

# Phosphorylation of cAMP Response Element-Binding Protein as a Downstream Read Out for Nicotinic Acetylcholine Receptor Activation in LUHMES Neuronal Cells

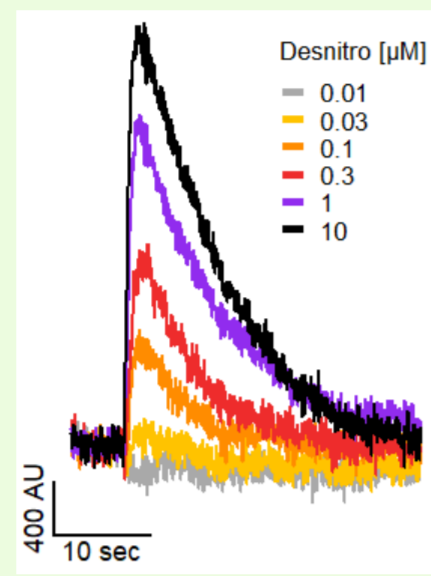
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## INTRODUCTION

Several neonicotinoids exert functional effects on human neuronal cells through the activation of nicotinic acetylcholine receptors, leading to subsequent Ca<sup>2+</sup>-influx. Such signals affecting nervous system development raise toxicological concerns (DNT) (see Fig.1).

Fig. 1.: Exemplary recordings of fluorescence signals showing calcium responses evoked by application of Desnitro-Imidacloprid (DN-IMI)



## OBJECTIVES

To better understand the potential downstream consequences of neonicotinoid exposure following activation of nicotinic acetylcholine receptors, we investigated changes in the phosphorylation of the cAMP response element-binding protein (CREB) in LUHMES-derived human neuronal cells. Phosphorylation of CREB at Ser133 (pCREB) plays a central role in the regulation of gene expression mediated by Ca<sup>2+</sup>-dependent intracellular signaling pathways, making it a valuable readout for early neurotoxic effects.

## MATERIAL & METHODS

**cAMP response element-binding protein (CREB) phosphorylation (Ser133)** was assessed using a bead-based sandwich immunoassay with target-specific capture and phospho-specific detection antibodies (see Fig.2). LUHMES cells were differentiated to DIV 9 (see Fig. 3) and treated with various stimulants across a range of cell densities and exposure durations to identify optimal assay parameters.

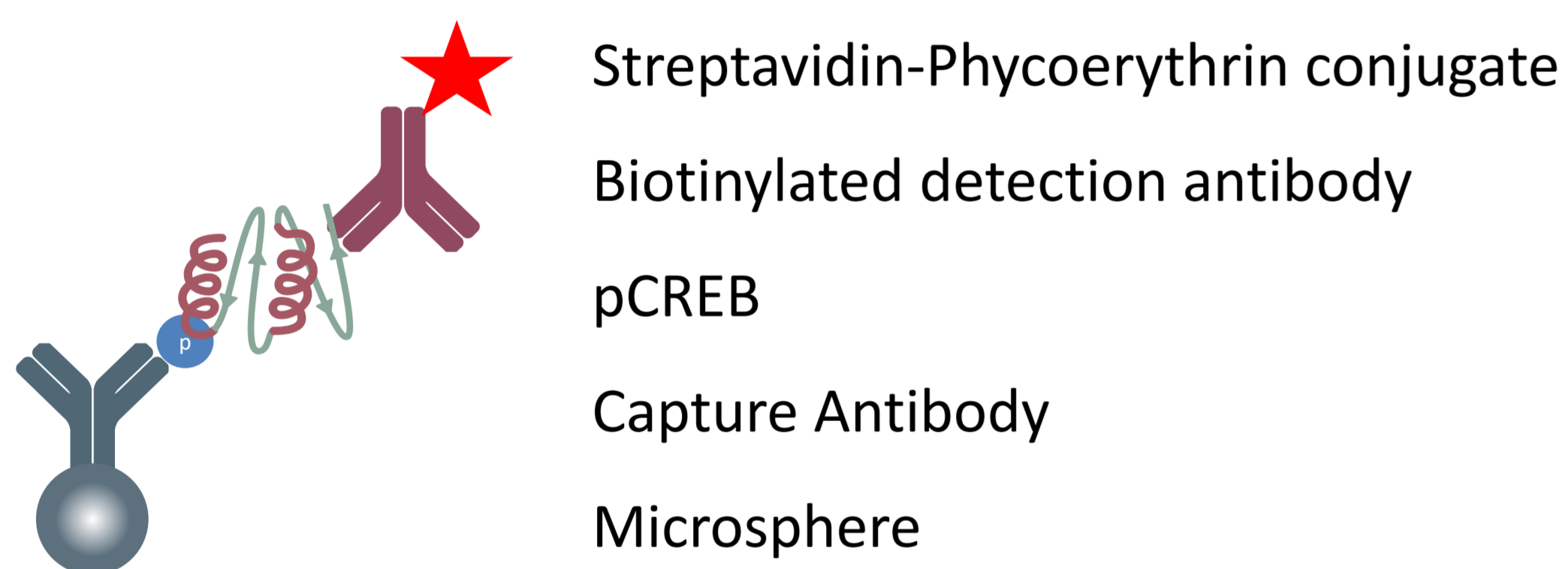


Fig. 2: Scheme of microsphere-based sandwich immunoassay

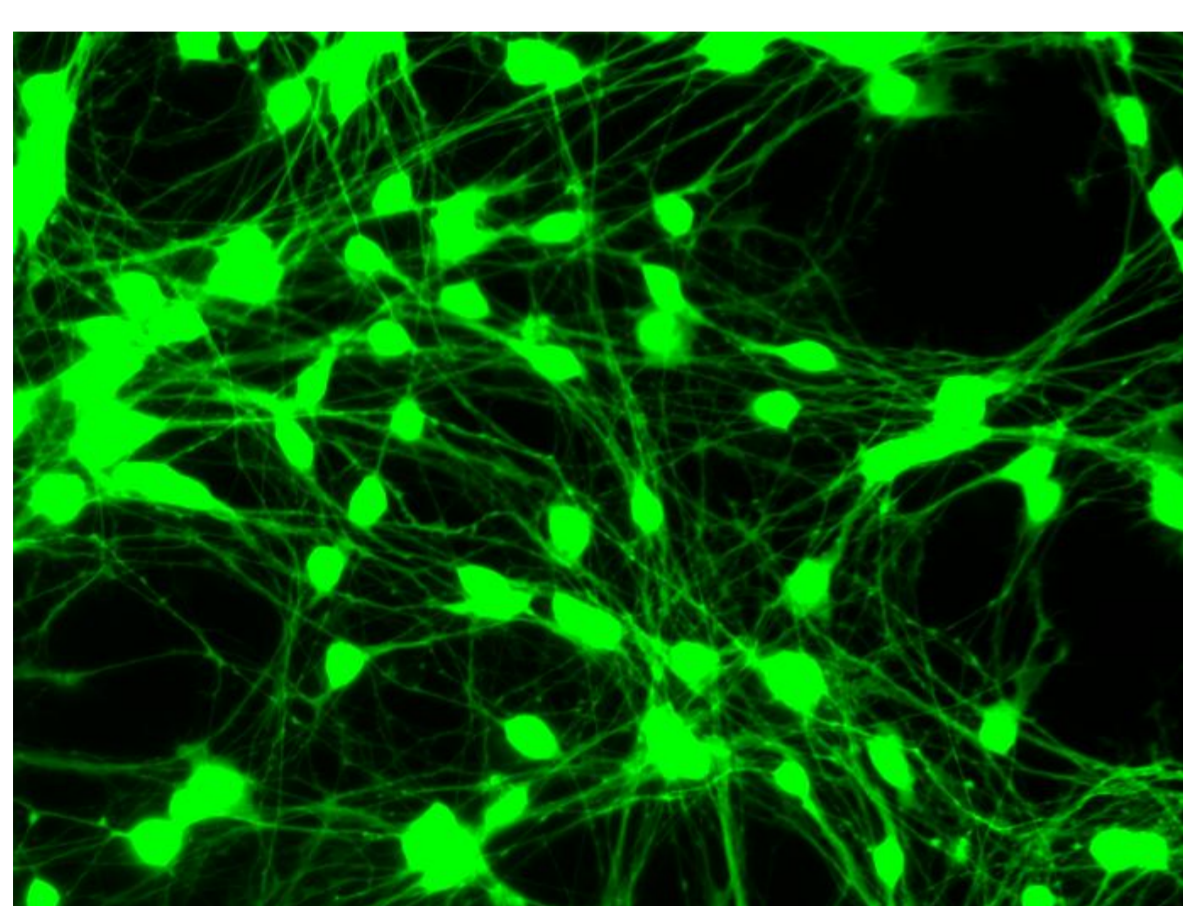


Fig. 3: LUHMES cells stained with Cal-520™ on day 9.

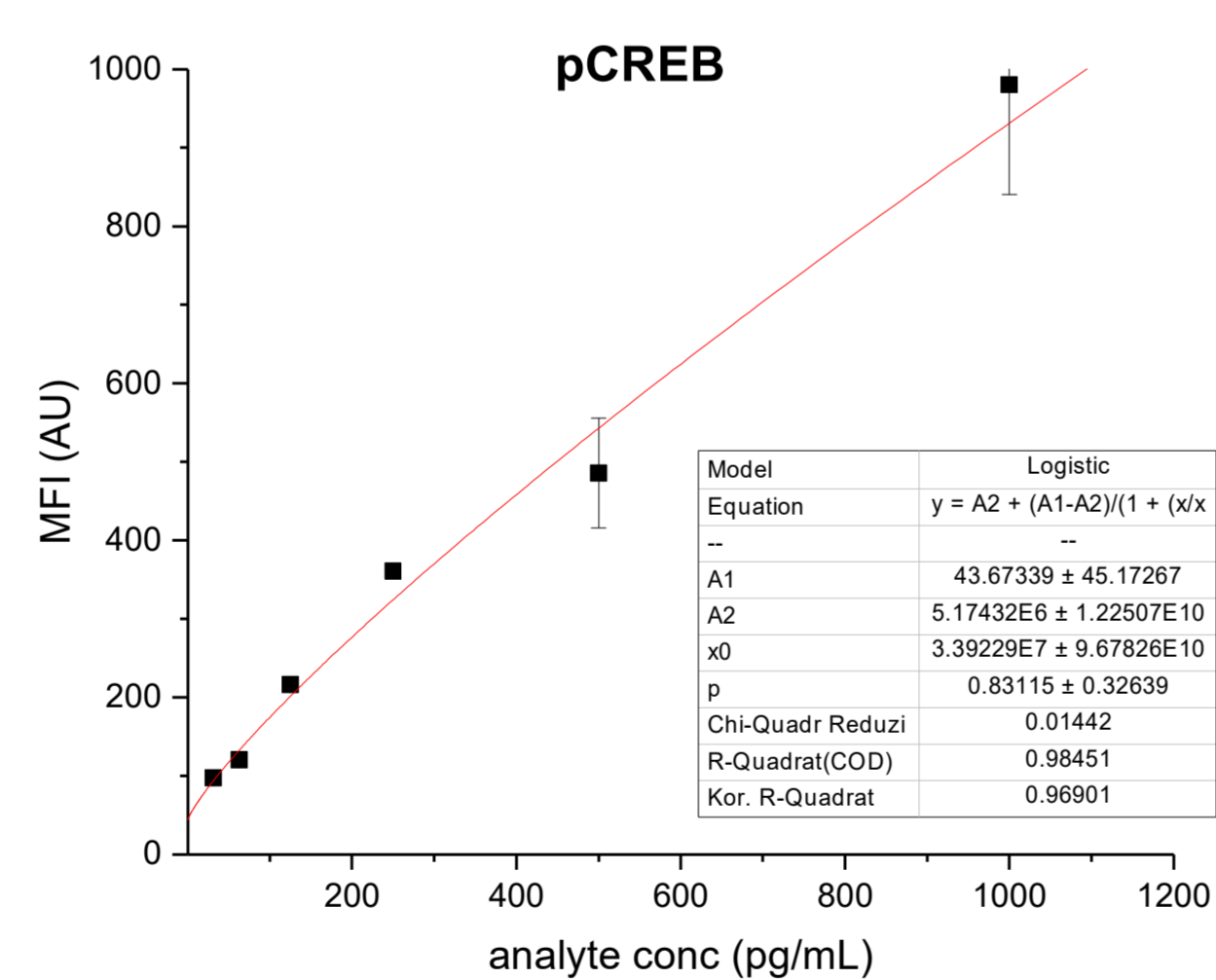


Fig. 4 Fluorescence signals from seven pCREB calibrators (black squares, N=2) were used to back-calculate signals from cell culture lysates.

## RESULTS

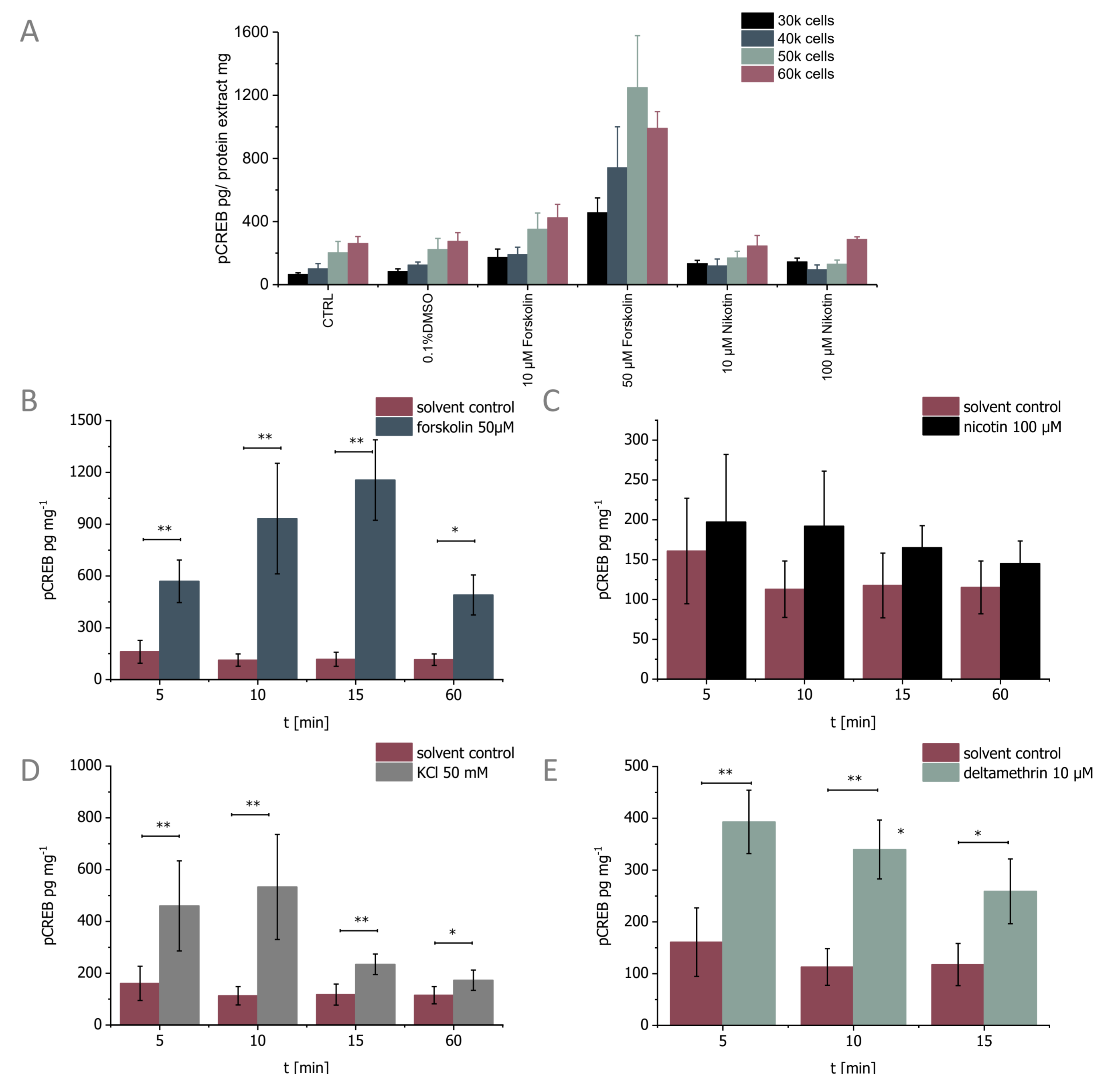


Figure 5: Different numbers of LUHMES cells were seeded and treated with 50 μM forskolin and nicotine for 15 minutes in order to determine the optimal cell density (A). For a time-course experiment, 50,000 LUHMES cells per well were seeded and treated with: 50 μM forskolin (B), 10 μM nicotine (E), 50 mM KCl (D) for 5, 10, 15 and 60 minutes and 10 μM deltamethrin for 5, 10 and 15 minutes.

The results show that the highest levels of phosphorylated CREB (pCREB) were observed at a cell density of 50,000 cells per well when adenylate cyclase was activated directly by forskolin (see Fig. 5A). Time-course analysis revealed stimulus-specific kinetics: forskolin elicited maximal CREB phosphorylation after 15 minutes (see Fig. 5B), whereas indirect stimuli, such as deltamethrin, KCl and nicotine, elicited maximal phosphorylation within five to ten minutes, followed by a progressive decline (see Fig. 5C-E).

## CONCLUSIONS & OUTLOOK

- Phosphorylation of CREB as downstream read out for Ca<sup>2+</sup> influx in LUHMES confirmed
- Nicotine increases pCREB
- We identified the optimal time point for readout by analysis of phosphorylation kinetics
- We will compare the potency and efficacy of nicotine with that of seven neonicotinoids and three of their metabolites