they can either promote elongation [24, 57, 60] or act as a stop signal and induce branching (see e.g. Refs. [9, 50]), but above all they act as attractive or repulsive agents, influencing not only growth but orientation [31, 45, 56]. Calcium signalling is a key player in these processes [18, 21, 26, 62], and the different responses to extracellular signals may depend on the specific neuronal type, but also reflect different spatiotemporal patterns of calcium signalling, in turn supported by different calcium mobilizing mechanisms. The growth cone is the leading edge of the extending neurite that senses the extracellular environment. Its behaviour and adaptation to the changing milieu are finely regulated by local changes in $[Ca^{2+}]_i$ (see e.g. Ref [18]), and, at least in part, in the expression of membrane and cytoskeletal proteins [32, 36], but the integration and crosstalk between signalling mechanisms at the periphery and at the soma are not completely understood. In particular, while evidence of retrograde signalling of transcription factors to the soma is available (see e.g. Ref. [10]), and a pioneering paper [7] suggested that the presence of the NGF at the soma was not necessary for neurite elongation, the role of somatic signals has not been extensively investigated to date.

Basic Fibroblast Growth Factor (FGF-2, or bFGF) is one of the best characterized neurotrophic factors involved in the control of neurite growth and orientation [41, 53]. In a well established model of peripheral neurons, cultured E7 chick ciliary ganglion neurons, we have shown that it induces long lasting calcium influx, dependent on the tyrosine kinase activity of FGFRs [13], and promotes neuritic growth by increasing the mean velocity of elongation of individual neurites [60]. The prototypical receptor for this agonist, FGFR1, is expressed in these neurons both in the somatic and in the neuritic compartment [16]. The purpose of this paper was to investigate the role of the

calcium signals activated by FGF-2 in the promotion of neurite growth, the specific contribution of signals at the soma and at the growth cone, and the calcium mobilizing mechanisms involved. To this end, taking advantage of compartmentalized cultures and of calcium imaging, we provide evidence that while the presence of FGF-2 at the soma plays a minor role, signalling at the growth cone is the main event responsible for the promotion of neurite outgrowth. On the other hand both at the growth cone and at the soma, calcium influx is involved in this action, through voltage dependent and TRPC (Transient Receptor Potential Canonical) channels. At the growth cone, the factor elicits a peculiar pattern of oscillations of $[Ca^{2+}]_i$, that can be strongly reduced by pharmacological blockers of the two channel families, suggesting a relationship between the onset of calcium oscillations and the neurite growth promoting action of FGF-2.

Methods

Dissociated cultures

Chick ciliary ganglia were dissected from E7 embryos and maintained in a chemically defined N2 medium [5] as previously described [13]. Briefly, ganglia were both enzymatically (0.06% trypsin, in cation-free phosphate-buffered saline (PBS), for 5 min at 37°C) and mechanically dissociated and resuspended in N2 medium. Nearly 15,000 cells were plated in the middle area of plastic dishes or glass coverslips coated with poly-D-lysine (PL; 100 μg/ml) and laminin (LN; 2 $\mu g/cm^2$) in N2 medium, alone or with added FGF-2 (20 ng/ml, Alomone Labs, Israel).

Organotypic cultures

Chick ciliary ganglia were obtained from E7 embryos and collected in PBS. Explants were cultured in rat tail collagen gel as previously reported [60] and plated in 12-multiwell plates previously treated with PL and LN. Briefly, ganglia were taken one by one in a small drop of PBS with a

Pasteur pipette and placed in the middle of an empty well with as little PBS as possible. A 15 µl drop of collagen gel mix was added on the top of each ganglion and kept at 37°C in a CO_2 incubator for 40 min until the gel had polymerized. Subsequently 1 ml of N2 medium was added to each well, alone or with added 20 ng/ml FGF-2.

Immunocytochemistry

For immunocytochemical experiments, dissociated cells were fixed in paraformaldehyde (PFA) 4% for 20 min at room temperature, whereas CG explants were fixed in methanol at –20°C for 10 min. For neurite outgrowth assays, both dissociated CG neurons and CG explants were stained with a monoclonal anti-NF68 antibody to take into account only the contribution of neurons. Chick CG neurons and CG explants fixed, respectively, after 24 and 48 hr of culture were both incubated overnight with the anti-NF68 antibody (1:300) at room temperature. On the following day, dissociated neurons were incubated with an anti-mouse IgG conjugated with the Cy3 fluorophore (1:1,000). In the case of organotypical cultures, the reaction was developed with a horse biotinylated antimouse IgG (Vector Laboratories, Burlingame, CA; 1:200) detected with the peroxidase-conjugated biotin-avidin complex (Vector Laboratories), followed by a brief incubation with 3,30-diaminobenzidine tetrahydrochloride (DAB; 15 mg/ml) and H_2O_2 (8 µg/ml).

Assessment of cell survival

Cell survival assays were performed on dissociated cultures. Cells were plated on 60-mm-diameter/2-mm-grid culture dishes (Corning Inc., Corning, NY), and two square fields of the grid in each dish were recorded by means of a CoolSNAP-Pro-color CCD camera (Roper Scientific/ Photometrics, Germany) connected to an inverted microscope (Olympus, IX50). Survival levels were assessed by counting neurons, in the same field, after 24 and 48 h of culture, and giving the values as percentages of the counts at 2 h.

Three independent experiments, in duplicate, were performed for each individual treatment.

Assessment of neurite outgrowth

In dissociated cultures, after immunocytochemical staining, neurite outgrowth was assessed by measuring the total area occupied by neurofilament-positive elements using the image processing program ImageJ [38] as previously described [60]. Briefly, for dissociated CG cultures, eight to ten fields (1,024 × 1,024 pixels, 16-bit gray scale) per culture dish were acquired by means of a Fluoview 200 laser scanning confocal microscope (Olympus) with a 50× oil immersion objective, at an excitation wavelength of 568 nm. Fields were selected by observing the DIC (Differential Interference Contrast) images and randomly choosing non overlapping fields. Contribution of neuronal somata was manually subtracted and the analysis was performed by applying to 16-bit images a threshold in order to obtain binary images (black pixels corresponding to neurites and white pixels to background). Total number of neurons was evaluated from DIC images of the same fields analyzed, and these values were used to normalize the total area occupied by black pixels (μm^2). Performing analysis on fluorescent images allowed to exclude the potential contribution of glial cell processes, without substantially losing any contribution from the neurites, as can be seen merging a DIC image with a fluorescent one of the same field (Suppl. Fig. 1).

As in previous experiments [16] analysis was performed on cultures at 24 h, since for longer times extensive neurite fasciculation could lead to incorrect evaluation of global neurite occupancy. Experiments were repeated three to four times in duplicate.

In the case of CG explants images were acquired in bright field mode with an inverted microscope Olympus IX50 equipped with a CoolSNAP-Pro CCD camera (Roper Scientific/Photometrics, Germany) and an image analysis software (Image Pro Plus 4.1 for Windows; Media Cybernetics). After subtraction of the area corresponding to the body of the whole explant, previously reconstructed from a collage of partial images (1,392 × 1,040 pixels, 8-bit gray scale), binary images were obtained again by applying a threshold. The percentage of black square pixels over the

total area (2,000 × 2,000 pixels) was used as an index of global neurite growth. As previously described [16, 60] analysis was performed at 48 h of culture.

Data are the average of three to six independent experiments, and five to twelve explants per condition were analyzed.

Compartmentalized cultures

In order to assess the relative contribution of FGF-2 signalling and of the downstream calcium fluxes at the somata and at the growth cone of the neurites, we performed a set of experiments with compartmentalized cultures, using Campenot chambers [7, 8] that allow to present FGF-2 (20 ng/ml) and the inhibitors to the cells in one or more compartments. Briefly, the plastic dishes were coated with 100 μg/ml PL and 2 μg/cm^2 LN, and 30,000 cells (obtained from E7 embryos) were plated in the central part of a three compartment chamber (Tyler Research, Canada), sealed with silicone grease to the bottom of the dish. To allow cells to adhere, chambers were kept for two hours at 37°C in a humidified atmosphere containing 5% CO_2. Afterwards, specific volumes of N2 medium supplemented with B27 (dilution 1:50; Gibco, Invitrogen Corporation, Carlsbad, CA, USA) were added into both central and side compartments.

Some Authors have used the average neurite length or the length of the longest neurites to quantify neurite growth (see e.g. Ref. [37, 54]). However, considering neurite length may not give the correct information on the effects of FGF-2 signalling on the global outgrowth of neuritic processes. Others have measured the global axonal density by means of Western Blot assessment of beta-tubulin levels [47]. We chose a different approach: in analogy with the protocols used to evaluate global neurite occupancy in dissociated and organotypic cultures, we measured the total area covered by neurites in each single track, by staining neurites with the anti-neurofilament antibody. Staining and acquisition protocols were the same as for organotypic cultures.

Three independent experiments (in duplicate) were performed for each treatment

Calcium imaging

$[Ca^{2+}]_i$ was monitored using the Ca^{2+} indicator dye FURA-2 acetoxymethylester (FURA-2AM, Molecular Probes, Inc.). Cells were loaded for 45 min at 37°C with 2 μM FURA-2AM in N2 medium and subsequently washed in Tyrode Standard solution (NaCl 154 mM, KCl 4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, HEPES 5 mM, glucose 5.5 mM, pH 7.34 with NaOH). After dye loading cells were transferred to a perfusion chamber (Bioptechs, USA) connected to a peristaltic pump and mounted on an inverted fluorescence microscope (Nikon TE-2000-S), a Xenon lamp illumination system and a CoolSNAP CCD camera (Roper Scientific/Photometrics, Germany). All experiments were performed at 37°C. A gravity microperfusion system, regulated by electrovalves, was employed to switch from the standard Tyrode solution to the same medium containing human recombinant FGF-2 (40 ng/ml) either with or without the inhibitors. Calcium measurements were performed exciting FURA-2AM for 0.5 s alternatively at 340 nm and 380 nm, with a dark interval of 1 s, and emission was recorded at 510 nm. Images were visualized on a computer with the dedicated acquisition software Metafluor (Universal Imaging Corporation, PA). In experiments performed at 24 h of culture, a 20× objective was used, and ROIs (Region of Interest) corresponding to the cell bodies were chosen. For simultaneous recordings from the soma and the growth cone, experiments were performed after 6 h of culture, when neurite extension was still limited and all the compartments could be observed by means of a 100× objective. One or at most two cells per dish could be recorded. Since at this short culture time cells could still be recovering from the dissociation procedure and some perturbation in the membrane could be expected, all cells were challenged with 40 mM KCl and non responsive cells were discarded. Raw data were analysed with IGOR Pro software: peak amplitudes of somatic signals are given as $\Delta R/R$ (increase in 340/380 ratio over the basal value).

Wavelet analysis of calcium signals

To give a quantitative evaluation of the changes in the oscillatory pattern of calcium signals at the growth cone following administration of FGF-2 and of the calcium channel blockers, an approach based on wavelet analysis was employed, using KYM 0.4 software (http://sourceforge.net/projects/kym/) and according to the formulation given in [40]. The original trace $f(t)$ was first detrended and denoised (see e.g. Fig. 6E, upper box), then its wavelet transform $W(a,b) = |a|^{-1/2} \int \psi^*[(t - b)/a]\, f(t)\, dt$ was computed, according to equation 2 of [40], as a function of time b and scale a (* denotes complex conjugation). Morlet function $\psi(t) \approx \pi^{-1/4} \exp(-0.5\, t^2) \exp(ist)$ was used as mother wavelet (s is a constant parameter; $s = 5$ in our case; see equations 3,4,5 of [40]). The resulting scaleogram $|W(t,v)|$ was plotted with an appropriate Lookup Table (LUT), after having renamed b as t and having done the substitution $v = s/(2\pi a)$, in order to get a representation of the signal in the time-frequency domain (see e.g. Fig. 6E, middle box). Then the index $J(t) \approx \int |W(t,v)|^2\, v\, dv$ was calculated as formally defined in equation 9 of [40] and plotted as a function of time (e.g. Fig. 6E, lower box), so providing a reliable estimate of the oscillatory activity of the signal $f(t)$. Finally, for each temporal window of interest (i.e. control, FGF-2 perfusion and addition of blockers) the average J (<J>) was calculated in order to estimate the increase of oscillatory activity in terms of ratio between activity during the treatments (FGF-2 or FGF-2 plus blockers) and activity in control conditions (ctrl): $r_J = \langle J \rangle_{drug} / \langle J \rangle_{ctrl}$ (as defined in equation 10 and 11 of [40]). By this way we could compare the average value of r_J during FGF-2 administration with its decrease in the presence of the blockers (see e.g. Fig. 6D), similarly to what we did with $\Delta R/R$ values obtained from somatic signals (see e.g. Fig. 6B).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Normality of the residual distributions has been verified through Shapiro–Wilk normality test; Levene's test showed variances to be quite homogeneous over each condition. For these reasons one-way ANOVA and

Tukey-corrected post hoc tests were a suitable method for significance assessment. Statistical analyses of control and experimental groups were performed using SPSS software.

If not otherwise specified, all chemicals and drugs were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Calcium dependence of neurite growth induced by FGF-2

In order to evaluate the role of calcium signals in the promotion of neurite growth induced by FGF-2, we cultured for 24 h dissociated neurons loaded with the membrane-permeant calcium chelator BAPTA-AM, at concentrations (1-5 μM) that we checked to have no influence on survival in dissociated cultures (FGF-2: 86.99 ± 6.35% at 24 h, 78.77 ± 8.86% at 48 h; FGF-2 + BAPTA 1 μM: 81.39 ± 1.33% at 24 h, 82.36 ± 4.94% at 48 h; FGF-2 + BAPTA 5 μM: 80.83 ± 1.49% at 24 h, 76.74 ± 3.06% at 48 h; $p > 0.05$). In these cultures, BAPTA at 1 μM reduced the FGF-2 induced increase in neurite growth and at 5 μM completely abolished it, while not influencing basal growth in the sole presence of poly-L-lysine and laminin (Figs. 1 and 2A).

Calcium influx from the extracellular medium through voltage-dependent channels has been reported to be involved in the promotion of neurite growth and orientation in several experimental models (see e.g. Refs. [23, 44, 56, 57]); to test their contribution to FGF-2 induced neurite growth in ciliary ganglion neurons, a pharmacological approach was employed. The two major types of voltage dependent calcium channels expressed in these cells, that collectively account for more than 90% of voltage activated calcium currents, are L- and N-type channels [14]. In the presence of 10 μM nifedipine (blocker of L channels) and 2 μM ω-conotoxin (blocker of N channels), FGF-2

dependent increase in neurite growth was significantly inhibited in dissociated cultures (where the inhibition was complete) and in whole ganglia, respectively at 24 h (Figs. 1 and 2B) and 48 h (Figs. 1 and 2C), providing evidence that calcium influx through voltage-dependent channels is a necessary step in the cascade of events linking FGFR1 activation to promotion of neurite elongation. Notably, at these concentrations the two blockers have been shown to have no effect on FGF-2 dependent neuronal survival [13].

E7 CG neurons in culture may show spontaneous electrical activity, that is enhanced by acute perfusion with FGF-2 [13]; in long-term culture with the factor many cells show calcium signals that are partially abolished by the voltage-dependent Na^+ channel blocker tetrodotoxin, TTX [2]. Therefore, we tested if blocking voltage dependent Na^+ channels could affect the promotion of neurite growth by FGF-2. In dissociated cultures at 24 h, 10 μM TTX had no effect both on FGF2-induced neurite growth (Figs. 1 and 2D) and neuronal survival ratios (FGF-2: 95.93 ± 2.53% at 24 h, 93.15 ± 2.85% at 48 h; FGF-2 + TTX 10 μM: 98.33 ± 1.61% at 24 h, 98.56 ± 1.95% at 48 h; $p > 0.05$).

It can be concluded that some additional mechanism(s) must be responsible for the FGF-2 induced depolarization of the membrane potential and subsequent activation of calcium VOCs. At the growth cone of extending axons, it has been shown that several neurotrophic factors and guidance molecules can activate calcium permeable cationic channels of the TRPC family [19, 27, 31, 45, 56]; the ensuing depolarization of the membrane potential may be sufficient to recruit voltage dependent calcium channels, thus further enhancing calcium influx [17, 56]. While no data are available for a similar role of FGF-2, we have previously shown [1] that the factor can induce a calcium influx at least partially dependent on TRPC1 activation in endothelial cells. In the light of these observations, we tested the role of this family of channels by using SKF 96365, a non selective blocker of TRPC channels.

At both 0.5 and 2 μM, SKF 96365 completely abolished the FGF-2 induced enhancement of neurite growth in dissociated cultures, while not influencing basal growth in control conditions (Figs. 1 and

2E); in organotypic cultures, with 2 μM SKF the inhibition was again specific and statistically significant, even if not complete (Figs. 1 and 2F). Notably, up to 2 μM, the blocker had no effect on FGF-2 dependent neuronal survival ratios (FGF-2: 98.71 ± 3.41% at 24 h, 101.51 ± 2.90% at 48 h; FGF-2 + SKF 0.5 μM: 104.18 ± 2.23% at 24 h, 106.13 ± 3.31% at 48 h; FGF-2 + SKF 2 μM: 108.29 ± 3.28% at 24 h, 110.41 ± 4.54% at 48 h; $p > 0.05$).

These data provide evidence that activation of TRPC channels is another key step in the signalling cascade downstream of FGF-2 controlling the growth of neuritic processes in cultured embryonic chick ciliary ganglion neurons.

Finally, calcium release from intracellular stores has been shown to play a role in neurite growth and orientation [28, 30]. In dissociated cultures of ciliary ganglion neurons, 10 nM thapsigargin (TG), an agent that irreversibly depletes intracellular stores, had no effect on neuronal survival ratios (FGF-2: 84.06 ± 1.93% at 24 h, 80.30 ± 3.08% at 48 h; FGF-2 + TG 10 nM: 85.86 ± 2.47%, 78.90 ± 3.64% at 48 h; $p > 0.05$) and on neurite outgrowth (Figs. 1 and 2G), both in control conditions and in the presence of FGF-2. Even at the relatively low concentration of 10 nM, in CG neurons at 24 h of culture, TG effectively induced calcium release from intracellular stores. The release was maximal, as a subsequent perfusion with 100 nM TG did not elicit any additional increase in $[Ca^{2+}]_i$ ($n = 65$; a representative trace is shown in Fig. 2H). When 2 mM of calcium were reintroduced in the extracellular solution, an increase in $[Ca^{2+}]_i$ could be observed, indicative of the activation of store operated calcium entry, SOCE [49]; remarkably, subsequent stimulation with FGF-2 elicited a further increase in $[Ca^{2+}]_i$.

Spatial compartmentalization of FGF-2 induced responses

A major issue, when dealing with the role of neurotrophic factors in growth and orientation of neuritic processes, is the understanding of the spatial specificity and subcellular localization of the

signals activated by the extracellular cue. While most data refer to changes in $[Ca^{2+}]_i$ at the growth cone (see e.g. Refs. [17, 52]), the role of the signals originated at the somatic compartment has been little studied. For this reason we employed compartmentalized cultures by using Campenot chambers [7, 8] (Fig. 3A).

Since protocols with Campenot chambers require the neurons to be cultured for several days, and since the effect of the addition of FGF-2 in the different compartments had to be compared with control conditions in which the neurons are cultured with the sole presence of adhesion molecules, a bias due to cellular death could not be excluded. For this reason, all experiments were performed with the B27 supplement [6] added to the N2 culture medium. In parallel experiments with dissociated cultures maintained up to 5 days in the presence of B27, with and without 20 ng/ml FGF-2, survival ratios were not significantly different in the two experimental conditions: in the presence of 20 ng/ml LN + B27, survival ratios were 74.74 ± 9.56% at 2 d, 45.43 ± 8.66% at 5 d; with added FGF-2, the ratios were 100.29 ± 5.00% at 2 d and 42.81 ± 5.29% at 5 d ($p > 0.05$).

In compartmentalized cultures, when FGF-2 was absent in all compartments, after 5 days in culture virtually no growth of neuritic processes could be detected in the lateral chambers. When the neurotrophic factor was added to any compartment, outgrowth of neurites in the lateral compartments could be detected starting from 3 days of culture (Fig. 3B). All subsequent analysis was performed after 2 additional days, i.e. at 5 days.

A rectangular region of 175 μm × 3500 μm was selected for each track, starting at 350 μm from the Teflon barrier (Fig. 3B). Results are shown in Fig. 3C, and can thus be summarized:

- when FGF-2 was present in the somatic compartment, a bilateral growth occurred, with modest total occupancy in both lateral compartments;
- when the factor was present in one lateral compartment, growth was significantly enhanced in this compartment, while a moderate growth could be detected also in the unstimulated compartment: the amount of the latter was not significantly different from what observed in the previous experimental

condition. These data provide a first evidence that FGF-2 is sensed by the growth cones of elongating neurites;

- when FGF-2 was present in both the central and one lateral compartment, global neurite growth in both compartments was slightly increased, but not significantly different from what observed with the factor in one lateral compartment only. Therefore, no synergic effect of FGF-2 presence at the somatic and neuritic compartments could be evidenced.

Characterization and localization of the calcium entry pathways controlling neurite growth

Compartmentalized cultures were next used to localize the contributions of the different calcium mobilizing mechanisms. All subsequent protocols were performed in the presence of 20 ng/ml FGF-2 in both the central and one lateral chamber (control condition) and neurite growth was measured in the chamber in which FGF-2 was present; the blockers were added either in the somatic or in the neuritic chamber or in both.

Addition of the voltage dependent calcium channel blockers nifedipine (10 μM) and ω-conotoxin (2 μM) either in the central or in the lateral compartment reduced global neurite growth by about 60-70% (Fig. 4A); when the blockers were present in both compartments, a comparable effect was observed (percentages of neurite occupation: CTRL: 17.64 ± 1.57%; Soma: 5.21 ± 0.52%; Neurites: 6.30 ± 1.70%; Soma+Neurites: 6.17 ± 1.00%).

The blocker of TRPC channels, SKF 96365 (2 μM), caused effects comparable to those induced by the VOCs blockers (Fig. 4B); the reduction of global neurite growth was between 50 and 60% (Percentages of neurite occupation: CTRL: 17.64 ± 1.57%; Soma: 9.12 ± 1.67%; Neurites: 5.79 ± 2.97%; Soma+Neurites: 7.67 ± 1.71%).

In both cases, no cooperative effect could be evidenced when the blockers were present in both the central and lateral compartments.

Spatial localization and specificity of FGF-2 induced calcium signals

To investigate the spatial localization of calcium signals elicited by FGF-2 and of the mechanisms involved in their generation, we simultaneously recorded from the soma and from the growth cone of the same cell. For this purpose, recordings were performed after about 6 hours of culture, in order to be able to visualize at high magnification neurons with relatively short neurites and identifiable growth cones (See Fig. 5A). By this approach, 1-2 neurons per dish, with 1-2 neurites each, could be analyzed.

In a previous paper [13] we used both 20 and 40 ng/ml in acute experiments, obtaining identical results. Here we opted for 40 ng/ml, since the perfusion pipette had to be positioned quite far from the cell, due to the critical adhesion at this early time of culture, and the actual concentration sensed by the cells could not be determined. We performed a set of control experiments with 20 ng/ml FGF-2: the percentage of responsive cells was 45% (9 out of 20 cells). In cells that did not respond to 20 ng/ml FGF-2, subsequent perfusion with the higher concentration failed to induce an increase in $[Ca^{2+}]_i$ (not shown); in responding cells, perfusion with 40 ng/ml FGF-2 did not elicit any additional response (Suppl. Fig. 2).

Subsequently, a total of 102 cells, with 133 neuritic processes, and 15 isolated neurites (whose soma was outside the field of view) was challenged with 40 ng/ml FGF-2. Responses were recorded in 47 somata (46.03%) and in 86 growth cones (58.10%). The increases in $[Ca^{2+}]_i$ showed a markedly different time course in the two compartments: at the soma the response was in most cases a smooth and gradual increase in $[Ca^{2+}]_i$, followed by either a maintained or a slowly decaying plateau (Fig. 5B, left), in accordance with the responses observed at 24 h [13, 61]. In some cases, the plateau was preceded by a transient phase, indicative of a possible contribution of release from intracellular stores, that has been previously [13] observed in a subpopulation of cells. At the growth cone, the factor usually elicited an oscillatory behaviour, in some cases superimposed on a steady increase

(Fig. 5B, right). This difference could reflect differences in cell geometry such as the surface/volume ratio; however, the picture is probably more complex, since in some instances it was possible to record signals from two growth cones of the same cell (the same cell of Fig. 5A), showing sharply different time courses of the responses (Fig. 5C, left and right): therefore, the spatial specificity of the responses to FGF-2 must be dependent, at least in part, from differences in the calcium mobilizing mechanisms activated in the different compartments.

On both cell bodies and growth cones, substitution of the standard Tyrode solution with another one containing 0.5 mM EGTA and 0 mM calcium completely and reversibly abolished the FGF-2 induced calcium signals (n = 4; Fig. 5D, left: soma; right: growth cone), pointing to a crucial and necessary role of calcium influx from the extracellular medium.

In some cells, spontaneous calcium oscillations could be observed at the growth cone (24.8%), and to a lesser degree (17.9%) at the soma. These cells responded to FGF-2 in percentages comparable to basally silent cells (Fig. 5E, left: soma; right: growth cone).

Finally, we tested the effects of channel blockers on the responses elicited by FGF-2 in the two subcellular compartments. In a previous paper [13] we reported that blockers of voltage-dependent calcium channels did not affect the plateau of the somatic response to FGF-2. The observation was based on data from a limited set of cells (n = 8). This issue is of particular relevance, since we have shown that block of somatic calcium VOCs significantly reduces FGF-2 induced neurite growth. Therefore, we preliminarily re-evaluated this aspect on a larger population of neurons after 24 h in culture, the same time of the recordings shown in [13].

The effects of the combined addition of the L- and N-type calcium channel blockers on the somatic response to FGF-2 were evaluated in 52 cells: a significant ($p < 0.01$) and reversible reduction of the plateau response could be observed in 69.2% of cells; the mean response amplitude ($\Delta R/R$) was 18.57 ± 2.27% before and 11.29 ± 1.55% after administration of the blockers. A typical recording is shown in the inset of Fig. 6A. Therefore, at 24 h, a component of the plateau response to FGF-2 at

the neuronal soma can be ascribed, in the majority of cells investigated, to calcium influx through voltage-dependent calcium channels.

In CG cultures at 6 h, when the VOCs blockers and SKF were presented to the cells prior to perfusion with the factors, no change in baseline $[Ca^{2+}]_i$ could be detected (n = 3; not shown). Acute application of the voltage dependent channel blockers ω-conotoxin (2 μM) and nifedipine (10 μM) affected the somatic and growth cone responses to FGF-2 in quite different ways. 15 cells, with a total of 19 neurites, and 4 isolated neurites were analyzed. At the soma, the effects were comparable to those observed at 24 h: a partial, but significant, and reversible reduction of the amplitude of the plateau response could be recorded in 9 out of 15 somata (Fig. 6A); ΔR/R before application of the blockers was 61.59 ± 16.35%, and in the presence of the blockers was 33.77 ± 13.55% (Fig. 6B).

In 15 out of 23 growth cones an effect was observed: in some cases the blockers caused a partial reduction of the amplitude of the response, but in general they affected the oscillatory behaviour in a more complex way (Fig. 6C). To extract quantitative information about the change in the pattern of oscillations induced by the blockers, an approach based on wavelet analysis [40] was employed, as described in the Methods section, taking r_J as an index of the change in the oscillatory activity. The average value of r_J during FGF-2 administration was 22.14 ± 7.57 and decreased to 6.87 ± 2.74 in the presence of the blockers (Fig. 6D, 6E).

When SKF 96365 was added to the medium during the response to FGF-2, the somatic and growth cone effects were remarkably similar to those observed with the VOCs blockers. 19 cells with a total of 21 neurites, and 4 isolated neurites were analyzed. The TRPC blocker reduced the response at 12 cell somata (Fig. 7A). Average ΔR/R at the soma was 61.89 ± 9.40% in the presence of FGF-2 and was reduced to 41.68 ± 7.79% following addition of the blocker (Fig 7B). In 18 out of 25 growth cones, a change in activity could be detected (Fig. 7C). Wavelet analysis provided evidence for a reduction in the oscillatory activity following perfusion with SKF 96365: average r_J was

22.90 ± 7.18 in FGF-2 and decreased to 8.47 ± 2.15 after addition of the blocker (Fig. 7D, 7E).

Discussion

The data presented in this paper provide new insight into the role of calcium signals activated in response to a neurotrophic factor, FGF-2, in the control of neurite growth and elongation, and point to a specific involvement of different subcellular compartments in this process.

Even if FGF-2 has been considered to be a target derived signal [46, 50], in our case no data are available to support this hypothesis; on the other hand, FGF-2 is expressed by ciliary ganglion neurons in dissociated cultures (A.G. and S.F., unpublished observation). Therefore, it is likely that in the CG it acts as a paracrine/autocrine factor, rather than as a target-derived one. It must be recalled that, even if our data are obtained from mixed cultures, we have previously shown that the role of FGF-2 is cell autonomous [61].

Taking advantage of compartmentalized cultures, we show that FGF-2 exerts a growth promoting action on the neurites. The signalling pathways activated by FGF-2 are not spatially restricted to the growth cone: somatic signals by themselves can induce moderate outgrowth, but only when FGF-2 is sensed by growth cones massive growth occurs. No cooperative effect could be detected when the factor was added in both central and one lateral compartment, suggesting that the signalling at the growth cone induces saturating activation of the machinery responsible for neurite elongation.

A key player in this context is calcium. Growth and orientation of neuritic processes have been shown to depend on calcium signals at the growth cone [18, 62], and to be affected by blockers of both voltage dependent calcium channels (see e.g. Ref. [57]) and of cationic channels of the TRPC family (see e.g. Refs. [28, 31, 56]). The available data provide a quite complex picture: activation of calcium VOCs has been associated with increased growth promoted by FGF-2 [57] and other molecules, including neurotransmitters [22, 44] and with the turning of growth cones towards chemoattractive guidance molecules [17]; on the other hand, it has been reported that their block

can rescue, and activation inhibit, neurite and axonal growth [39, 42, 51]. Interestingly, calcium influx through this class of channels seems not to be a universal prerequisite: in *Xenopus* spinal cord explants, a mixture of calcium VOCs blockers did not affect axonal growth rate [28].

TRPC channels are a more recent entry into the neuritic/axonal growth affair, but the findings are similarly diverging: while the data of their involvement in growth cone turning behaviour point to a chemoattractant role, both promotion and inhibition of growth rate have been reported. For instance, conflicting results have been reported for the role of TRPC5 in axonal growth of hippocampal neurons [11, 19]; it has been shown to promote neurite growth in a neuroblastoma cell line [58] and to inhibit it in PC12 cells [25]. Remarkably, Jacques-Fricke et al. [28], reported that blocking TRPC channels reduced neurite growth rate, while blocking TRPV and stretch activated calcium permeable channels enhanced it. We obtained information on the specific calcium mobilizing pathways activated by FGF-2 by means of a pharmacological approach. While the specificity of the VOCs blockers is well established, the case for SKF-96365 is more controversial [3, 33, 43, 48]. However, most of the nonspecific effects, in particular block of other members of the TRP superfamily or of L-type voltage-dependent calcium channels, are detectable at doses almost two orders of magnitude above 2 μM, the higher concentration used in our experiments. Therefore, the selectivity of the pharmacological tool used in the present work can be considered sufficient to prove the involvement of TRPC channels in the signals and biological effects downstream the activated FGF receptor. A more direct approach, such as silencing of the channel proteins, is not easily performed on these primary cultures, and due to the high redundancy of the different members and their complex heteromerization patterns, only multiple silencing could give useful and reliable answers.

Both classes of blockers significantly reduced neurite growth induced by FGF-2. Data from dissociated and organotypic cultures have been confirmed by those on compartmentalized cultures: when applied either in the central or in one lateral compartment the blockers markedly reduced the global growth of neuritic processes. The effects of the two classes were quite similar, and did not

completely abolish neurite growth (probably underlying the partial contribution of the two pathways); on the other hand, other calcium mobilizing mechanisms may be activated by FGF-2. Moreover, the effects were comparable when the blockers were added either in the central compartment or at the neurites, and no additivity could be evidenced when they were present in both.

Calcium release from intracellular stores has been reported to play a role in this context, too [17, 28]. In our case no effects could be observed in neurons cultured for 24 h following irreversible depletion of the stores by TG.

To investigate the spatial specificity of calcium signalling in neurons challenged with a neurotrophic factor, we performed simultaneous recordings of FGF-2 induced changes in $[Ca^{2+}]_i$ from the growth cone and the soma. To perform this challenging protocol, we had to image calcium signals at high resolution in cells showing relatively short neurites, so that both compartments could be visualized in the same field, i.e. in neurons at about 6 h of culture. In these experimental conditions, FGF-2 elicited long lasting calcium signals in a substantial percentage of neurons, with specific time courses in the two compartments: a rather stable plateau at the soma (comparable to signals recorded at 24-48 h [13], and trains of oscillations, in most cases superimposed on a steady increase in $[Ca^{2+}]_i$, at the growth cone. By means of a quantitative analytical approach based on wavelet analysis we show that channel blockers cause a substantial reduction in the peculiar oscillatory behaviour at the growth cone, where the growth promoting effect of FGF-2 is more marked.

It is of relevance that the information provided by the different experimental protocols was obtained at quite different culture times, from 6 h in acute experiments to 1-5 days for assessment on neurite growth: the substantial agreement between observations at different time scales strengthens the validity of the results.

A final, and relevant, issue is: why blocking calcium channels at the soma, where FGF-2 has a moderate effect on neurite growth, gave the same reduction observed when the blockers were

applied to the neurites? The hypothesis of a retrograde signal could help to explain this observation: when the factor is presented at the growth cone, by some way the information is transferred to the soma, where recruitment of voltage dependent and independent calcium channels activates the machinery responsible for neurite outgrowth. Thus, blocking the channels at the somatic compartment impairs the process, even if the neuritic compartment is not perturbed. Retrogade signals have been reported by several Authors for the prototypical neurotrophin, NGF [55, 34] but also for FGFs [4, 15, 35].

The balance between local and global calcium signals in developing neurons, and the mechanisms by which they can propagate from one compartment to another, has been addressed in a few recent papers. In cortical neurons Hutchins and Kalil [26] have observed local and global calcium signals in elongating axons and their collaterals, dependent on calcium VOCs activation. Interestingly, no changes in $[Ca^{2+}]_i$ were detected at the soma. Guan et al. [20], have shown that extracellular cues, such as the repellent Slit-1, act on neuronal migration when applied at the growth cone and not at the soma: the agonist elicits a local calcium increase that retrogradely propagates to the soma. Finally, Yamada et al. [59] found that glutamate did induce axonal retraction in dentate granule cells when applied at the somatodendritic area but not when presented at the growth cone; the response was mediated by a calcium wave originating at the soma and propagated anterogradely to the growth cone, while an increase in $[Ca^{2+}]_i$ at the latter site was without effect. Therefore, it appears that different forms of signal propagation, dependent on specific calcium mobilizing mechanisms, can modulate the motility of the somatic and growth cone compartments.

In our case, some form of crosstalk between the soma (or more precisely, the perisomatic compartment) and the growth cone appears to exist: while the oscillations at the growth cone are localized signals and are likely involved in the massive growth observed when the factor is present only at the periphery, the steady component can represent the global signal that activates downstream neurite growth-promoting mechanisms dependent on perisomatic calcium influx.

In conclusion, by taking advantage of a set of different experimental approaches we have provided a coherent, even if not conclusive, picture of the interplay between the calcium influx mechanisms activated by a neurotrophic factor at different subcellular compartments and their involvement in the promotion of neurite growth. The most relevant findings can be summarized as follows:

- FGF-2 promotion of neurite growth in GC neurons is mainly dependent on calcium influx;
- somatic signals play a role in this process, but maximum growth can be observed only with FGF-2 at the growth cone;
- both calcium VOCs and TRPC channels are involved. Blockers of each of the two channel families can completely abolish the oscillations at the growth cone, thus indicating that a complex interplay between voltage dependent and independent calcium channels is necessary for the activation of this peculiar response. On the other hand, the sustained component appears to be a good candidate for the global signal that can transfer information between the periphery and the soma.

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Figure legends

Figure 1. Evaluation of global neurite growth in dissociated and organotypic cultures.

Images of dissociated (upper row) and organotypic (lower row) cultures of E7 chick CG neurons, respectively at 24 and 48 h of culture, in control conditions (P+LN), with FGF-2 alone and in the added presence of BAPTA and of the various inhibitors of calcium mobilizing mechanisms. Staining procedures as described in the Methods section. Scale bars: A 5 μm, B 20 μm.

Figure 2. Effects of blockers of different calcium mobilizing pathways on neurite growth at 24 h in dissociated (A, B, D, E, G) and at 48 h in organotypic (C, F) cultures of E7 chick CG neurons.

A. In dissociated neurons, incubation with 1-5 μM BAPTA-AM did reduce global neurite growth dependent on FGF-2 without affecting growth in the sole presence of adhesion molecules. Values of neurite occupation were: CTRL (P+LN): 471.61 ± 16.45 μm^2; FGF-2: 610.86 ± 22.79 μm^2; BAPTA 1 μM: 426.04 ± 17.41 μm^2; BAPTA 5 μM: 403.72 ± 21.99 μm^2; FGF-2 + BAPTA 1 μM: 492.42 ± 21.18 μm^2; FGF-2 + BAPTA 5 μM: 415.30 ± 15.06 μm^2. ** $p < 0.01$ vs. CTRL condition; ++ $p < 0.01$ vs. FGF-2 condition.

B. 2 μM ω-conotoxin and 10 μM nifedipine (CTX +NIFE) reduced FGF-2 induced neurite growth in dissociated cultures. Values of neurite occupation were: CTRL: 621.42 ± 45.33 μm^2; FGF-2: 869.30 ± 86.90 μm^2; CTX+NIFE 608 ± 43.66 μm^2; FGF-2 + CTX+NIFE: 497.17 ± 25.27 μm^2. ** $p < 0.01$ vs. CTRL; ++ $p < 0.01$ vs. FGF-2 condition.

C. Comparable effects of the two Ca^{2+} VOCs blockers were observed in organotypic cultures. Percentages of neurite occupation were: CTRL: 14.23 ± 3.21 %; FGF-2: 42.45 ± 4.39 %; CTX+NIFE: 12.06 ± 2.31 %; FGF-2 + CTX+NIFE: 29.30 ± 2.71 %. ** $p < 0.01$ vs. CTRL; + $p < 0.05$ vs. FGF-2 condition.

D. Incubation with tetrodotoxin (TTX), blocker of Na^+ VOCs, had no significant effect on neurite growth in dissociated cultures. CTRL: 544.85 ± 21.10 μm^2; FGF-2: 743.72 ± 36.09 μm^2; TTX: 598 ± 22.36 μm^2; FGF-2 + TTX: 698.59 ± 22.84 μm^2. ** $p < 0.01$ vs. CTRL; # $p < 0.05$ vs. TTX condition.

E. Neurite occupation promoted by FGF-2 in dissociated cultures was reduced by the TRPC channel blocker SKF 96365. CTRL: 623.70 ± 67.81 μm^2; FGF-2: 918.71 ± 81.93 μm^2; SKF 0.5 μM: 586.81 ± 50.39 μm^2; SKF 2 μM: 517.72 ± 43.50 μm^2; FGF-2 + SKF 0.5 μM: 519.34 ± 27.99 μm^2; FGF-2 + SKF 2 μM: 575.53 ± 35.95 μm^2. ** $p < 0.01$ vs CTRL; ++ $p < 0.01$ vs. FGF-2 condition.

F. Comparable effects of SKF 96365 were observed in organotypic cultures. CTRL: 8.73 ± 1.42 %; FGF-2: 35.91 ± 2.68 %; SKF 0.5 μM: 8.53 ± 1.64 %; SKF 2 μM: 5.13 ± 1.20 %; FGF-2 + SKF 0.5 μM: 35.27 ± 3.71 % ; FGF-2 + SKF 2 μM: 22.13 ± 3.54 %. ** $p < 0.01$ vs CTRL; + $p < 0.05$ vs FGF-2 condition.

G. Interfering with calcium release form intracellular stores by preincubation of dissociated cultures with 10 nM thapsigargin (TG), an inhibitor of SERCA calcium pumps, had no significant effect on neurite growth. CTRL: 612.45 ± 29.68 μm^2; FGF-2: 838.40 ± 62.80 μm^2; TG: 562.29 ± 20.85 μm^2; FGF-2 + TG: 761.88 ± 32.42 μm^2. ** $p < 0.01$ vs CTRL; ## $p < 0.01$ vs TG.

H. 10 nM TG induced saturating calcium release from intracellular stores in CG neurons in dissociated cultures at 24 h, when added to a nominally calcium free solution with 0.5 mM EGTA. Subsequent stimulation with 100 nM TG did not elicit any additional increase in $[Ca^{2+}]_i$. Reintroduction of 2 mM calcium in the extracellular medium evoked an increase in $[Ca^{2+}]_i$; further stimulation with 40 ng/ml FGF-2 was still able to evoke an additional increase in $[Ca^{2+}]_i$.

Figure 3. Compartmentalized cultures: the contribution of somatic and neuritic signalling activated by FGF-2 to global neurite growth.

A. Schematic drawing of the Campenot chambers used in our experiments.

B. Images of a few tracks in a lateral compartment of a Campenot chamber at 5 days of culture, with neurites, stained with the anti neurofilament antibody, extending along them. In gray is depicted the field selected for analysis. Scale bar: 500 μm.

C. Neurite occupancy in the left (L) and right (R) lateral compartments of Campenot chambers when 20 ng/ml FGF-2 was present either at the somatic or at one lateral compartment, or in both. Percentages of occupation were: FGF-2 in the central compartment, L 2.75 ± 1.11 %, R 2.39 ± 0.66 %; FGF-2 in R lateral compartment, L 0.91 ± 0.24 %, R 12.15 ± 2.90 %; FGF-2 in both central and R lateral compartment, L 2.21 ± 1.39 %, R 17.63 ± 1.57 %. * $p < 0.05$ and ** $p < 0.01$ vs. L and R with FGF-2 in the central compartment, vs. L with FGF-2 in the R compartment, and vs. L with FGF-2 in both central and R compartment.

Figure 4. Effects of addition of the calcium channel blockers in the different compartments on FGF-2 induced neurite growth.

A. 2 μM ω-conotoxin and 10 μM nifedipine (CTX+NIFE) significantly reduced neurite growth when added in either the central compartment or in the lateral compartment, or in both. CTRL: 20 ng/ml FGF-2 alone. In all protocols, FGF-2 was present in both the central and one lateral compartment. Percentages of neurite occupation were: CTRL: 17.64 ± 1.57 %; Blockers on soma: 5.21 ± 0.52 %; Blockers on neurites: 6.30 ± 1.70 %; Blockers on soma and neurites: 6.17 ± 1.00 %. ** $p < 0.01$ vs. all other conditions.

B. The TRPC channel blocker SKF-96365 exerted effects comparable to those of the VOCs blockers. Percentages of neurite occupation: CTRL: 17.64 ± 1.57 %; SKF on soma: 9.12 ± 1.67 %; SKF on neurites: 5.79 ± 2.97 %; SKF on soma and neurites: 7.67 ± 1.71 %. * $p < 0.05$ vs. all other conditions.

Figure 5. Calcium signals at the soma and at the growth cone activated by acute application of 40 ng/ml FGF-2 in CG neurons at 6 h of culture.

A. Image obtained by Intensity Modulated Display (IMD) mode of a neuron with two neurites. The neuron was loaded with Fura-2 and challenged with 40 ng/ml FGF-2. Scale bar 5 μm.

B. Left: a representative trace showing a somatic long lasting plateau increase in $[Ca^{2+}]_i$ in response to FGF-2. Right: trace from the growth cone of the same cell. Perfusion with the factor induced the onset of an oscillatory behaviour. Responding somata were 47 of 102 (46.03%); responding growth cones 86 of 148 (58.10%).

C. Recording from the two growth cones of the neuron shown in A. Left: growth cone marked with +; right: growth cone marked with *.

D. Perfusion with a low calcium extracellular solution reversibly abolished responses to FGF-2 both at the soma (left) and at the growth cone (right). N = 4.

E. An example of a cell showing spontaneous oscillations of $[Ca^{2+}]_i$ both at the soma (left) and at the growth cone (right). Subsequent perfusion with 40 ng/ml FGF-2 induced a change in $[Ca^{2+}]_i$ in both subcellular compartments. Percentages of cells showing spontaneous oscillations: 17.9 % at the soma, 24.8 % at the growth cone.

Figure 6. Blockers of calcium VOCs affect the FGF-2 induced calcium signals.

A. A representative trace showing the reduction of the somatic plateau response to 40 ng/ml FGF-2 by the blockers of N- and L- type calcium channels (2 μM ω-conotoxin and 10 μM nifedipine). Inset: a representative trace showing the effects of the calcium VOCs blockers on FGF-2 induced somatic increases in $[Ca^{2+}]_i$ at 24 h of culture.

B. Average reduction of the plateau response to FGF-2 by the calcium VOCs blockers. N = 9; * $p < 0.05$; paired-samples t-test.

C. Complex effects of the VOCs blockers on the oscillatory response to FGF-2 at the growth cone of the same neuron.

D. Average increase of oscillatory activity relative to basal behaviour, r_J, during administration of FGF-2 and after addition of the VOCs blockers. N = 15; * $p < 0.05$; paired-samples t-test.

E. Wavelet analysis of the oscillatory activity at the same growth cone as in C in basal conditions, following administration of 40 ng/ml FGF-2 and subsequent addition of the calcium VOCs blockers. Upper box: original trace (in blue) and the same signal after a detrending and denoising procedure (in red). Middle box: plot of the scaleogram representing the wavelet transform of the red trace in the upper box. Lower box: plot of the activity index J as a function of time. Vertical red lines in the upper and lower boxes mark the changes in the extracellular solution indicated in C.

Figure 7. Effects of the TRPC blocker, SKF-96365, on FGF-2 induced calcium signals.

A. A representative trace showing the reduction of the somatic plateau response to 40 ng/ml FGF-2 by SKF 96365.

B. Average reduction of the plateau response to FGF-2 by SKF 96365. N = 12; ** $p < 0.01$; paired-samples t-test.

C. Complex effects of the TRPC blocker on the oscillatory response to FGF-2 at the growth cone.

D. Average increase of oscillatory activity relative to basal behaviour, r_J, during administration of FGF-2 and after addition of SKF 96365. N = 18; * $p < 0.05$; paired-samples t-test.

E. Wavelet analysis of the oscillatory activity at the same growth cone as in C in basal conditions, following administration of 40 ng/ml FGF-2 and subsequent addition of SKF 96365. Upper box: original trace (in blue) and the same signal after a detrending and denoising procedure (in red). Middle box: plot of the scaleogram representing the wavelet transform of the red trace in the upper box. Lower box: plot of the activity index J as a function of time. Vertical red lines in the upper and lower boxes mark the changes in the extracellular solution indicated in C.

Figure1
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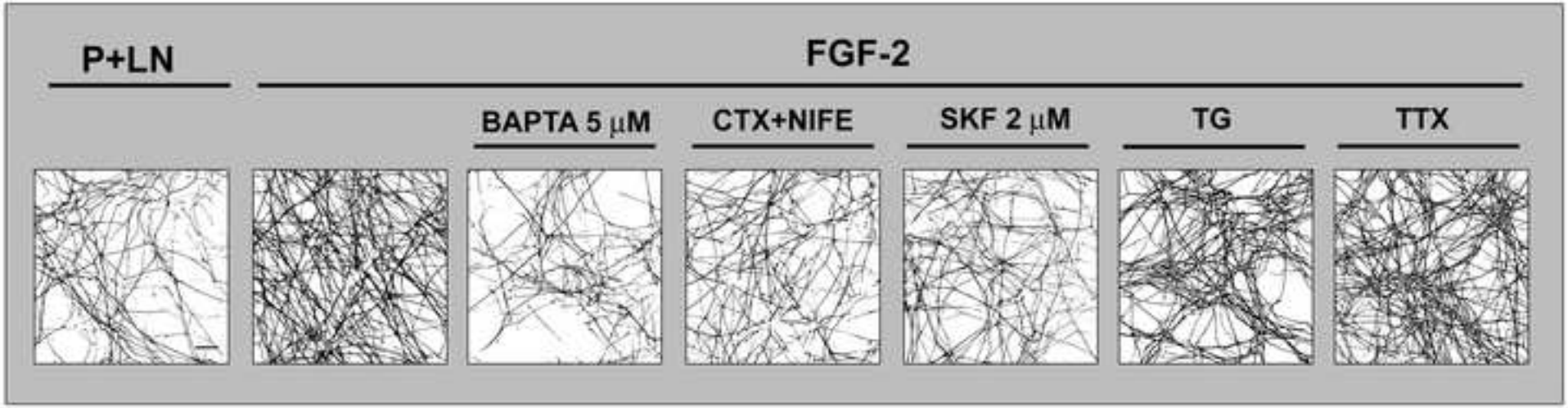

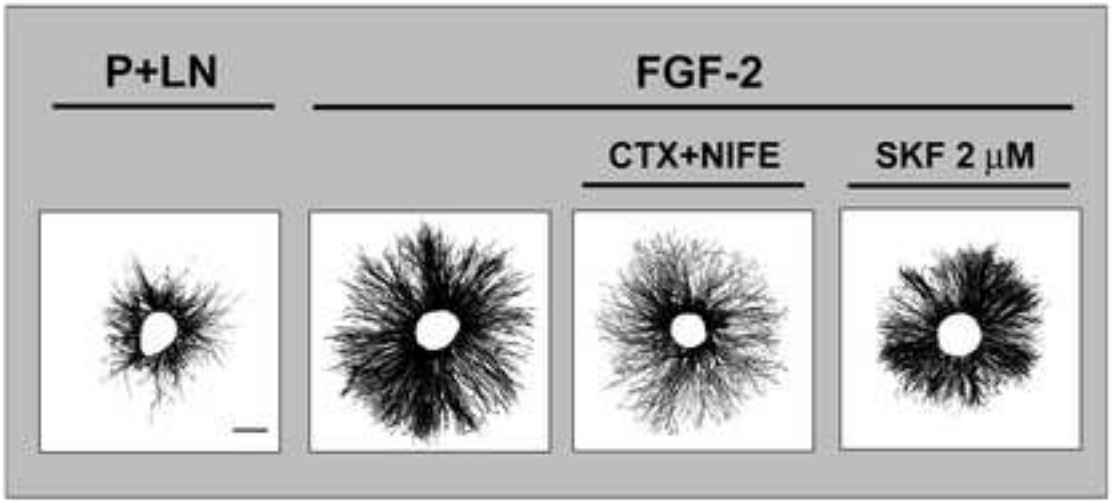

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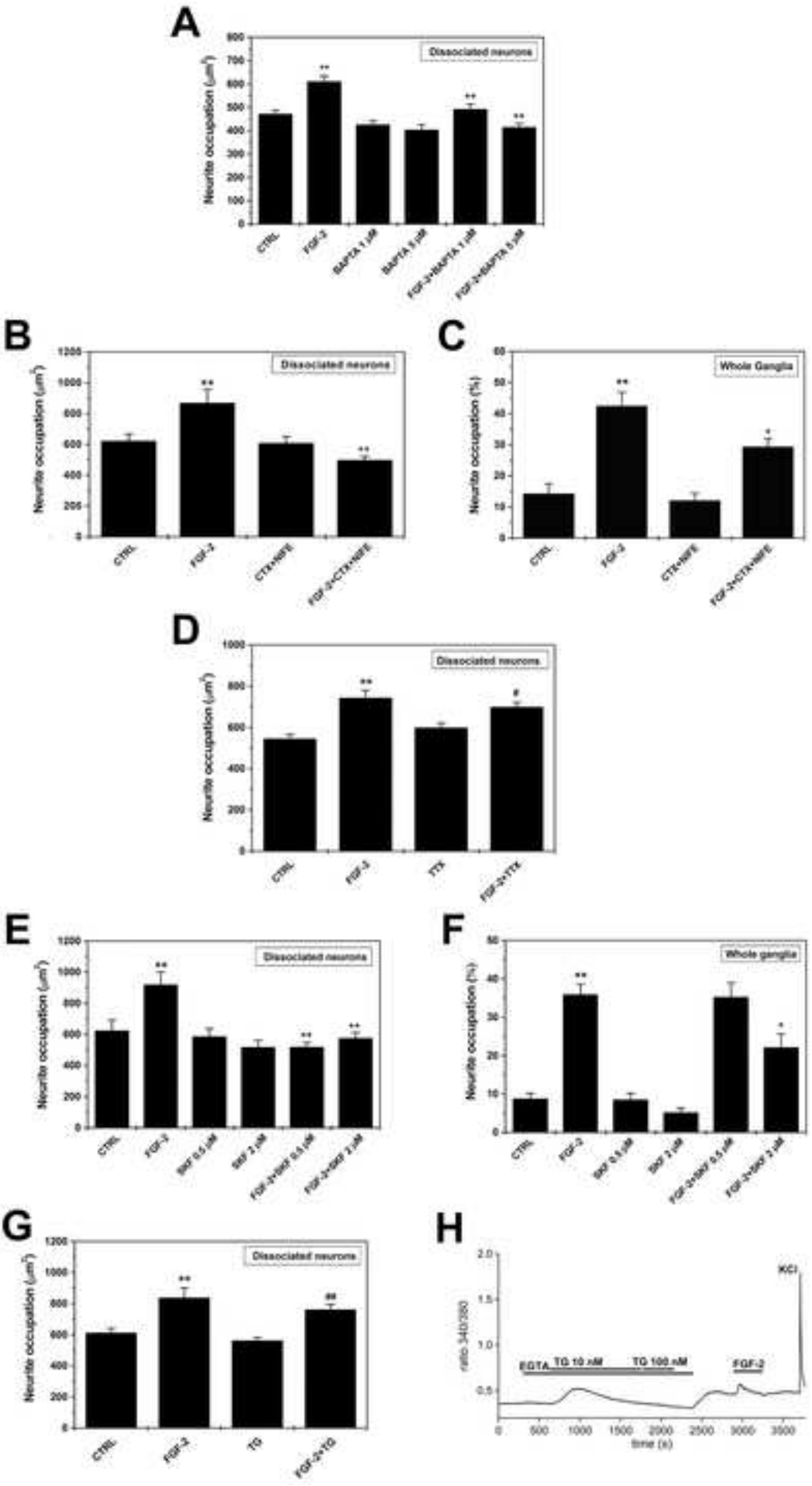

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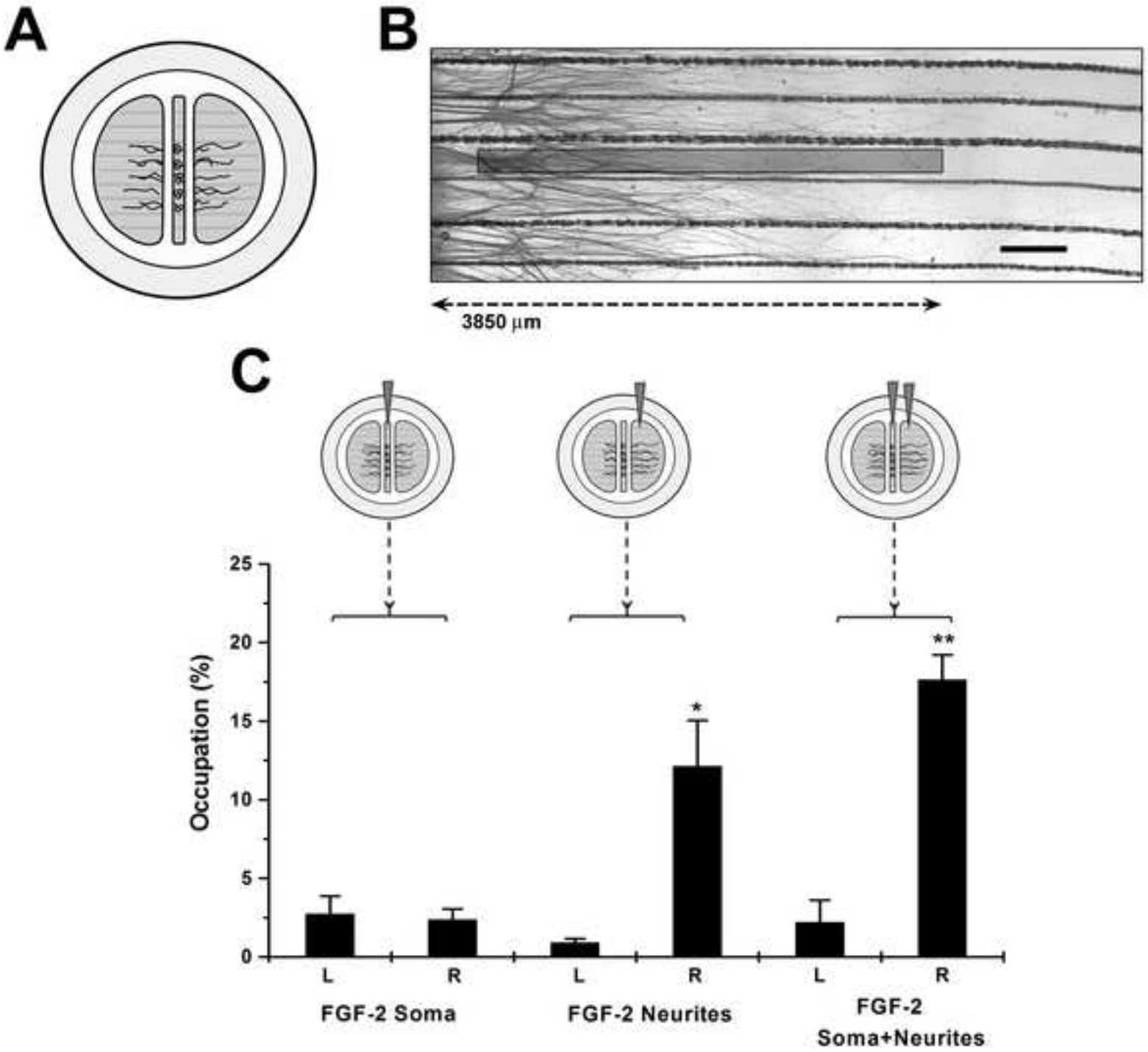

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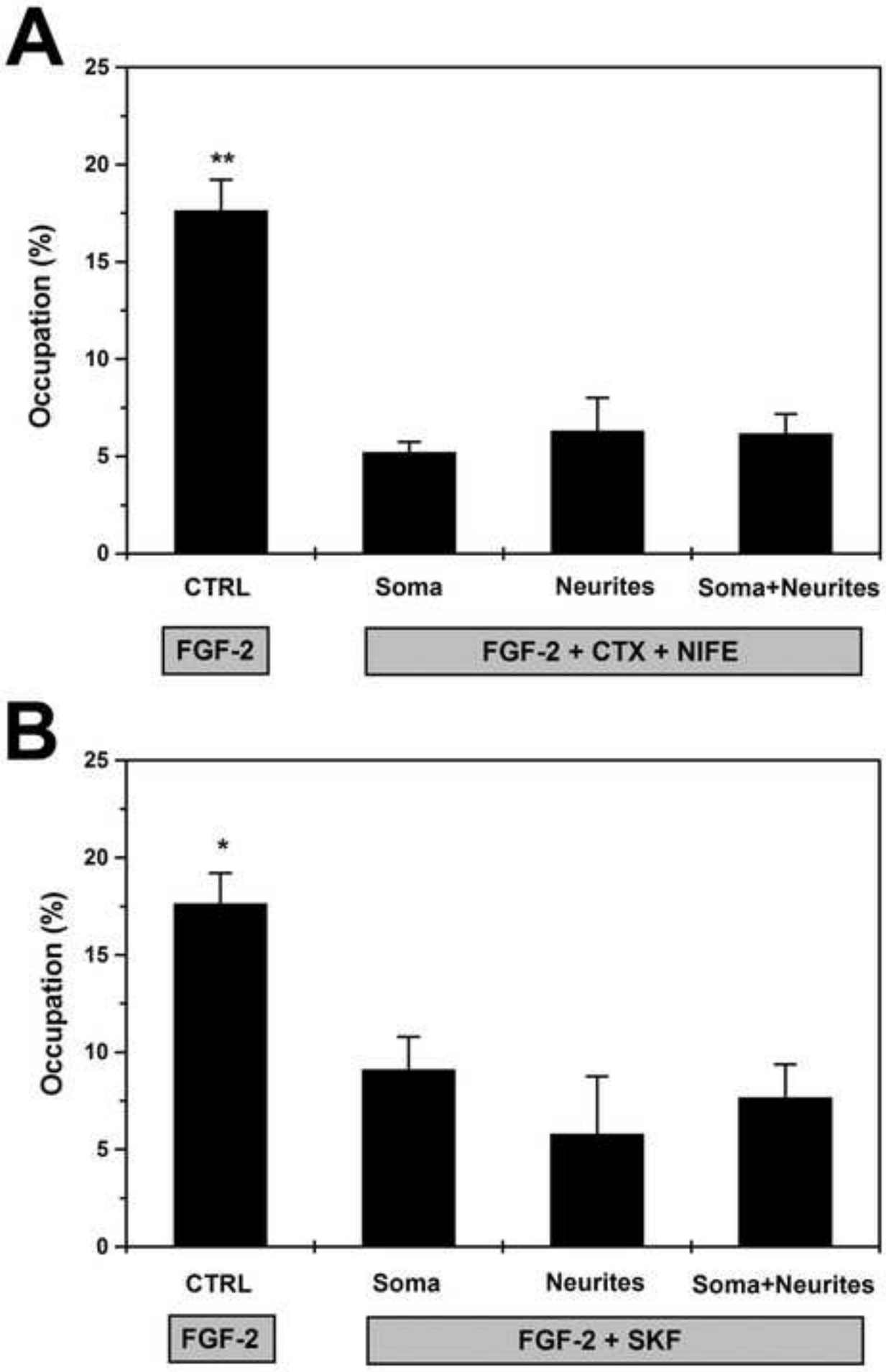

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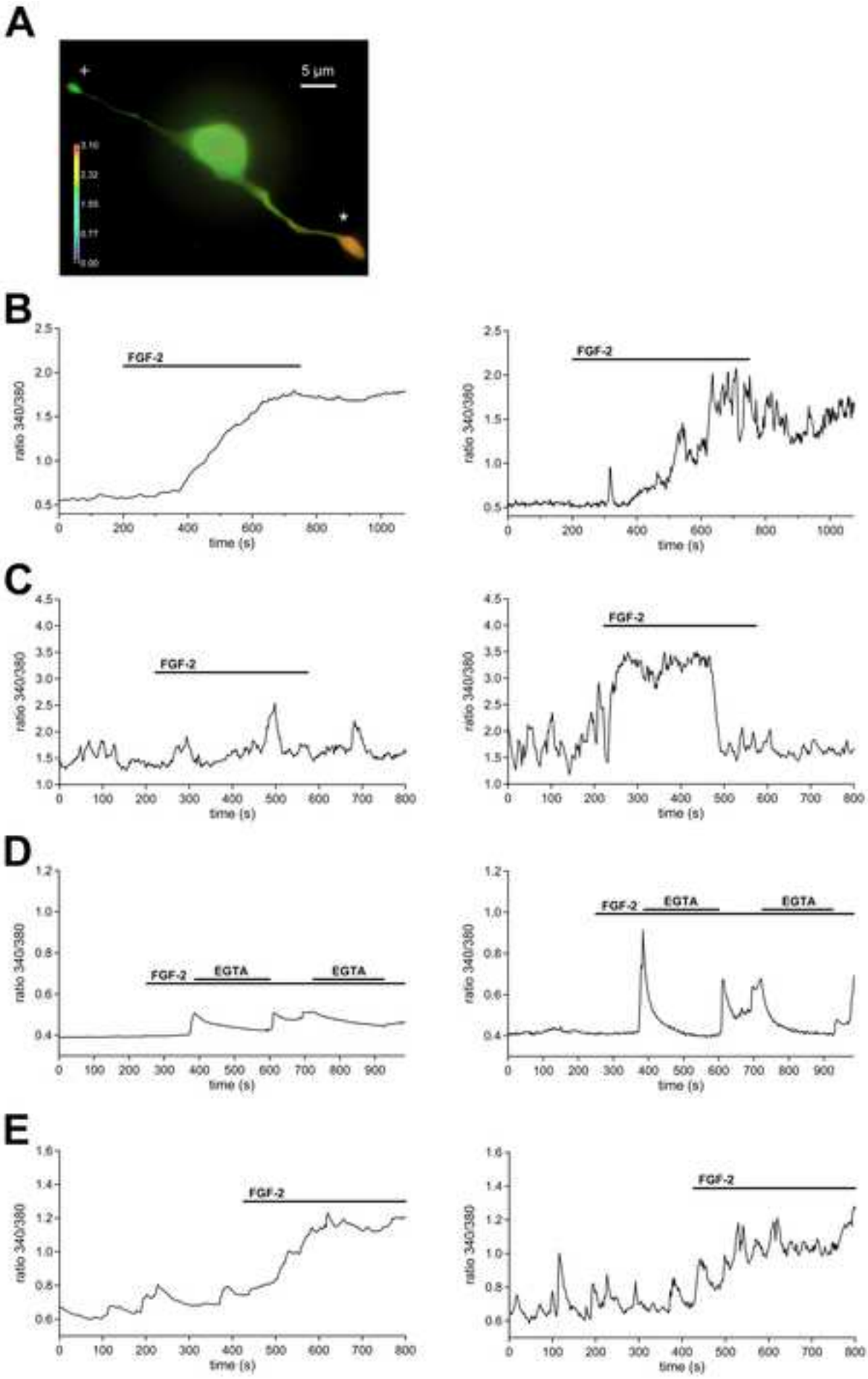

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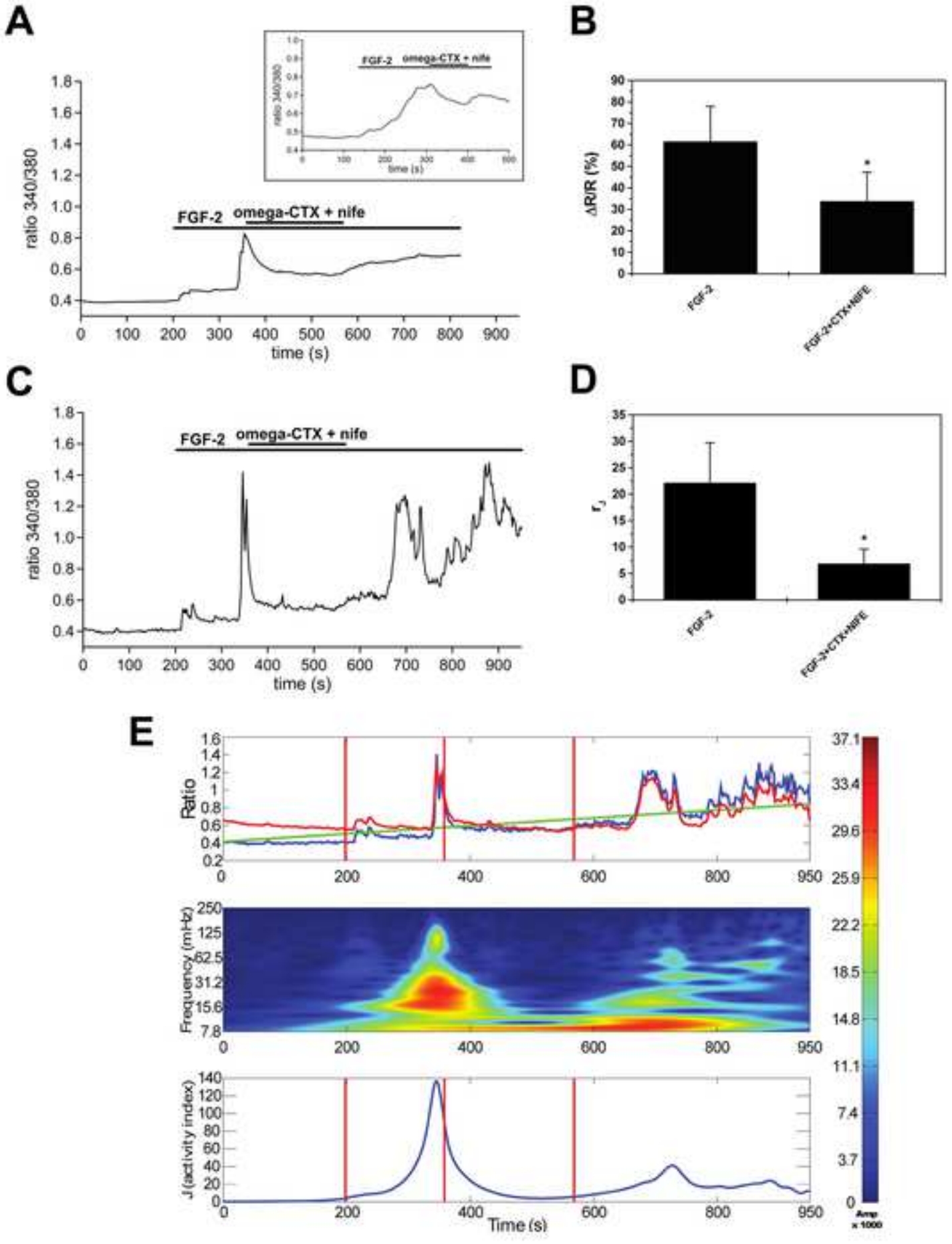

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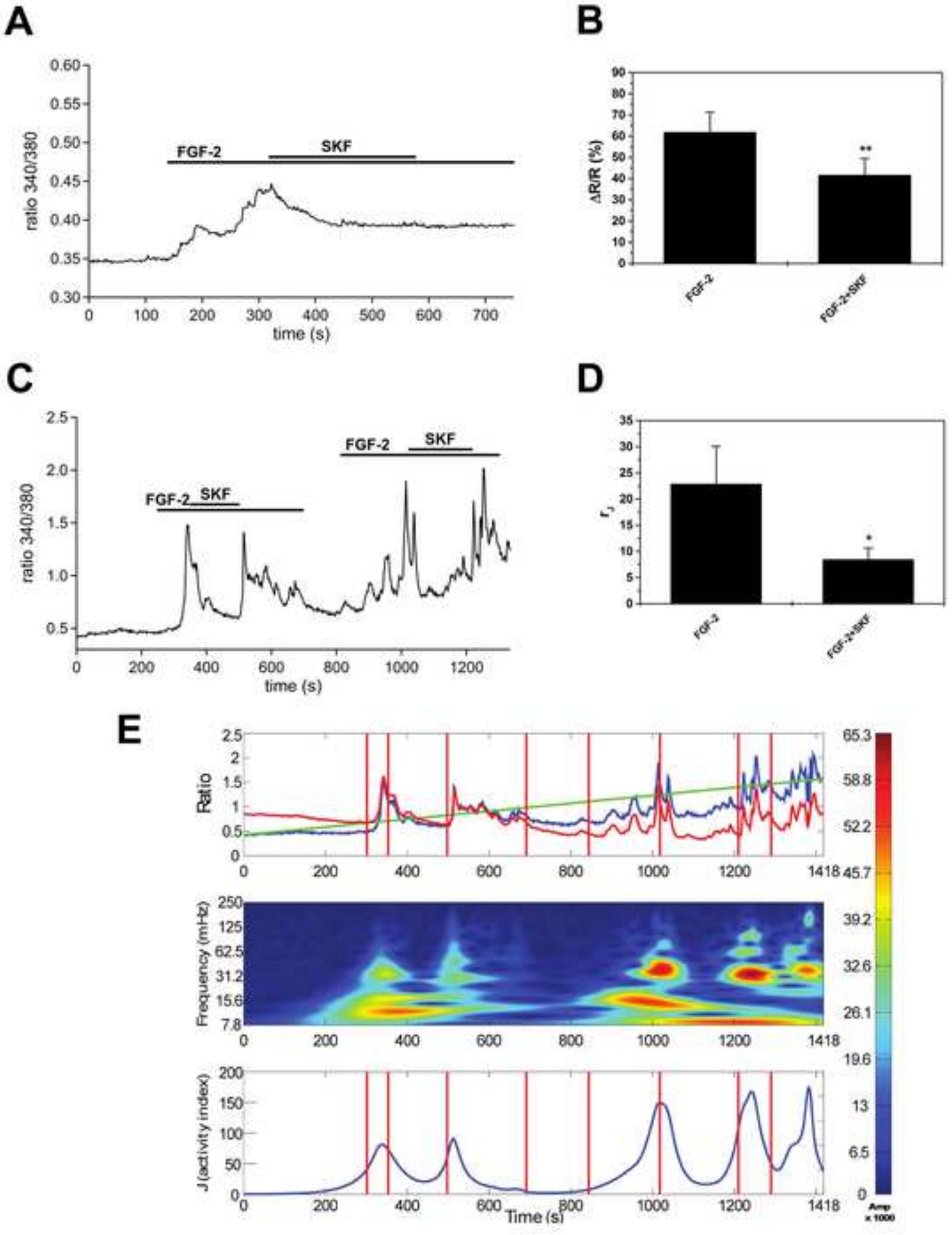

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