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The effects of antidepressants on cyclic AMP-response element-driven gene transcription in a model cell system

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ABSTRACT

The effects of the antidepressant drugs clomipramine (CLOM), desipramine (DMI), tianeptine (TIAN) and of norfluoxetine (NORF, the active metabolite of fluoxetine), were investigated in CHO cells expressing human β_2 adrenoceptors and a secreted placental alkaline phosphatase (SPAP) reporter gene to determine their actions on cyclic AMP-driven gene transcription.

After 18 h of exposure, CLOM, DMI and NORF, but not TIAN, had biphasic effects on 1 μ M isoprenaline-stimulated SPAP production with concentrations between 10 nM and 1 μ M enhancing the maximal (E_{\max}) SPAP response, without changing EC_{50} values, but higher concentrations produced marked inhibitory effects.

At nanomolar concentrations, CLOM and DMI increased expression of phospho-CREB (cyclic AMP response element binding protein). NORF was less effective but did significantly increase phospho-CREB at a concentration of 200 nM. TIAN had no effect. None of the antidepressants had any effect on CREB expression, nor on the accumulation of cyclic AMP.

After prolonged exposure (7–21 days) to a low concentration (200 nM) of the antidepressants, the enhanced E_{\max} values for SPAP production evident after 18 h were not maintained but CLOM and DMI induced a significant leftward shift in the isoprenaline EC_{50} after a 7-day period of treatment and this was sustained at the 21 day time point. TIAN did not produce any significant changes.

The results demonstrate that, *in vitro*, some but not all antidepressants can modify gene transcription via monoamine and cyclic AMP-independent mechanisms. The *in vivo* adaptive responses to TIAN probably involve alterations in different gene sets to those affected by other antidepressants.

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1. Introduction

The mechanism of action of the majority of antidepressant drugs (AD) is widely believed to depend, initially, upon acute increases in the synaptic availability of monoamine transmitters at key sites in the brain resulting from reuptake inhibition. However, the delayed clinical response to AD treatment implies that adaptive changes must occur in neuronal circuits involved in affective behaviours for these drugs to be effective.

In recent years, there has been a focus on the involvement of the cyclic AMP (cAMP) signal transduction system in the molecular adaptations occurring during prolonged AD exposure. For example, Thome et al. [1] showed that chronic, but not acute, treatment of rats with a number of clinically effective ADs significantly increased cAMP-controlled gene transcription, as well as the phosphorylation (and activation) of the transcription factor, cAMP response element binding protein (CREB), in several limbic brain regions. It is suggested

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that increased expression of cAMP-driven neurotrophic factor genes protects stress-vulnerable neurones from environmental challenges, including increased hypothalamic–pituitary–adrenal axis activity, and that this might be the genesis of the antidepressant effect [2]. Although it is possible that AD-induced changes in cAMP signalling and, consequently, in downstream gene transcription, are the result of prolonged increases in synaptic monoamines, it is also conceivable that antidepressants might have other mechanisms of action. Indeed, the atypical AD tianeptine (TIAN) increases monoamine transport, thereby reducing synaptic availability of neurotransmitter [3,4].

To avoid the complications of network interactions *in vivo*, and to rule out the involvement of monoamines, some studies have employed relatively simple *in vitro* cell models to investigate alternative mechanisms of AD action, e.g. [5,6]. In a previous study we reported the effects of the AD desipramine (DMI) on gene transcription using a non-excitable Chinese hamster ovary (CHO) cell line transfected with the human β_2 -adrenoceptor and a secreted placental alkaline phosphatase (SPAP) reporter gene which effectively represents an *in vitro* model of the cAMP signalling pathway [7]. DMI treatment at μM concentrations led to reductions in SPAP gene expression which were not apparently the result of changes in any element of the cAMP transduction system, leading to the conclusion that the AD inhibits transcription at a point downstream of CREB phosphorylation. These data were somewhat surprising given the increased CREB phosphorylation and gene expression *in vivo* reported by Thome et al. [1]. To address this apparent discrepancy, in the present study, we have examined the effects of different clinically effective ADs, including the atypical agent tianeptine, over a wider range of concentrations to look for common mechanisms that might underlie their therapeutic efficacy. We report that clomipramine (CLOM), DMI and norfluoxetine (NORF), the major metabolite of the selective serotonin reuptake inhibitor (SSRI) fluoxetine, have biphasic effects, enhancing CREB phosphorylation and gene transcription at low concentrations but, as previously reported, inhibiting transcription in the μM range. The atypical AD TIAN was without effect.

2. Materials and methods

2.1. Materials

Cell culture reagents were from Sigma Chemicals (Poole, Dorset, UK) except foetal calf serum (FCS) which was from PAA Laboratories (Teddington, Middlesex, UK). [^3H]-adenine and [^{14}C]-cAMP were obtained from Amersham International (Buckinghamshire, UK). Western blotting reagents were supplied by BIORAD. Primary antibodies (anti-CREB and anti-phospho-CREB) were supplied by Cell Signalling technologies (New England Biolabs), secondary antibody (goat anti-rabbit HRP-conjugated) was supplied by DAKO, UK. Emulsifier Scintillator Plus (scintillation fluid) was from Packard Biosciences (Perkin-Elmer, UK). All other reagents were supplied by Sigma Chemicals (Poole, Dorset, UK) unless otherwise stated.

2.2. Cell culture

Chinese hamster ovary (CHO) cells stably transfected with the human β_2 -adrenoceptor (at an initial expression level of 310 fmol/mg protein) and a secreted placental alkaline phosphatase (SPAP) reporter gene (CHO β_2 SPAP; [8]), were grown in Dulbecco's modified Eagles medium/nutrient mix F12 (1:1) supplemented with 2 mM L-glutamine and 10% foetal calf serum (FCS) at 37 °C in an atmosphere of 5% CO $_2$:95% air. The SPAP gene is transcriptionally controlled by a promoter region containing six cyclic AMP response elements (CREs).

2.3. Measurement of SPAP

Assays were performed on confluent cells grown in 24 well cluster dishes according to the method described by Cullen and Malim [9] and McDonnell et al. [8]. Briefly, cells were grown to confluency on 24 well cluster dishes before being serum starved in serum-free Dulbecco's modified Eagles medium/nutrient mix F12 (1:1) supplemented with 2 mM L-glutamine only, serum-free medium (SFM), for 24 h prior to assaying. Medium was aspirated from the 24-well plates and replaced with SFM-containing antidepressants at the required concentrations and cells were incubated for the required length of time. On the day of assay, the medium was aspirated and replaced with 1 ml per well fresh SFM. Isoprenaline at appropriate concentrations was added to relevant wells and dishes were replaced in the incubator for 5 h. Medium was aspirated from all wells and replaced with 300 μl per well fresh SFM. Dishes were replaced in the incubator for a further 1 h. Twenty microlitres of samples from each well were transferred into 96 well cluster dishes which were then either frozen at –20 °C until required, or heated at 65 °C for 30 min (inactivates endogenous alkaline phosphatases). Two hundred microlitres of *p*-nitrophenol phosphate (PNPP) reaction substrate was added to each well before incubating dishes for a final hour at 37 °C. Hydrolysis of PNPP by SPAP was measured at 405 nm and data were converted to SPAP concentration in mU ml^{-1} using the equation shown below

$$[\text{SPAP}] (\text{mU ml}^{-1}) = \frac{A}{t \times 18.5 \times V}$$

where A is the measured optical density at 405 nm, t the time with substrate (60 min) and V is the volume of sample.

2.4. [^3H]-cyclic AMP accumulation

Cyclic AMP assays were conducted according to the single column separation method reported by Alvarez and Daniels [10] using cells grown to confluency in 24 well cluster dishes. Where appropriate, for 18 h preincubations, antidepressants were applied to the cells at the same time as [^3H]-adenine (1 μCi per well) for overnight labelling.

Experiments were performed in Krebs' Henseleit buffer (KHB) warmed to 37 °C. Medium was aspirated from all wells and cells were washed with 1 ml per well KHB, before being replaced with 500 μl per well KHB containing 10 μM rolipram. Plates were incubated for 15 min at 37 °C. Antidepressants and isoprenaline were added to wells in 5 μl additions from 100-fold

concentrated stock solutions and plates were incubated for a further 15 min at 37 °C before adding 50 µl 4.4 M HCl to stop reactions and permeabilise cell membranes. Plates were refrigerated for 30 min at 4 °C to allow for complete permeabilisation and column preparation (600 µg dry acid alumina per column). Fifty microlitres of supernatant from each well was transferred into 5 ml scintillation vials with 3.5 ml scintillation fluid before placing in a refrigerated scintillation counter and counting for total ^3H . Fifty microlitres of [^{14}C]-cAMP standard was then added to each well and plates were gently swirled to mix well contents. Well contents were then transferred onto individual, pre-prepared dry acid alumina columns. Columns were washed with 5 ml 5 mM HCl, then 1 ml 0.1 M ammonium acetate, discarding all eluate. Columns were then placed over 20 ml scintillation vials and washed with 3.5 ml 0.1 M ammonium acetate, collecting all eluate. Ten microlitres of scintillation fluid was added to each vial before placing in a refrigerated liquid scintillation counter and counting for ^3H and ^{14}C .

[^3H]-cAMP production was expressed as a percentage of [^3H]-adenine incorporation, assumed to be equivalent to total ^3H .

2.5. Western blotting

On the day of experiment, cells were lysed in 300 µl lysis buffer containing complete mini protease inhibitor cocktail (Roche), then centrifuged at $15,000 \times g$ at 4 °C for 10 min. Supernatants (cytosolic fractions) were transferred into new microfuge tubes (10 µl samples were used for protein determinations). The remaining pellets (nuclear fraction) were resuspended into 150 µl lysis buffer. Twenty microlitres of samples were used for protein determination using the Lowry method [11]. To both fractions, equal volumes of 2× Laemmli solubilisation buffer containing 2.5% bromophenol blue were added.

The nuclear fraction was very viscous especially after addition of solubilisation buffer. The samples were sonicated for 5–10 s to shear DNA and reduce the sample viscosity. Both fractions were boiled at 95 °C using a Grant Instruments (Cambridge) hotplate for 5 min, and then centrifuged at $15,000 \times g$ at 4 °C. The samples were used immediately or stored at –20 °C until needed.

Western blotting was carried out according to manufacturer's instructions using the following antibodies: primary antibodies, phospho-CREB (ser 133) (polyclonal) and CREB antibody (polyclonal) were supplied by Cell Signalling Technology (New England Biolabs, UK). The secondary antibody, goat-antirabbit horseradish peroxidase (HRP)-conjugated, was supplied by DAKO, UK.

Briefly, 10% SDS-PAGE gels were prepared for use with the BIORAD Mini Protean III kit. On each gel lane 2 contained 10 µl BIORAD Kaleidoscope markers, lanes 4–9 contained samples and lanes 1, 3 and 10 were empty. Gels were run for 5–10 min at 100 V, V constant (until the markers start to separate), then at 200 V, V constant for ~45 min or until the dye front ran off the gel. Gels were transferred on to nitrocellulose membranes, again according to manufacturer's instructions, 100 V for 1 h. Membranes were blocked in blocking buffer (5% (w/v) Marvel, non-fat dried milk powder, in Tris-buffered saline, containing 0.1% Tween-20 [TBS-T]), for 1 h at room temperature. Membranes were then washed in TBS-T for 30 min before

incubating with primary antibody (1:2000 in blocking buffer) overnight at 4 °C, with gentle shaking. Primary antibody solution was removed and membranes washed for 30 min in TBS-T, at room temperature, before incubating with secondary antibody (1:2000 in blocking buffer) for 1 h at room temperature, again with gentle shaking. Secondary antibody solution was discarded and membranes washed for a final time in TBS-T for 30 min, at room temperature. Washed membranes were exposed to ECL™ detection solution for 1 min before being quickly blotted dry on filter paper and wrapped in SaranWrap™. Wrapped membranes were placed in an A4 size X-ray cassette and exposed to Hyperfilm ECL™ autoradiography film for up to 15 min. Films were developed using an AGFA autodeveloper. The developed Western blot films were scanned using a GS-710 Imaging Densitometer (Bio-Rad) on transmission mode using the Quantity One software package for image analysis.

2.6. Statistical analysis

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) using the SF ANOVA computer program with a $p < 0.05$ indicating a significant difference. Concentration/response curve fitting was performed by non-linear regression using the GraphPad Prism computer program (GraphPad, San Diego, CA, USA).

3. Results

CHO β_2 SPAP cells were preincubated with 20 µM CLOM, DMI, NORF and TIAN for 18 h before being stimulated with isoprenaline. In previous studies [7] the isoprenaline response was shown to be competitively inhibited by the β_2 adrenoceptor antagonist ICI 118551 with a KD of 0.9 nM confirming that it is mediated by the transfected receptor. Fig. 1 shows that, as previously reported for DMI [7], all antidepressants, except for TIAN, significantly reduced maximum isoprenaline-stimulated SPAP production compared to the control ($E_{\text{max}} = 19.8 \pm 0.9 \mu\text{U/ml}$) with E_{max} values of 4.5 ± 0.4 ,

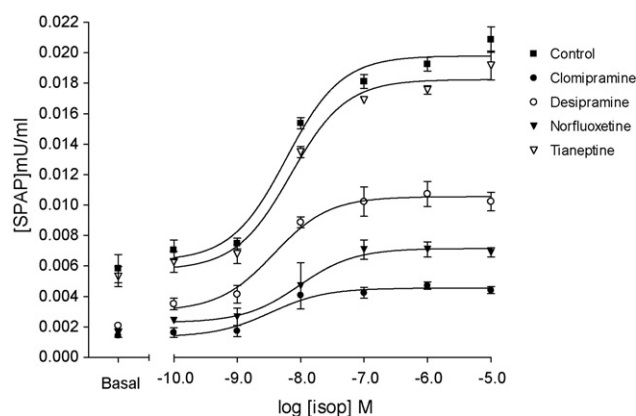


Fig. 1 – Concentration–response curves for isoprenaline-stimulated SPAP production in CHO β_2 SPAP cells pre-incubated for 18 h in the presence of 20 µM CLOM, DMI, NORF or TIAN. Data points are means \pm S.E.M. of three separate experiments each conducted in triplicate.

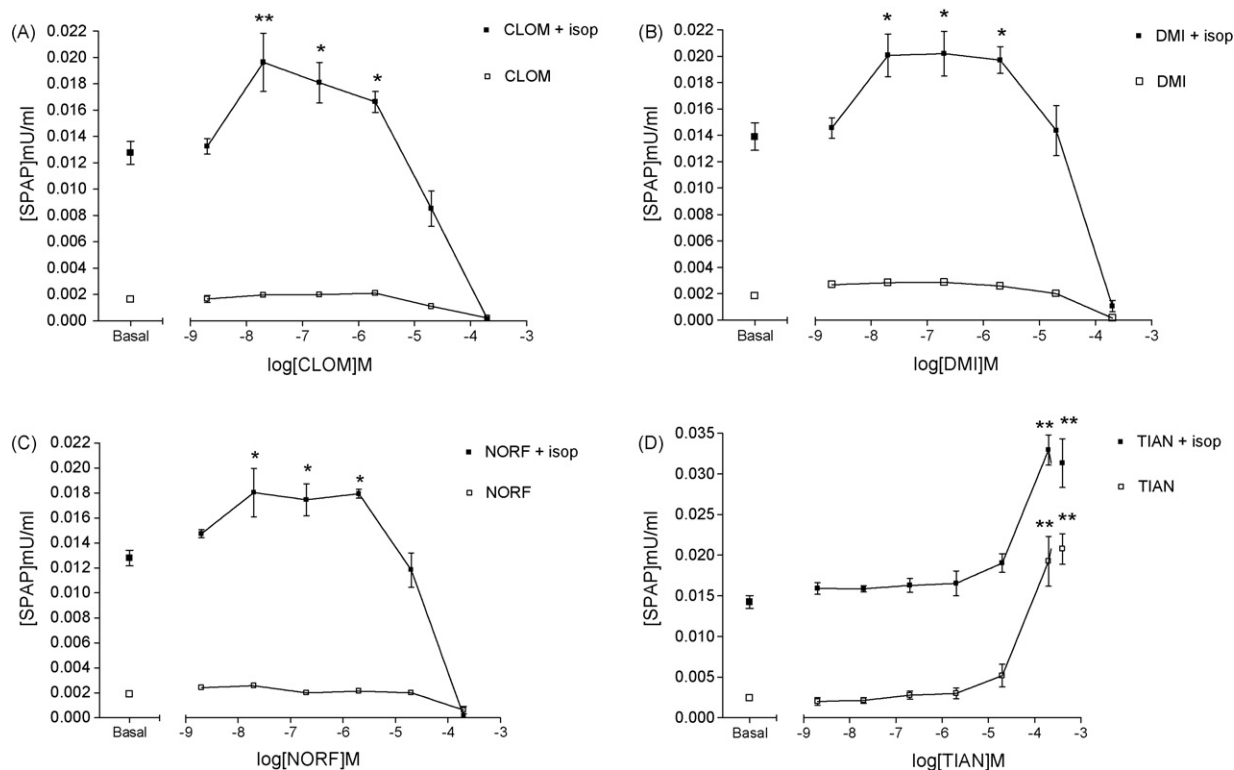


Fig. 2 – Concentration–response curves for isoprenaline-stimulated SPAP production in CHO β_2 SPAP cells pre-incubated for 18 h in the presence of increasing concentrations of (A) CLOM, (B) DMI, (C) NORF, (D) TIAN. Data points are means \pm S.E.M. of three separate experiments each conducted in triplicate. * $p < 0.05$; ** $p < 0.01$, ANOVA and Dunnett's multiple comparison test with respect to isoprenaline alone.

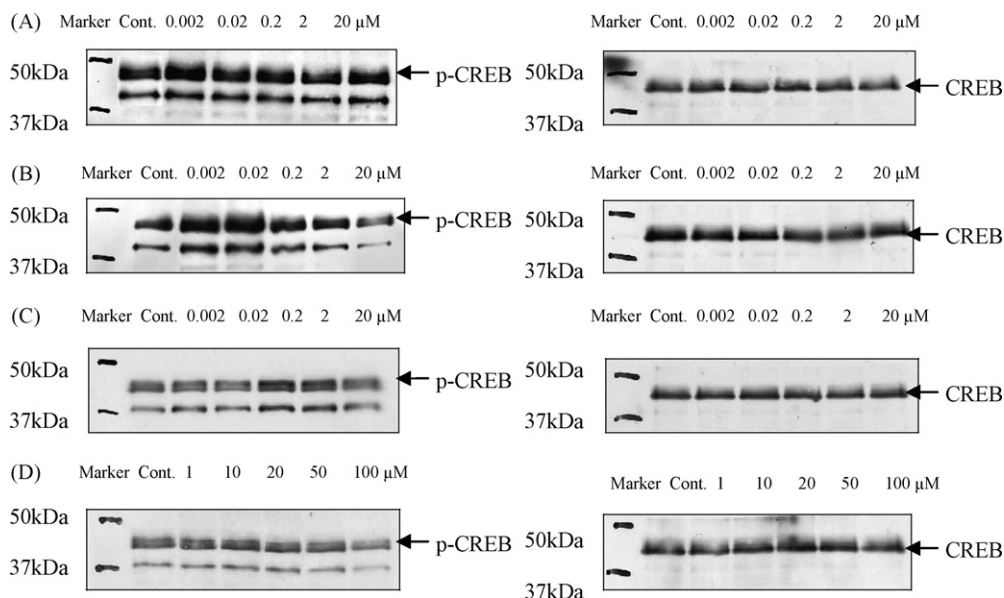


Fig. 3 – Western blots for CHO β_2 SPAP cell lysates pre-incubated for 18 h with increasing concentrations of CLOM (A), DMI (B), NORF (C) or TIAN (D) and treated with isoprenaline (1 μ M) for 2 h before harvesting and lysing. Protein bands at ~ 43 kDa correspond to either CREB (right) or p-CREB (left). Data are representative of a single experiment, repeated on two further occasions with essentially identical findings.

10.6 ± 0.8 , 7.2 ± 0.9 $\mu\text{U/ml}$ for CLOM, DMI and NORF, respectively. The E_{max} value for TIAN was 18.3 ± 1.2 $\mu\text{U/ml}$. Similarly, CLOM, DMI and NORF, but not TIAN-treated cells, showed a significant reduction in the basal levels of SPAP production (1.4 ± 0.2 , 2.1 ± 0.3 , 1.6 ± 0.5 and 5.3 ± 0.1 $\mu\text{U/ml}$, respectively) compared to the control levels (5.8 ± 0.2 $\mu\text{U/ml}$). There were no significant differences from control (5.95 ± 3.4 nM) in the EC_{50} values for isoprenaline in the presence of CLOM (3.36 ± 4.4 nM), DMI (3.77 ± 4.2 nM), NORF (9.50 ± 1.9 nM) or TIAN (6.96 ± 3.9 nM).

In order to determine the concentration dependence of the effects of the ADs on CREB/CRE-mediated gene transcription, CHO β_2 SPAP cells were incubated with increasing concentrations of the drugs for 18 h before stimulating with 1 μM isoprenaline. The results are shown in Fig. 2. At concentrations between 10 nM and 1 μM , CLOM, DMI and NORF enhanced isoprenaline-stimulated SPAP production, with higher concentrations producing inhibitory effects similar to those shown in Fig. 1. CLOM, DMI and NORF had no significant effects in the absence of isoprenaline but, at concentrations of 20 μM and above, TIAN alone stimulated SPAP production to a level greatly in excess of that due to 1 μM isoprenaline. TIAN also enhanced the response to isoprenaline when the two drugs were applied in combination.

Isoprenaline stimulated [^3H]-cAMP accumulation with an EC_{50} value of 3.0 ± 0.3 nM. Forskolin (10 μM) produced a maximal cAMP production comparable to that of isoprenaline. The ADs were preincubated with the cells overnight in the presence of the [^3H]-adenine used to label the nucleotide pools. At concentrations ranging from 2 nM to 20 μM (and up to 100 μM in the case of TIAN), none of the ADs had any significant effect on [^3H]-cAMP accumulation either alone or in the presence of isoprenaline (data not shown).

We have previously reported that DMI at a concentration of 20 μM had no effect on CREB phosphorylation in CHO β_2 SPAP cells. However, having observed the effects on gene transcription at lower concentrations, the effects of the ADS on expression of CREB and phospho-CREB were examined over the same concentration ranges as used for the SPAP gene transcription studies. CLOM and DMI increased expression of phospho-CREB at concentrations in the nM range, whereas higher levels were ineffective (Figs. 3 and 4). NORF was less effective but did significantly increase phospho-CREB at a concentration of 200 nM. TIAN had no effect apart from a tendency to reduce phospho-CREB expression at 100 μM . None of the ADs had any effect on CREB expression (densitometric analysis not shown).

The effects of prolonged exposure to the antidepressants on CREB/CRE-mediated gene transcription were also investigated to model more closely the long term treatments employed in the clinic. A low drug concentration (0.2 μM) was employed because this enhanced gene transcription after 18 h. There were no changes in the maximal effects of isoprenaline following exposure to any of the drugs for 7, 14 or 21 days (Fig. 5, Table 1). However, CLOM and DMI (Fig. 5) induced a significant leftward shift in EC_{50} after a 7-day period of treatment and this was sustained at the 21-day time point. NORF produced a small, but not significant, leftward shift in EC_{50} after 7 and 14 days, whereas TIAN did not produce any significant change in EC_{50} compared to the control value (Table 2).

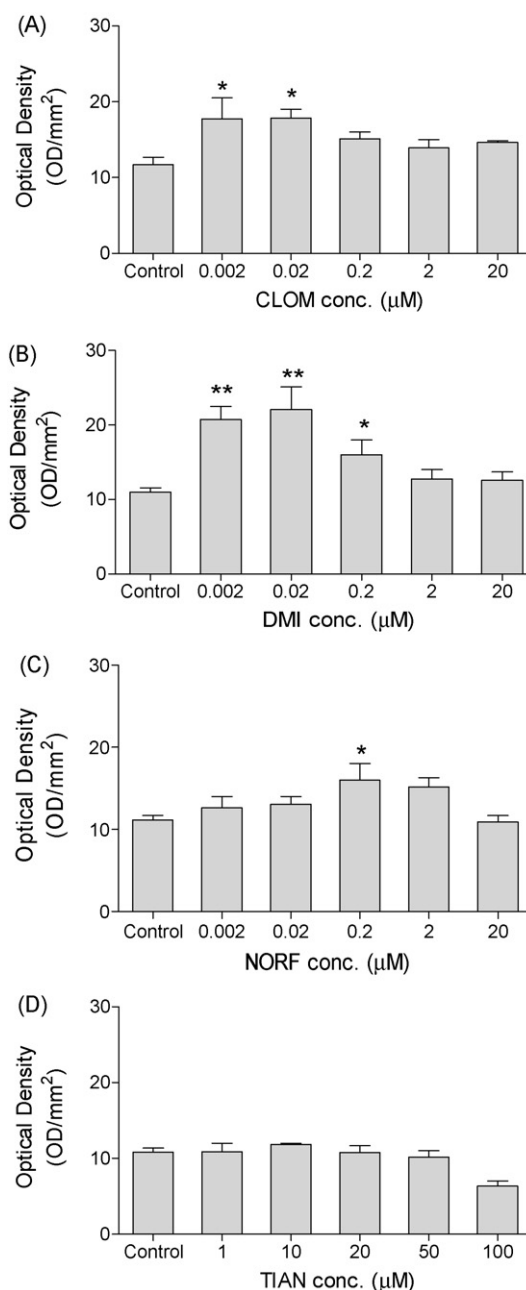


Fig. 4 – Densitometric analysis of Western blots for phospho-CREB in CHO β_2 SPAP cell lysates pre-incubated for 18 h with CLOM (A), DMI (B), NORF (C) and TIAN (D). Results are presented as means \pm S.E.M. of three independent experiments. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's multiple comparison test. * $p < 0.05$ and ** $p < 0.01$.

4. Discussion

In a previous study using the same CHO β_2 SPAP model cell system as in the present investigation it was reported that a range of antidepressants inhibited CRE-driven transcription of the SPAP gene in the absence of any effects on the cAMP signalling system [7]. Given that a variety of *in vivo* studies

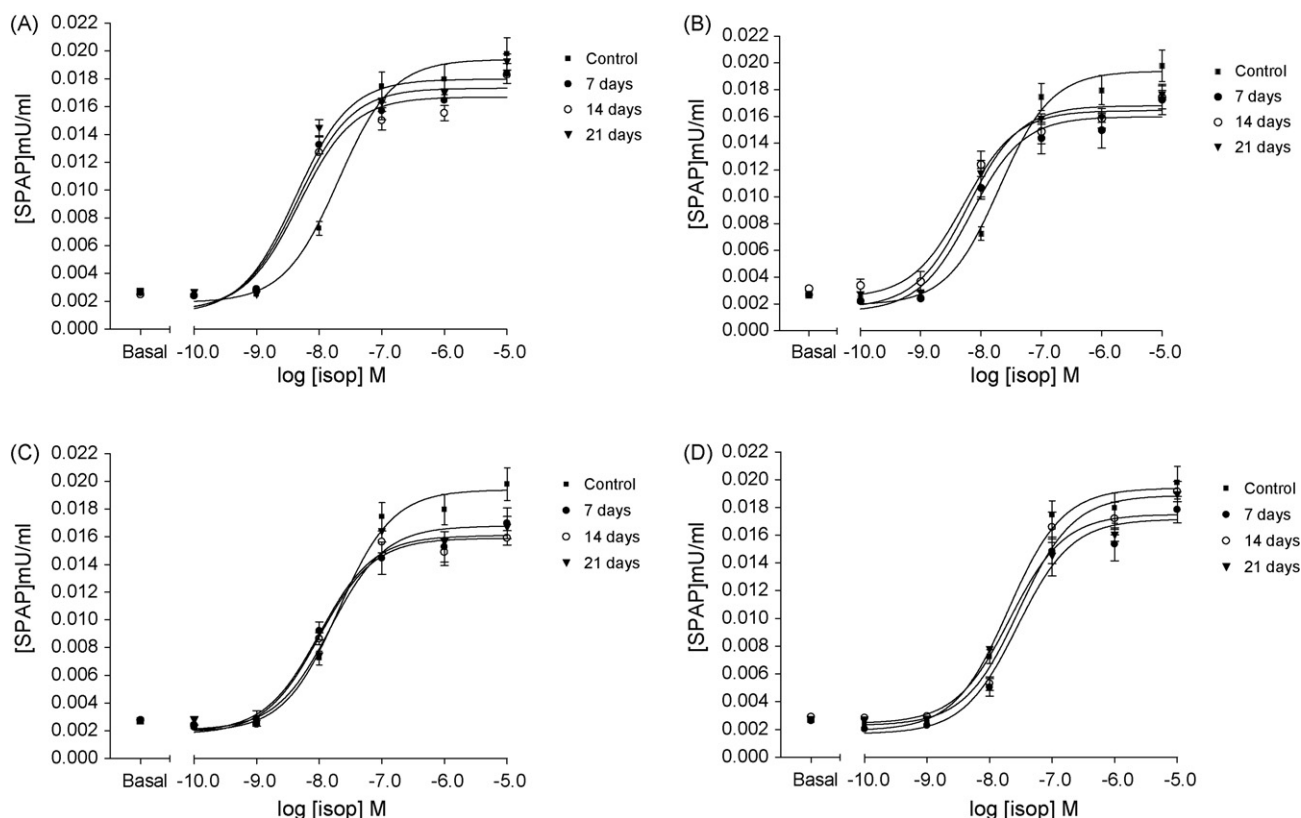


Fig. 5 – Concentration–response curves of isoprenaline-stimulated SPAP production in CHO β_2 SPAP cells pre-incubated in the presence of (A) 0.2 μ M CLOM, (B) 0.2 μ M DMI, (C) 0.2 μ M NORF and (D) 0.2 μ M TIAN for 18 h, 7 days, 14 days and 21 days. Data points are means \pm S.E.M. from three individual experiments each conducted in triplicate.

[1,12,13] have demonstrated increases in CREB phosphorylation and enhanced transcription of downstream genes, including cell survival factors such as BDNF, in limbic areas of rodent brain following repeated treatment [14–16], it was difficult to see the relevance of these observations to the mechanism of action of the antidepressants.

In the present study, however, a re-evaluation of the concentration dependency of the drugs' effects has demonstrated that, for CLOM, DMI and the active fluoxetine metabolite NORF, there are biphasic effects, such that at lower concentrations, in the nanomolar or low micromolar

range, SPAP gene transcription is elevated (Fig. 2). As observed previously, at higher concentrations, CLOM, DMI and NORF all reduced isoprenaline-stimulated reporter gene transcription (Figs. 1 and 2). The effects of the atypical tricyclic antidepressant TIAN were quite different in that there were no effects at nanomolar concentrations but marked enhancements of transcription at concentrations in excess of 20 μ M (Fig. 2).

The enhancements of transcription by low concentrations of CLOM, DMI and NORF were matched by similar increases in the expression of the transcription factor phospho-CREB over

Table 1 – Effects of prolonged antidepressant exposure on maximal SPAP responses to isoprenaline

Antidepressants	SPAP E_{\max} (μ U/ml)		
	7 days treatment	14 days treatment	21 days treatment
CLOM	17.3 \pm 0.5	16.7 \pm 0.4	17.9 \pm 0.5
DMI	16.0 \pm 0.6	16.5 \pm 0.5	16.8 \pm 0.5
NORF	16.1 \pm 0.5	15.9 \pm 0.4	16.8 \pm 0.4
TIAN	17.15 \pm 0.6	18.9 \pm 0.6	17.5 \pm 0.5
No drug			19.4 \pm 1.6

SPAP E_{\max} (μ U/ml) calculated from the concentration–response curves of isoprenaline-stimulated SPAP production in CHO β_2 SPAP cells incubated in the presence of 0.2 μ M CLOM, DMI, NORF or TIAN for different periods of time. There was no significant difference between any of the treatments and the control which consisted of cells incubated in the absence of antidepressant (no drug) for 21 days. The starts of different length incubations were staggered such that all cells were harvested and analysis conducted at the same times. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's multiple comparison test.

Table 2 – Effects of prolonged antidepressant exposure on EC_{50s} of SPAP responses to isoprenaline

Antidepressants	Isoprenaline EC ₅₀ (nM)		
	7 days treatment	14 days treatment	21 days treatment
CLOM	4.5 ± 2.8 [#]	4.7 ± 1.6 [#]	4.2 ± 3.9 [#]
DMI	6.7 ± 3.2 [#]	5.2 ± 3.5 [#]	6.0 ± 1.9 [#]
NORF	10.0 ± 3.7	10.1 ± 4.2	15.0 ± 3.7
TIAN	27.7 ± 5.4	29.2 ± 1.9	21.0 ± 6.9
No drug			20.0 ± 4.5

EC₅₀ (nM) values calculated from the concentration–response curves for isoprenaline-stimulated SPAP production in CHOβ₂SPAP cells incubated in the presence of 0.2 μM CLOM, DMI, NORF or TIAN for different periods of time. [#] indicates a significant difference (*p* < 0.05) from the control value derived from cells incubated in the absence of antidepressant (no drug) for 21 days. The starts of different length incubations were staggered such that all cells were harvested and analysis conducted at the same times. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's multiple comparison test.

the same concentration range and it is tempting to suggest that the primary response to the drugs is an increase in CREB phosphorylation resulting in activation of the SPAP reporter gene. It is difficult to see how either the reduced transcription due to higher concentrations of CLOM, DMI and NORF or the elevation in the presence of TIAN could be related to cAMP-driven CREB phosphorylation given the absence of any correlating change in phospho-CREB expression. The mechanism of these effects must either lie downstream, possibly at the level of the transcription-controlling complex of co-activators or be mediated by independent means. For example, we have recently shown that CLOM, in the micromolar concentration range, acts as a mitochondrial inhibitor via direct binding to complex III of the respiratory chain and it is conceivable that this metabolic effect could influence gene transcription [17]. Given that the inhibitory changes were evident in the maximal responses with no alterations in EC₅₀, this at least indicates that the effects are not due to competitive inhibition by the antidepressants at the level of the receptor.

The enhancement of CREB phosphorylation and CRE-driven SPAP gene transcription by CLOM, DMI and NORF cannot be attributed to modulation of adenylyl cyclase or phosphodiesterase activity since none of the drugs altered cAMP accumulation. It is, however, possible that the antidepressants are able to modify CREB phosphorylation at the level of cyclic AMP-dependent protein kinase A (PKA) and it has been demonstrated that chronic, but not acute, treatment of rats with various SSRI antidepressants induced activation of PKA in the cerebral cortex via increasing the binding of cAMP to the regulatory II subunit of PKA [18,19]. Similar results have also been obtained with chronic administration of TCAs [20]. The increased activation of PKA in response to antidepressant treatment is potentially important since it was reported that β-adrenoceptor-linked cAMP-dependent PKA activity was reduced in human fibroblasts from patients with major depression [21]. The present observations are consistent with those of Koch et al. [22], who reported an increase in phospho-CREB without any changes in the total CREB in Jurkat cells (a human t-cell lymphoma) after chronic treatment with imipramine (10 μM), but not with fluoxetine (4 μM) although the active metabolite NORF was not employed in that study.

CREB can also be activated by kinases other than PKA including, CaMK II, p90RSK and p38 MAP kinase, all of which phosphorylate CREB at serine 133 [23], and it is conceivable

that the antidepressant drugs could interact with one or more of these enzymes to increase CREB phosphorylation. For example, chronic treatment of rats with the antidepressants DMI and reboxetine up-regulated CaMK II in neuronal cell bodies of the hippocampus [24] and fluvoxamine and DMI significantly increased the kinase activity in presynaptic vesicles of frontal/prefrontal cortex [25]. Prolonged *in vitro* exposure to imipramine, DMI, CLOM, and maprotiline has also been suggested to modify 5HT₂ receptor function in C6 glioma cells via a CaMK II-dependent mechanism [26] indicating that the effects of the antidepressants do not rely on complex brain circuitry modifications but can reflect rather more direct actions on target cells. Very recently, Duman et al. [27] implicated MAP kinase activation in the action of DMI and sertraline since the behavioural effects of these antidepressants were attenuated by a MAP kinase kinase (MEK) inhibitor. Again, direct effects of the antidepressants on target cells are implied by the finding that fluoxetine activates the extra-cellular signal-regulated-protein kinase (Erk) and p38 mitogen-associated protein (MAP) kinase cascades in cultured astrocytes [28] resulting in increased expression of the genes for the neurotrophins brain-derived nerve factor (BDNF) and glial-derived nerve factor (GDNF), in line with fluoxetine's *in vivo* effects.

In the present study, chronic exposure of the CHOβ₂SPAP cells to a low concentration (0.2 μM) of the tricyclic antidepressants CLOM and DMI did not cause a sustained increase in the maximal response to isoprenaline (as seen after 18 h) but did induce a significant leftward shift in the isoprenaline concentration/response curve, after a 7-day period of treatment, which was maintained up to 21 days. NORF produced a small but non-significant reduction in EC₅₀ after 7-day period treatment but TIAN was again without effect. In a previous study control cells cultured in the absence of antidepressants for periods from 18 h to 21 days also showed no changes in the responses to isoprenaline [38].

These changes in isoprenaline potency cannot be explained by a direct interaction of the antidepressants with the β₂-adrenoceptor since they were not seen acutely. However, an increase in coupling of the receptor to its downstream effectors would be expected to enhance potency with regard to the reporter gene's transcription. Such a change in post-receptor signalling could be a result of changes in the cAMP transduction pathway or at the level of gene transcription. This suggestion is consistent with studies which have

demonstrated that antidepressants upregulate cAMP signaling pathways after chronic antidepressant treatment at several sites, including increased Gs protein activation of adenylyl cyclase [29], increased PKA activation [30] and increased CREB phosphorylation [1].

Given the biphasic nature of the concentration/response relationships for CLOM, DMI and NORF *in vitro* it is important to know whether drug levels achieved *in vivo* would be predicted to enhance or inhibit gene transcription. For example, in a clinical trial of panic disorder treatment with CLOM, after 14 days of escalating dosing up to a mean of 95 mg/day, the total serum level of CLOM and its major metabolite was 209 ng/ml [31] i.e. about 0.7 μ M; just above the range at which enhancements of transcription were seen in the present study. However, CLOM concentrations are reported to be approximately 10-fold higher in the brain than in the blood after repeated dosing in rats [32] bringing them into the inhibitory range as predicted by *in vitro* testing. Although it is naive to expect to be able to closely correlate *in vitro* with *in vivo* responses, the complex nature of the response perhaps explains partially why there is such a controversial relationship between antidepressant levels and the therapeutic response [31].

TIAN had a very different response profile to the other antidepressants. This might suggest, since the changes in CRE-driven gene transcription are not a universal property of antidepressants, that they are irrelevant to the clinical action of the drugs. However, it is equally possible that there is a common effect of antidepressant drugs that can be achieved via different routes. It is broadly accepted that depression involves stress-induced remodelling of sensitive brain regions and that antidepressants can reverse this [33–35]. Multiple neurotransmitter systems contribute to remodelling, including excitatory amino acids, serotonin, and GABA, working synergistically with glucocorticoids. TIAN, in common with other antidepressants, reverses the process but, it is proposed, through its actions on excitatory amino acids rather than monoamines [36]. Additionally, in common with other antidepressants, TIAN could have direct effects on gene transcription in target cells in stress-vulnerable brain regions as suggested by its inhibitory effects *in vitro* on glucocorticoid-driven transcription in a fibroblast cell model [37].

5. Conclusions

In summary, the data presented show that a number of clinically effective antidepressants, acting at a local cellular level, modulate CRE-driven gene transcription in a biphasic, concentration-dependent fashion independent of monoamines or changes in cyclic AMP; these changes are maintained, in a modified form, for a prolonged period of time. If these effects are reflected *in vivo* it might help to explain the variations in individual patient responses to antidepressants.

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