Project PNCDI2 Idei contract 164/2007
Director: Conf. Dr. Alexandru Babes

1. Effect of GDNF (glial cell-derived neurotrophic factor) on the co-expression of temperature-sensitive TRP (transient receptor potential) channels in cultured sensory neurons from the rat

1.1. Introduction

TRP channels are involved in the transduction of thermal, mechanical and chemical painful stimuli. Six channels from the TRP super-family are known to detect changes in ambient temperature and they are known as thermoTRP channels: TRPV1, TRPV2, TRPV3 and TRPV4 (Caterina et al., 1997; Ahluwalia et al., 2002; Peier et al., 2002; Watanabe et al., 2002) are gated by heat, while TRPM8 (Peier et al., 2002; McKemy et al., 2002) and TRPA1 (or ANKTM1, Story et al., 2003) are activated by cold stimuli.
TRPM8 is a non-selective cation channel expressed in dorsal root and trigeminal ganglion neurons. It is activated by moderate cooling (with a temperature threshold of ~25 °C) and chemical compounds such as menthol, eucalyptol and icilin.
TRPA1 is an ion channel activated by irritant compounds such as mustard oil, cinnamon oil, the active compound in garlic (alicin), acrolein, etc... being also modulated by endogenous factors like bradykinin. TRPA1 activation by cold and its role in noxious cold sensing are still a matter of debate. On one hand it appears that in heterologous expression systems, TRPA1 is activated by noxious cold temperatures, with a threshold of ~17 °C (Story et al. 2003; Bandell et al., 2004), while other scientists were unable to confirm the activation of TRPA1 by cold (Jordt et al., 2004; Nagata et al., 2005), or could not find evidence to support the involvement of TRPA1 in cold sensing in cultured DRG neurons (Babes et al., 2004).
TRPV1 is a non-selective cation channel activated by capsaicin (an irritant agent in hot chili peppers) and noxious heat (>42 °C). TRPV1 is involved in inflammation and has a role in inflammatory heat hyperalgesia. Numerous inflammatory mediators sensitize TRPV1: bradykinin, prostaglandin E2, NGF, ATP, acid pH, etc...
The aim of this project was to characterize the effect of GDNF (glial cell line-derived neurotrophic factor) on thermoTRP channels TRPM8, TRPA1 and TRPV1 in sensory neurons from the rat kept in culture in serum-free medium.

1.2. Materials and methods:

Dorsal root ganglia (DRG) from spinal levels L1 to S1 were dissected out from Wistar rats. Individual neurons were obtained after enzymatic treatment and trituration and kept in pimary cultures in serum-free medium. Half of the neurons were chronically treated with GDNF 100 ng/ml, while the other half were control neurons.
We have used the technique of calcium microfluorimetry to investigate the effect of GDNF. Cells were loaded with the fluorescent calcium indicator Calcium Green 1-AM and several parameters were monitored: the change in intracellular calcium concentration ($[Ca^{2+}]_i$), the thermal threshold of activation by cooling (the thermal stimulus was a 50 s

cooling ramp from 32 to 17 °C) and the changes in various fractions of neuronal sub-populations. The experimental protocol consisted in the application of a cooling ramp, then the same cooling ramp in the presence of (-)-menthol, followed by the application of allyl isothiocyanate (AITC or mustard oil) and capsaicin.

1.3. Results

After incubation with GDNF, the level of co-expression of TRPM8 and TRPA1 increased significantly, from 3% in control conditions to 9% of the total neuronal population ($\chi 2$ test, $p < 0.05$) (fig. 1.1. A). Moreover, the fraction of TRPM8 neurons also expressing TRPA1 has also increased (fig. 1.1. B). Similar results were obtained regarding the co-expression between TRPM8 and TRPV1, with an increase in the degree of co-expression in the total population (fig. 1.1. C) as well as within the TRPM8-expressing subgroup (fig. 1.1. D).

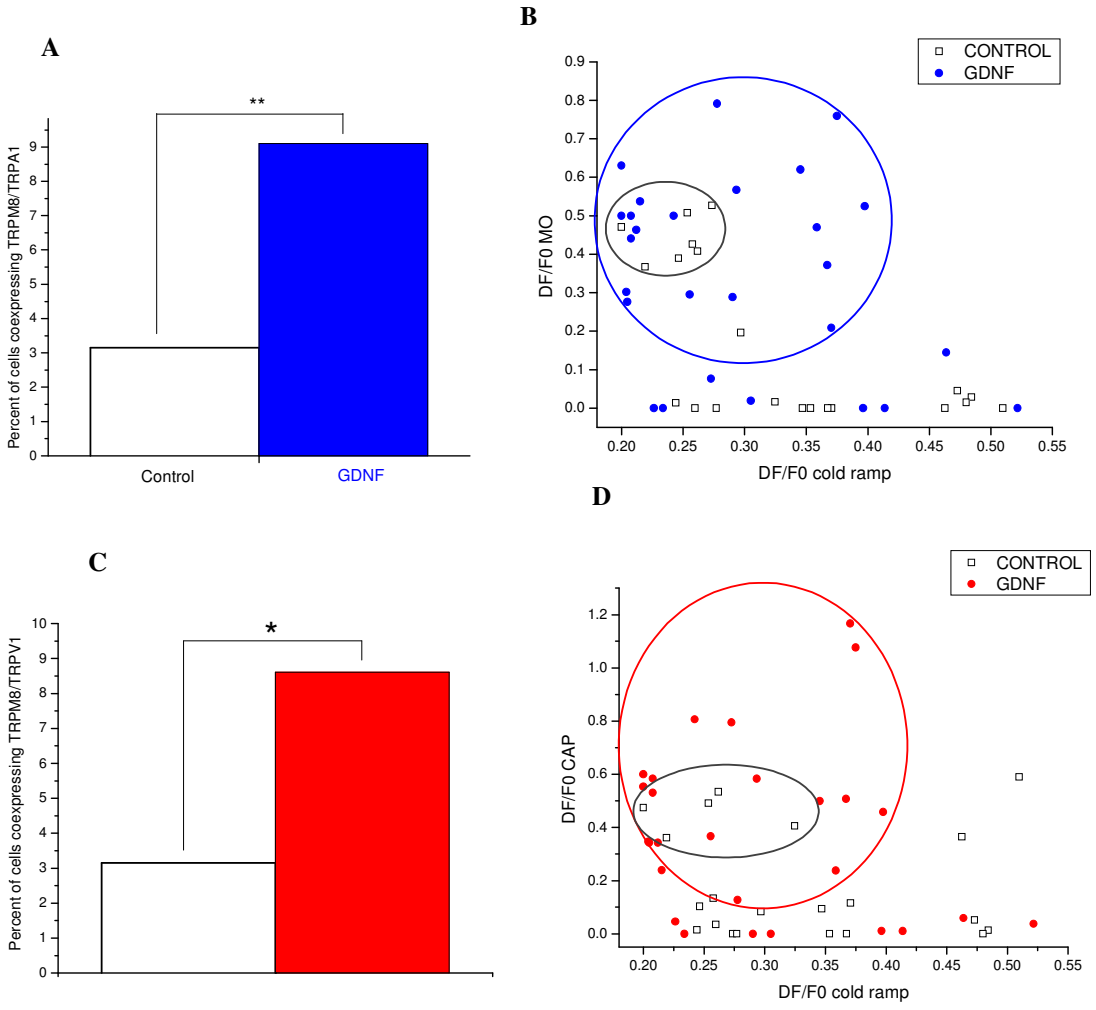

Figure 1. 1. A. Degree of co-expression TRPM8-TRPA1 in the total neuronal population. **B**. The fraction of TRPM8 neurons that also express TRPA1 is increased after GDNF treatment. **C.** Degree of co-expression TRPM8-TRPV1 in the total neuronal population. **D**. The fraction of TRPM8 neurons that also express TRPV1 is increased after GDNF treatment.

Another observation was that the number of neurons that express the TRPA1 receptor has increased after GDNF treatment (from 35% to 47%, $p<0.05$) (fig. 1.2A). Moreover, the TRPA1/TRPV1 co-expression was increased from 35% to 54% ($p<0.01$). Within the population of capsaicin-sensitive neurons (most likely TRPV1-expressing), GDNF induced an increase in the number of TRPA1-expressing cells (fig. 1.2B).
In the control group, only 2% of neurons express all three channels investigated in this study (TRPM8/TRPA1/TRPV1). In the case of GDNF-treated cells, the fraction of these neurons was increased to 8.5% ($p < 0.05$). There was no significant effect of GDNF in what concerns the amplitude of the responses to thermal and chemical agonists and also no effect on the thermal thresholds for activation by cooling.

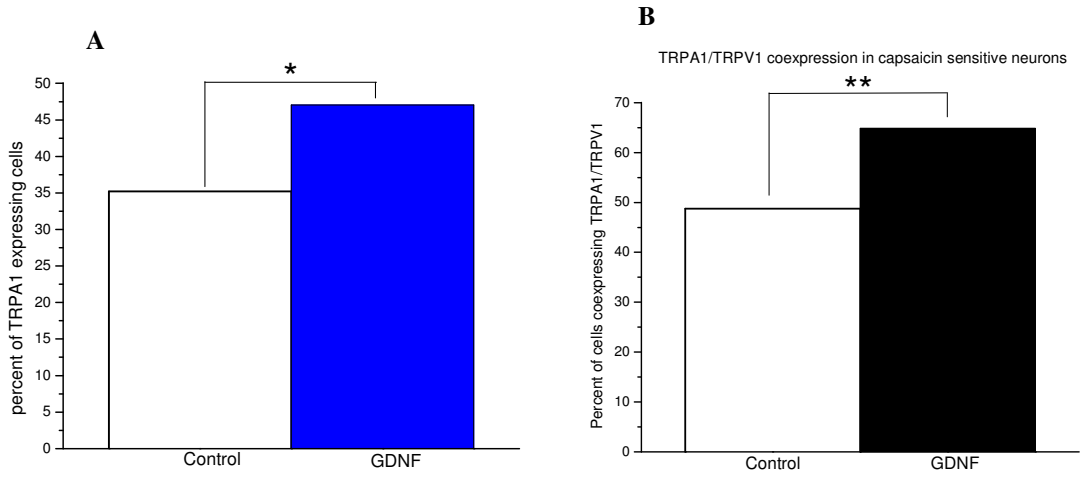

Figure 1.2. A. GDNF treatment significantly increased the fraction of sensory neurons expressing TRPA1; **B.** Within the subpopulation of capsaicin-sensitive neurons (TRPV1-expressing) GDNF increased the level of co-expression with TRPA1.

1.4. Conclusions

In conclusion, GDNF induced an increase in the level of co-expression of TRPM8, TRPA1 and TRPV1 channels, which indicates a possible involvement in the alteration of function and sensitivity of nociceptive neurons through a change in the expression and interaction between TRP channels. This result is significant, considering the role of TRP channels in acute and chronic pain signaling.
The increased level of expression of TRPA1 and of TRPA1/TRPV1 co-expression after GDNF treatment represents a possible mechanism of allodyina and hyperalgesia oin inflammatory states.

2. Transient transfection of HEK293 cells with human TRPV1

2.1. Introduction

An important objective of our project for 2008 was to set up the method of amplification and purification of plasmid DNA, as well as to be able to transfect human embryonic kidney cells (HEK293) with thermoTRP channels (particularly TRPV1, TRPV2 and TRPA1). This molecular biology approach was successfully carried out for the first time in our laboratory. We have used the HEK293 cell line kindly donated by Prof. Peter McNaughton from Cambridge University. For the preliminary experiments we have used the clone of human TRPV1, kind gift from Dr. Gordon Reid from University College Cork.

2.2. Materials and methods

2.2.1. Amplification and purification of plasmid DNA

In order to amplify the TRPV1 clone (incorporated into the pcDNA3.1 plasmid) we have used TOP10 competent bacteria, gift from our collaborators from the Cantacuzino Institute in Bucharest (dr. Geza Szegli). Using the same conditions, we have also amplified the GFP (Green Fluorescent Protein) clone, a marker gene that is used to identify successfully transfected cells. Bacteria were incubated with ~50 ng DNA on ice. After 20 min bacteria were transformed using a thermal shock (40 s at 42 °C) and then LB medium (LB Broth, Sigma) was added and the bacteria were left for 1 h in the shaker incubator at 37 °C. Following this treatment, bacteria were plated on Luria Agar (Sigma) in the presence of ampicillin and left overnight at 37 °C. The following day the presence of ampicillin-resistant bacterial colonies was identified both for the TRPV1 and the GFP dishes. We then proceeded to grow small scale (5 ml) bacterial cultures in liquid medium (LB). Cultures were kept at 37 °C for 8 hours with shaking. Well defined volumes of the small scale cultures were used to initiate large scale cultures (50 ml), which were kept overnight (12 h) at 37 °C with shaking. The following day plasmid DNA was purified from large scale cultures using the QIAfilter plasmid DNA kit from Qiagen. The concentration of plasmid DNA obtained was measured spectrofotometrically.

2.2.2. Transfection of HEK293 cells using the calcium phosophate co-precipitation method

In order to obtain functional expression of the TRPV1 channel in mammalian HEK293 cells we used the simple but efficient method of calcium phosphate co-precipitation. Briefly, a solution containing the plasmid in the presence of a high calcium concentration was added drop wise over an equal volume of a solution of Hepes Buffered Saline (HBS) 2x. As a consequence we obtained a suspension of very fine particles of calcium phosphate precipitate to which DNA molecules adhered. Petri dishes with low density cultures of HEK293 cells (1:20 after confluence) were incubated for 8 h with this precipitate suspension at 37 °C and 5% CO2. Following incubation cells were washed with culture medium and the transfection solution was replaced with normal culture medium. Transfection efficiency was monitored using fluorescence microscopy, by quantifying the fluorescent cells (expressing GFP following transfection, fig. 2.1.). In

order to increase the probability that all fluorescent cells (GFP-expressing) should also express TRPV1, a ratio of 1:10 TRPV1:GFP was used for the co-transfection with the two plasmids.

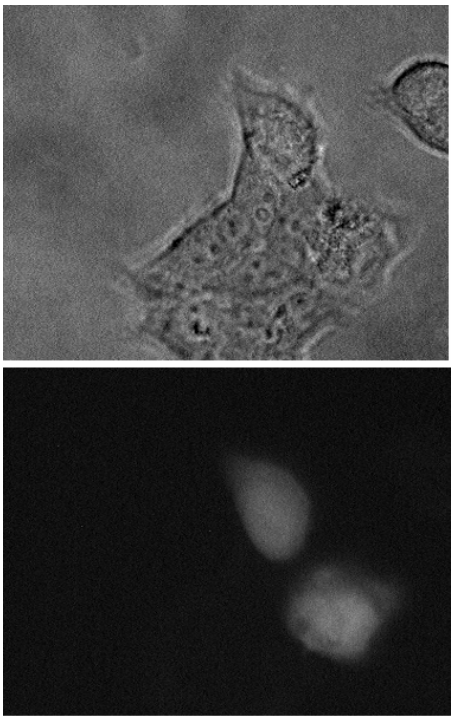

Fig. 2.1. The image of the same optic field of HEK293 cells transiently transfected with TRPV1 and GFP in phase contrast microscopy (upper picture) and in epifluorescence (lower picture). Two fluorescent cells (thus GFP-expressing) can be seen in the lower picture.

2.3. Results

Follwing successful transfection of HEK293 cells with GFP and TRPV1 (fig. 2.1) we investigated the activity of human TRPV1 in these cells using two different methods: calcium microfluorimetry and the patch clamp technique.

In the first case, cells were transfected with TRPV1 only (the presence of GFP would have interfered with the fluorescence of the calcium indicator), after which they were plated on glass coverslips and incubated with the fluorescent calcium indicator Calcium Green-1 AM. Then, the coverslips were placed on the fluorescence microscope Olympus IX-70 and the cells were stimulated with capsaicin (50 nM) and noxious heat (>43 °C). As control cells we have used not-transfected HEK293 cells which were stimulated in the same conditions. Not-transfected cells displayed no response to either capsaicin or noxious heat. In contrast to the control cells, a large fraction of transfected cells responded to both capsaicin and the thermal stimulus with a substantial and reproducible increase in the intracellular calcium concentration (fig. 2.2). As it can be seen in fig. 2.2, the cells were stimulated twice with capsaicin (50 nM, 30 s) and once with noxious heat. The figure shows four different HEK293 cells which have responded to all three stimuli, demonstrating that they have expressed TRPV1 following the transfection procedure.

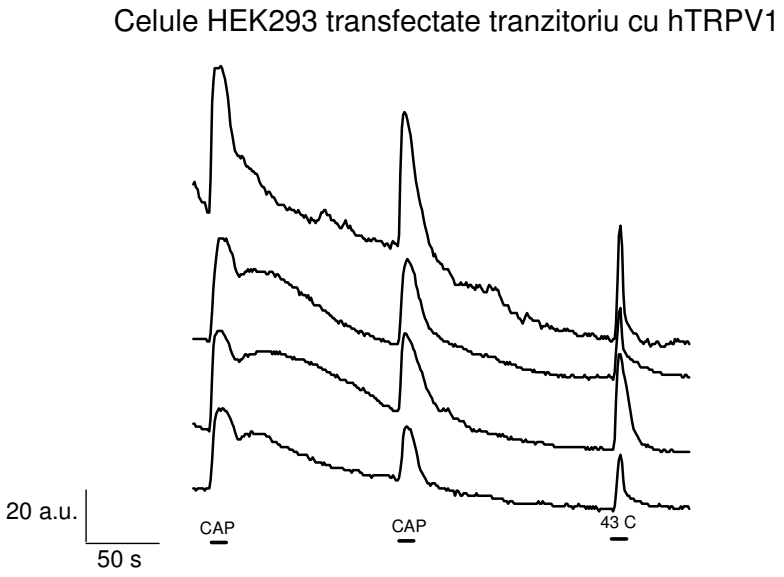

Fig. 2.2. Fluorescence traces of Calcium Green-1 AM in four different HEK293 cells expreswsing hTRPV1following transient transfection. It can be seen that all cells respond to the specific TRPV1 agonist capsaicin and to stimulation with noxious heat.

In the case of patch clamp experiments, the cells were co-transfected with TRPV1 and GFP (in a 10:1 ratio) in order to allow for visual identification of successfully transfected cells.

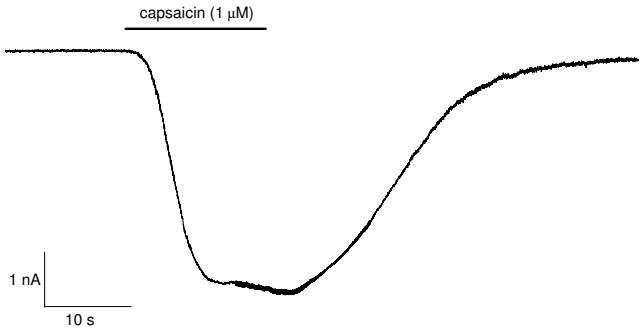

Fig. 2.3. Inward current activated by capsaicin (1 μM, 15 s) in a HEK293 cell transiently transfected with hTRPV1 voltage-clamped at -60 mV.

As in the case of calcium imaging experiments, not-transfected (control) cells did not show any response (change in membrane current) upon application of capsaicin in the whole-cell configuration at a holding potential of -60 mV. In the same conditions, hTRPV1-transfected cells responded with an inward current (fig. 2.3). These results

confirm the functional expression of human TRPV1 channel in HEK293 cells following transient transfection using the calcium phosphate co-precipitation method.

2.4. Conclusion

Following these experiments we can claim that we successfully obtained recordings from hTRPV1 channels transiently expressed in HEK293 cells (of human origin). This ability will allow us to investigate other channels of interest (particularly TRPV2 and TRPA1) in the same conditions. The heterologous expression system, although less physiological than the native system (cultured DRG neurons), has the advantage that it allows the recording of channel activity in isolation, that is in the absence of other channels or receptors which may perturb the function of the channel of interest. Moreover, this type of experiment will allow us to co-express TRP channels with receptors for inflammatory mediators (bradykinin, prostaglandin E2, histamine, serotonin) and to investigate in detail the modulation of these channels in inflammatory pain states.