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Three-factor models versus time series models: quantifying time-dependencies of interactions between stimuli in cell biology and psychobiology for short longitudinal data

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Signal integration determines cell fate on the cellular level, affects cognitive processes and affective responses on the behavioural level, and is likely to be involved in psychoneurobiological processes underlying mood disorders. Interactions between stimuli may subjected to time effects. Time-dependencies of interactions between stimuli typically lead to complex cell responses and complex responses on the behavioural level. We show that both three-factor models and time series models can be used to uncover such time-dependencies. However, we argue that for short longitudinal data the three factor modelling approach is more suitable. In order to illustrate both approaches, we re-analysed previously published short longitudinal data sets. We found that in human embryonic kidney 293 cells cells the interaction effect in the regulation of extracellular signal-regulated kinase (ERK) 1 signalling activation by insulin and epidermal growth factor is subjected to a time effect and dramatically decays at peak values of ERK activation. In contrast, we found that the interaction effect induced by hypoxia and tumour necrosis factor-alpha for the transcriptional activity of the human cyclo-oxygenase-2 promoter in HEK293 cells is time invariant at least in the first 12-h time window after stimulation. Furthermore, we applied the three-factor model to previously reported animal studies. In these studies, memory storage was found to be subjected to an interaction effect of the beta-adrenoceptor agonist clenbuterol and certain antagonists acting on the alpha-1-adrenoceptor / glucocorticoid-receptor system. Our model-based analysis suggests that only if the antagonist drug is administer in a critical time window, then the interaction effect is relevant.

Keywords: three-factor models; interactions between stimuli; crosstalk between signalling pathways; transcriptional activation.

1. Introduction

In the past, research in cell biology has been dominated by the idea of linear stimulus–response pathways that relate intracellular cues to specific genes. In contrast, modern-day cell biology has revealed that gene expression is frequently regulated by multiple cues that are integrated on the level of signal transduction pathways and on the DNA promoter level (Bokes *et al*., 2009; Kholodenko *et al*., 2012; Frank *et al*., 2012b). Experimental evidence indicates that the regulation of gene expression by extra-cellular stimuli often involves crosstalk between signalling pathways and interactions between transcription factors.

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Crosstalk and interacting transcription factors can lead to the cooperative activation of genes and may give rise to 'synergistic' cell–cell-interaction effects (Lenbury *et al*., 2000). In this context, cooperative activation means that if a gene is activated cooperatively by two extra-cellular stimuli A and B, then the expression level of that gene induced by the combined stimulation with A and B is higher than the expected level obtained by summing up the expression levels induced by the individual stimulations with either A or B. Such greater-than-additive effects have been referred to as 'synergy' effects (Ptashne & Gann, 1997; Levine, 2010; Frank *et al*., 2012a). In what follows, we will refer to them as interaction effects in order to use a more technical term. Interaction effects induced by two stimuli A and B can be tested using two factor models (Slinker, 1998).

For example, with the help of two factor models, it has been shown that ceftazidime interacts with certain cytokines to inhibit the intercellular replication of the bacteria *Burkholderia pseudomallei* (Propst *et al*., 2010). Likewise, it has been found that gene expression of the follicle-stimulating hormone-β is induced by the combined stimulation with activin and progesterone or GnRH stimulation and is subjected by an interaction effect between the two stimuli (Coss *et al*., 2007; Thackray *et al*., 2010). Two factor models have been used to show that collagen production is affected by an interaction effect between hypoxia treatment and stimulation with the basic fibroblast growth factor (Gunja & Athanasiou, 2010). Likewise, interleukin-6 gene expression in tumour cells is regulated by an interaction effect between inflammatory signals and activation of the aryl hydrocarbon receptor (DiNatale *et al*., 2010) (see the comparison in Fig. 4(a) presented in DiNatale *et al*., 2010 between the vehicle and tetrachlorodibenzo-p-dioxin condition). Interaction effects in signalling networks not only can be observed on the cellular level but their implications on the behavioural level can be quantified as well. For example, stress-related memory storage has been found to be regulated by beta-1-adrenoceptors and glucocorticoid receptors in postsynaptic sites of the basolateral amygdala (Ferry *et al*., 1999; Roozendaal *et al*., 2002) and involves an interaction effect. Moreover, it has been shown that explorative animal behaviour is subjected to the interaction between extracellular signal-regulated kinase (ERK) 1 signalling and the impact of amphetamine (Engel *et al*., 2009).

Taking the boarder perspective, the cellular-neural system is typically exposed to a plenitude of input signals (e.g. training cues, stress signals, signals induced by medication). On the single-cell level, these signals contribute to a cellular environment that is rich on extra-cellular stimuli. These stimuli are integrated in signal transduction networks and on the promoter level and may give rise to interaction effects. Interaction effects can determine the structural composition of the cellular-neural system, e.g. by affecting cell fate and synaptic plasticity. On the other hand, as mentioned above, interaction effects determine cognitive functions (e.g. memory storage (Sandi & Rose, 1994; Ferry *et al*., 1999; Roozendaal, 2000; Roozendaal *et al*., 2002)) and affective responses (e.g. explorative animal behaviour (Engel *et al*., 2009)) and are likely to be involved in the emergence and treatment of mood disorders (Anacker *et al*., 2011; Joels, 2011). In particular, the glucocorticoid receptors antagonists such as mifepristone have been shown to be effective in the treatment of mood disorders and in particular as antidepressant (DeBattista & Belanoff, 2006; Anacker *et al*., 2011; Joels, 2011; Frank, 2013). Therefore, an understanding of interaction effects involving glucocorticoid receptors antagonists is of clinical relevance.

While two factor models are useful tools to uncover the statistical significance of an interaction effect, two factor models are limited in their applicability because they cannot account for variations in the degree of an interaction effect over the course of different time epochs. In the present study, we overcome this limitation by applying three-factor models to experimental data of transcriptional activity. In Section 3, we briefly discuss three-factor models and compare them with time series models that might be used as well in order to reveal time-dependencies of interaction effects. Subsequently, in Section 3 we will present a re-analysis of previous published data by means of three-factor models. For the regulation of

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ERK by insulin and epidermal growth factor (EGF) we will demonstrate that the interaction effect in the early and late transient response is of the same magnitude but decreases in magnitude significantly at the peak response (Example 3.1). Moreover, we will study the interaction effect between hypoxia and tumour necrosis factor-alpha (TNFα) stimulations. The TNFα is in particular important for our understanding of the inflammatory pathway (see, e.g., Seymour & Henderson, 2001). For the cooperative regulation of the Cyclo-Oxygenas-2 (COX2) promoter, we show that the interaction effect is practically invariant over relative long periods of 12 hours; the interaction effect however can only be observed at relative high levels of stimulations (Example 3.2). Finally, a series of animal studies will be addressed in which it has been shown that memory consolidation is subjected to various interaction effects (Example 3.3). Finally, for Examples 3.1 and 3.2 we will conduct also a time-series modelling approach. However, as we will argue in the theory section (Section 2), while the time series modelling approach can provide additional information that goes beyond the information provided by three-factor models, time series modelling is not a suitable tool to reveal time-dependencies of interaction effects in the case of very short longitudinal data.

2. Three-factor models versus time series models for short longitudinal data

Two-factor models are designed to explain cell responses Y_{jk} for a control condition (coded by $j = k = 0$), under stimulus A ($j = 1$, $k = 0$) or stimulus B ($j = 0$, $k = 1$), and under the impact of both stimuli ($j = k = 1$). A benefit of two factor models is that they can capture interaction effects between the two stimuli A and B. Such interaction effects can be visualized in terms of means plots (Keppel & Wickens, 2004). In general, a means plot shows the sample mean values for each stimulation condition (e.g. see Section 3). While a means plot is a useful tool for visualizing interaction effects, a means plot analysis must be completed by testing whether an interaction effect is statistically significant.

The impact of time on an interaction effect can be tested using a three-factor ANOVA (Fisher, 1925; Keppel & Wickens, 2004). In this case, the two stimuli correspond to two factors of the three-factor model and the time variable represents the third factor. Cell responses are described by the variable Y_{jkt}, where $t = 1, \ldots, T$ denotes the time points being observed. While three-factor models account for three possible two-way interaction effects, in our context, of particular importance is the fact that three-factor models exhibit the three-way interaction term. This three-way interaction term captures the interaction between time and the two-way interaction effect induced by stimuli A and B. Three-factor models are in particular suitable to study the impact of time on stimulus–stimulus interactions in the case of short longitudinal data. In order to illustrate this point, let us consider time series models as alternative to three-factor models.

Among various time series models, autoregressive models (Diggle, 1990; Madsen, 2008) are of particular interest for our purposes of studying effects of time. The reason for this is that autoregressive models exhibit model parameters that do not only correspond to the magnitude of a set of scores but also inform us about the stability of the dynamics of a cellular or behavioural response (Diggle, 1990; Madsen, 2008). More precisely, autoregressive models can describe stable and unstable dynamical systems. In doing so, they address an aspect that is not covered by three-factor models. However, in order to estimate these stability-related model parameters a minimal number of observation is required. This is a crucial issue for short longitudinal data sets. The model that requires the smallest data set is the autoregressive model of order 1 (AR-1) and reads (Diggle, 1990; Madsen, 2008)

$$Y_{jk}(t+1) = \mu_{jk} + a_{1,jk}(Y_{jk}(t) - \mu_{jk}) + \epsilon_{jk}(t). \tag{2.1}$$

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Here, Y_{jk} is the observable at T discrete time points $t = 1, 2, \ldots, T$. The indices j and k capture the levels of the stimuli A and B. The parameter μ_{jk} corresponds to the mean of the observed scores and can directly be estimated by taking the average across a given longitudinal data set. The variable ϵ_{jk} describes a noise term given in terms of a normally distributed random variable with zero mean. The parameter $a_{1,jk}$ is the first autoregressive coefficient and provides information about the stability of the dynamics under consideration. For $|a_{1,jk}| \geq 1$ the stochastic dynamics is unstable. In contrast, for $|a_{1,jk}| < 1$ the dynamics has an asymptotically stable fixed point at $Y_{ij} = \mu_{ij}$. In addition to this qualitative distinction, for $|a_{1,jk}| < 1$ the degree of stability increases when $|a_{1,jk}|$ becomes smaller. That is, in the deterministic case, the relaxation towards the fixed point becomes faster when $|a_{1,jk}|$ becomes smaller. For short time series, the coefficient $a_{1,jk}$ can be estimated via the ordinary least square fit (OLS) estimator defined by (Hayakawa, 2007)

$$a_{1,jk,estim} = \frac{\sum_{t=2}^{T} z_{jk}(t) z_{jk}(t-1)}{\sum_{t=2}^{T} z_{jk}(t-1)^2}, \; z_{jk}(t) = Y_{jk}(t) - \mu_{jk}, \tag{2.2}$$

where $z_{jk}(t)$ is the centred set of observations with time average equal to zero. However, the estimator does not make sense for very short data sets composed only of two observations. In order to see this, we consider the case $T = 2$ which leads to

$$a_{1,jk,\mathrm{estim}} = \frac{z_{jk}(2)}{z_{jk}(1)}. \tag{2.3}$$

However, for $T = 2$ we have $z_{jk}(1) = d$ and $z_{jk}(2) = -d$ with $d = (Y_{jk}(1) - Y_{jk}(2))/2$. Therefore, the estimator yields $a_{1,jk,estim} = -1$ irrespective of the numerical values $Y_{jk}(1)$ and $Y_{jk}(2)$. Therefore, in order to estimate the parameters of the AR-1 from a longitudinal data set via OLS estimator, the data set must satisfy a minimal length requirement of three observations. A slightly alternative approach uses differences rather than centred data (Hayakawa, 2007). In this case, $z_{jk}(t)$ are defined by $z_{jk}(t) = Y_{jk}(t) - Y_{jk}(t-1)$. In order to apply an equation similar to (2.2), we need at least two difference scores $z_{jk}(2)$ and $z_{jk}(3)$. Therefore, the minimal length requirement is three observations again. For sake of completeness, note that (2.2) for the difference scores reads

$$a_{1,jk,\mathrm{estim}} = \frac{\sum_{t=3}^{T} z_{jk}(t) z_{jk}(t-1)}{\sum_{t=3}^{T} z_{jk}(t-1)^2}. \tag{2.4}$$

Let us mention two more methods for estimating $a_{1,jk}$. The first is the Yule–Walker method (Diggle, 1990), which estimates the coefficients of autoregressive coefficients from moments and correlation coefficients obtained by time averaging across a given longitudinal data set. For the AR-1, the estimator of $a_{1,jk}$ reads

$$a_{1,jk,\mathrm{estim}} = \mathrm{Corr}_{jk}(1), \tag{2.5}$$

where $\mathrm{Corr}_{jk}(1)$ is the lag-1 correlation coefficient of the scores $Y_{jk}(t)$. For a longitudinal data set with two observations only, we have

$$\mathrm{Corr}_{jk}(1) = \frac{z_{jk}(1) z_{jk}(2)}{z_{jk}(1)^2 + z_{jk}(2)^2} \tag{2.6}$$

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with $z_{jk}(1) = d$ and $z_{jk}(2) = -d$ and $d = (Y_{jk}(1) - Y_{jk}(2))/2$. Consequently, the correlation coefficient equals $\mathrm{Corr}_{jk}(1) = -0.5$ irrespective of the scores $Y_{jk}(1)$ and $Y_{jk}(2)$ and likewise the estimators (2.5) yield $a_{1,jk,\mathrm{estim}} = -0.5$ in any case. We arrive again at the conclusion that for applying the Yule–Walker method to estimate $a_{1,jk}$ there is a minimal length requirement of three observations. As alternative to the OLS and the Yule-Walker method, the coefficient $a_{1,jk}$ may be estimated by means of an appropriately defined maximum-likelihood estimator (MLE). From (2.2), it follows that the centred data set with $z_{jk}(t) = Y_{jk}(t) - \mu$ satisfies

$$z_{jk}(t+1) = a_{1,jk} z_{jk}(t) + \epsilon_{jk}(t). \tag{2.7}$$

Since ϵ_{jk} has a normal distribution, the conditional probability density $P(z_{jk}(t)|z_{jk}(t-1))$ is a normal distribution

$$P(z_{jk}(t)|z_{jk}(t-1)) = \frac{1}{\sqrt{2\pi\sigma_{jk}^2}} \exp\left\{-\frac{(z_{jk}(t) - a_{1,jk} z_{jk}(t-1))^2}{2\sigma_{jk}^2}\right\}, \tag{2.8}$$

where σ_{jk}^2 is the variance of ϵ_{jk}. Likewise, the likelihood Λ to observe a 'path' $z_{jk}(1), \ldots, z_{jk}(T)$ of T observations given the model parameter $a_{1,jk}$ and σ_{jk}^2 is given by

$$\Lambda(a_{1,jk}, \sigma_{jk}^2) = \prod_{t=2}^{T} P(z_{jk}(t)|z_{jk}(t-1)). \tag{2.9}$$

The MLEs of $a_{1,jk}$ and σ_{jk}^2 are the parameters that maximize the likelihood Λ for a given path (Box *et al.*, 1994; Frank, 2010). If there are only two observations, the likelihood function reduces to

$$\Lambda(a_{1,jk}, \sigma_{jk}^2) = P(z_{jk}(2)|z_{jk}(1)). \tag{2.10}$$

The likelihood becomes maximal with $\Lambda = 1/\sqrt{2\pi\sigma_{jk}^2}$ for $a_{1,jk} = z_{jk}(2)/z_{jk}(1)$ irrespective of σ_{jk}^2. Therefore, we arrive at the estimator $a_{1,jk,estim} = z_{jk}(2)/z_{jk}(1)$, which corresponds to the OLS estimator discussed above. As shown above, we have $a_{1,jk,estim} = -1$ irrespective of $Y_{jk}(1)$ and $Y_{jk}(2)$. Consequently, the MLE method cannot be applied to a short data set composed of $T = 2$ observation and requires (just as the OLS and Yule–Walker method) a minimal length of $T = 3$ observations.

Having considered a time–discrete time series model, we may have a brief look at time–continuous time series models. Such models are typically defined by stochastic differential equations. In this context, a benchmark model is the model of an Ornstein–Uhlenbeck process defined by (Risken, 1989; Frank, 2005)

$$\mathrm{d}Y_{jk}(t) = -a_{jk}(Y_{jk}(t) - \mu_{jk})\mathrm{d}t + \mathrm{d}\xi. \tag{2.11}$$

In the absence of the noise term $\mathrm{d}\xi$, the model has a fixed point at $Y_{jk} = \mu_{jk}$. The parameter a_{jk} defines the rate of exponential growth or decay out of the fixed point μ_{jk}. If $a_{jk} > 0$, the fixed point is stable and the parameter a_{jk} is the decay rate. For $a_{jk} < 0$ the fixed point is unstable and $|a_{jk}|$ corresponds to the exponential growth rate. The variable ξ is a Wiener process with variance $2g_{jk}^2$. Accordingly, $d\xi$ is the infinitesimal increment of the Wiener process. For $a_{jk} > 0$, the model parameters may be estimated via

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MLE method again. First of all, μ corresponds to the mean of the process and may be estimated using time averaging. Introducing the centred variable $z_{jk}(t) = Y_{jk}(t) - \mu_{jk}$ again, (2.11) becomes

$$\mathrm{d}z_{jk}(t) = -a_{jk} z_{jk}(t)\mathrm{d}t + \mathrm{d}\xi \tag{2.12}$$

and the conditional probability density (transition probability density) reads (Risken, 1989; Frank, 2005)

$$P(z_{jk}(t_2)|z_{jk}(t_1)) = \frac{1}{\sqrt{2\pi W_{jk}}} \exp\left\{-\frac{(z_{jk}(t_2) - z_{jk}(t_1)\exp\{-a_{jk}\tau\})^2}{2W_{jk}}\right\} \tag{2.13}$$

with $t_2 > t_1$, $\tau = t_2 - t_1$, and $W_{jk} = g_{jk}^2(1 - \exp\{-2a_{jk}\tau\})$. In line with our previous considerations on the AR-1 process, it can be shown that if there are only two observations available, then the likelihood function Λ reads $\Lambda(a_{jk}, g_{jk}^2) = P(z_{jk}(t_2)|z_{jk}(t_1))$. The function becomes maximal if

$$a_{jk} = -\frac{1}{\tau}\log\left(\frac{z_{jk}(t_2)}{z_{jk}(t_1)}\right) \tag{2.14}$$

irrespective of the numerical value of g_{jk}. However, this leads to a contradiction because $z_{jk}(t_2)/z_{jk}(t_1) = -1$ (see above). Therefore, the model parameters cannot be estimated from two observations only. Rather, there is a minimal length requirement again of three observations.

In summary, time series models may be used to determined via a model-based approach whether or not there is an interaction effect of two stimuli A and B on the dynamics underlying the observed cellular or behavioural response. However, for some fundamental time series models, the autoregressive models and the linear stochastic differential equations, there is a minimal length requirement to estimate the model coefficients, which is given by three observations. Consequently, in order to check whether or not an interaction effect changes over time, epochs of three observations may be analysed. Different schedules of epoch analysis are possible that can be distinguished by the degree of overlap between the epochs. The most conservative epoch analysis features zero overlap. Accordingly, we may decompose a longitudinal data set composed of $T = 6$ observations into two epochs $Y_{jk}(1)$, $Y_{jk}(2)$, $Y_{jk}(3)$ (epoch 1) and $Y_{jk}(4)$, $Y_{jk}(5)$, $Y_{jk}(6)$ (epoch 2). In doing so, we can obtain AR-1 estimates for μ_{jke} and $a_{1,jke}$, where the index $e = 1, 2$ indicates the epoch. The least conservative epoch analysis uses epochs with two scores overlap. From a data set with $T = 4$ observations the epochs $Y_{jk}(1)$, $Y_{jk}(2)$, $Y_{jk}(3)$ (epoch 1) and $Y_{jk}(2)$, $Y_{jk}(3)$, $Y_{jk}(4)$ (epoch 2) can be constructed and the parameters μ_{jke} and $a_{1,jke}$ can be estimated in both epochs. Importantly, the epoch analysis allows us to study the time-dependency of a stimulus–stimulus interaction effect on the model parameters μ_{jk} and $a_{1,jk}$.

Three-factor models and time series models allow us to study stimulus–stimulus interaction effects on responses observed in cell biology and psychobiology. The model-based analysis via time series models can provide more information than the analysis via three-factor models. However, in order to determine time-dependencies of interaction effects, time series models in general require a minimal number of observations. In contrast, three-factor models are not subjected to such a requirement. In fact, a three-factor model can determine a time-dependency effect for longitudinal data sets composed of just two observations. For the class of autoregressive models and for the Ornstein–Uhlenbeck model, we found that there is a minimal length requirement of at least four observations for testing whether or not stimulus–stimulus two-way interaction effects vary over time. More precisely, using the AR-1 ('the shortest' model of all autoregressive models) or alternatively the Ornstein–Uhlenbeck model and taking

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the least conservative epoch analysis we found that a longitudinal data set of $T = 4$ observations may be decomposed into two overlapping epochs of three observations. For each epoch the model parameters may be estimated separately. The model parameters thus obtained may then be compared across the epochs.

In the subsequent section we will study stimulus–stimulus interaction effects observed in previously published experimental studies. From these studies, short longitudinal data sets composed of three observations are available. Therefore, the time-dependency will be studied only by means of three-factor models. Moreover, the model coefficients of the AR-1 will be determined in order to determine whether or not there are stimulus–stimulus interaction effects on the model parameters. Note that the time-dependency of these interaction effects on the AR-1 parameters cannot be studied because the available data sets do not meet the minimal length requirement for doing so.

3. Examples and applications

EXAMPLE 3.1 Interaction effect of ERK regulation by insulin and EGF factor is subjected to variations in time

Borisov *et al*. (2009) studied the interplay between insulin and EGF signalling networks on the activation of ERK. Human embryonic kidney 293 cells (HEK293 cells) were exposed to either 100 nM insulin, 0.1 nM EGF, or to a combination of both stimuli and the activation of phosphorylated ERK was measured at 1.5, 5, and 15 min after stimulation. The original data are shown in Fig. 1(a) (controls were at zero; data not shown).

We re-analysed the data from this study as follows. First, all data were submitted to a three-factor ANOVA using SPSS (a standard statistical software package) to obtain a rigorously quantitative analysis. Second, means plot graphs were generated to aid the interpretation of the ANOVA results. Means plots of the data are shown in Fig. 1(b) averaged across time and in Fig. 1(c, d) for individual time slots. Clearly visible in panel B are the individual impacts of insulin and EGF across all time points. Insulin increased ERK activation irrespective of the presence or absence of EGF. Likewise, EGF increased ERK activation irrespective of insulin levels. The ANOVA test showed that these two (main) effects were statistically significant ($p < 0.01$ for insulin; $p < 0.001$ for EGF). As shown in the 1.5- and 15-min subplots of Fig. 1(c), the impact of EGF was amplified under insulin (or vice versa: the impact of insulin was magnified by the impact of EGF). In fact, when averaging across all time points (Fig. 1(b)), the ANOVA test showed that the two-way interaction between insulin and EGF was significant ($p < 0.001$). However, the interaction effect was dramatically reduced at the 5-min time point relative to the 1.5- and 15-min time points. Interestingly, at that time point ERK activation was at its maximum. Consequently, the inspection of the means plots suggests that the magnitude of the interaction effect varied across time or as a function of ERK activation. This time-dependency is highlighted by the three-dimensional graph shown in panel (d).

In fact, the ANOVA test yielded a significant three-way interaction between time, insulin and EGF ($p < 0.001$). This three-way interaction tells us that the impact of the concomitant factor 'time' changed the interaction between insulin and EGF in a statistically significant sense, which supports similar conclusions drawn earlier (Borisov *et al*., 2009). As a by-product of these considerations, we tested whether the 'strength' of the interaction was different for the 1.5- and 15-min observations using a three-factor ANOVA again but without the data from the 5-min time slot. In this case, the three-way interaction was not significant at a significance level of 0.05 indicating that the 'strength' or 'degree' of the interaction between insulin and EGF was approximately the same for the early 5- and late 15-min observation points.

Using the model-based analysis with respect to the AR-1 defined by (2.2) the model parameters μ and a_1 were determined. Note that for the control condition the parameter a_1 could not be determined because

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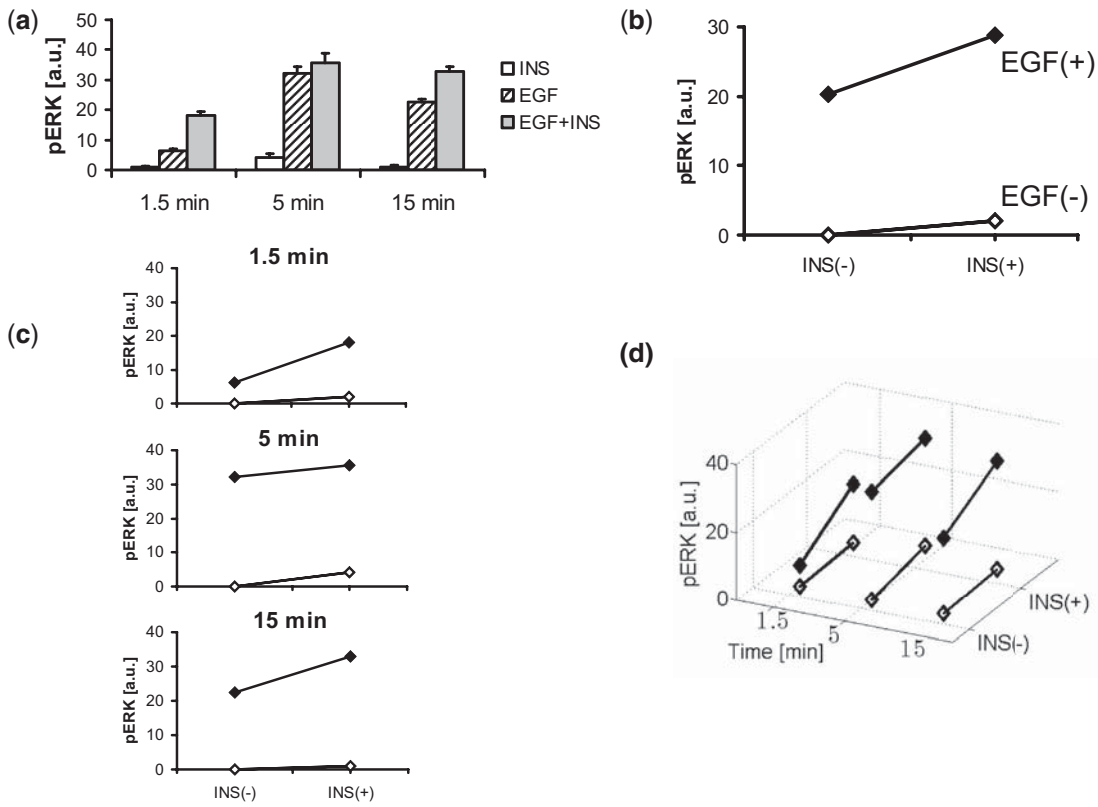

FIG. 1. ERK responses to insulin and EGF. Phosphorylated ERK measured in HEK293 cells stimulated with 100 nM insulin or 0.1 nM EGF or both ligands for 1.5, 5, or 15 min. (a) Data as reported in Borisov *et al.* (2009). Error bars represent SDs. (b) Means plot for time averaged responses. (c) Means plots for the three time points shown in panel (a). (d) Three-dimensional representation of the sub-panels shown in (c) emphasizing the three-dimensional character of a three-factor ANOVA model. Notation: 'INS'=insulin.

TABLE 1 *OLS estimates of AR-1 parameters (means and SDs) for the ERK response observed in Borisov* et al. *(2009)*

	CTR	INS	EGF	EGF + INS
μ	0 (0)	2.0 (0.7)	20.3 (1.5)	28.8 (2.1)
a_1	—	−0.80 (0.05)	−0.41 (0.01)	−0.28 (0.05)

as mentioned above all controls showed ERK responses of zero magnitude. That is, the fluctuations out of zero were negligibly small. Table 1 shows the parameter estimates. Note that the parameter μ corresponds to the averages across all time points of the ERK responses as shown in Fig. 1(b). They have been discussed in detail above. A two-way ANOVA on the parameter μ showed consistent results with the three-way ANOVA reported above. Accordingly, the main effects of insulin ($p < 0.01$) and EGF ($p < 0.001$) were significant and the two-way interaction effect between insulin and EGF was significant

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as well ($p < 0.001$). The a_1 parameter was largest in the amount for the insulin stimulation and assumed the smallest value for the combined stimulation with insulin and EGF. A single factor ANOVA with stimulation type (three levels) as factor showed that the effect of the stimulation type on the parameter a_1 was significant ($p < 0.05$). Post-hoc analysis using individual t tests showed that the differences between all conditions were statistically significant ($p < 0.05$ for insulin versus EGF, insulin versus combined stimulation and EGF versus combined stimulation). Since the parameter a_1 provides information about the stability of the underlying dynamical model, the model-based analysis suggests that overall (i.e. when averaged over all three observation times) the mechanistic system producing the ERK response showed the largest degree of stability under the combined stimulation and showed the smallest degree of stability under stimulation with insulin alone. Consequently, while the analysis of the model parameter μ was already covered by the three-factor ANOVA analysis discussed above, the analysis of the parameter a_1 yielded some new pieces of information.

EXAMPLE 3.2 Transcriptional activity of COX2 promoter is subjected to an interaction effect between hypoxia treatment and TNFα stimulation and is characterized by a time-invariant interaction effect.

In a recent study (Bruning *et al.*, 2012), the transcriptional activity of COX2 promoter in HEK293 cells was measured under the control of either hypoxia or TNFα or both stimuli. To this end, HEK293 cells transfected with a Gaussia luciferase reporter under the control of a fragment of the COX2 promoter were exposed to either 21% O_2 (normoxia) or 1% O_2 (hypoxia) with or without stimulation by 1 ng/ml TNFα, and Gaussia luciferase was measured every 3 h. We re-analysed the data set as follows. First, 1-h-rates of transcriptional activity for the time windows 3–6 h, 6–9 h, 9–12 h were computed from 3-hour data-increments. Second, all data were submitted to a three-factor ANOVA. Third, means plots for the three time windows were computed from the data to obtain a graphical interpretation of the ANOVA.

Figure 2(a) displays the transcription rates observed under the four aforementioned stimulation conditions. Figure 2(b) represents the data of panel (A) in terms of means plots for the three observation periods. In all subplots of Fig. 2(b) an interaction effect is clearly visible. In fact, the two-way interaction between the oxygen level (normoxia and hypoxia) and the TNFα stimulation was statistically significant ($p < 0.01$). However, the three-way interaction between observation periods and the interaction effect was not significant at a significance level of 0.05. In other words, although visual inspection reveals that the angle between the connecting lines varies across the three subplots of Fig. 2(b), these variations are considered as by-chance-effects.

In a subset of $n = 3$ replications, HEK293 cells were also stimulated with 0.1 ng/ml TNFα under normoxia and hypoxia (data not reported in Bruning *et al.* (2012)). The data from these weak TNFα stimulations are shown in Fig. 2(c) (as dashed lines) together with the data from Fig. 2(b). From Fig. 2(c) we see that the increase of the TNFα level from 0 to 0.1 to 1 ng/ml amplified the hypoxic impact in all three time windows. In order to quantify this observation, we submitted all data to a three-factor ANOVA (using mean substitution by subgroups for missing data). The ANOVA showed that the two-way interaction between oxygen level and TNFα was statistically significant again ($p < 0.001$). Again, the three-way interaction between time and the interaction effect was not statistically significant. However, visual inspection of Fig. 2(c) suggests that the oxygen treatment and the 0 and 0.1 ng/ml TNFα levels did not produce an essential interaction effect (almost parallel connecting lines). In contrast, for the 0.1 and 1.0 ng/ml TNFα levels the means plots suggest the presence of an interaction effect. Therefore, we tested the significance of two-way interaction effects separately using only the 0 and 0.1 ng/ml TNFα levels, on the one hand, and the 0.1 and 1.0 ng/ml TNFα levels, on the other. The interaction effect was not significant ($p > 0.05$) in the former case but it was significant ($p < 0.05$) in the latter case supporting our hypothesis that the interaction effect emerges only at higher TNFα doses and is not present at relatively

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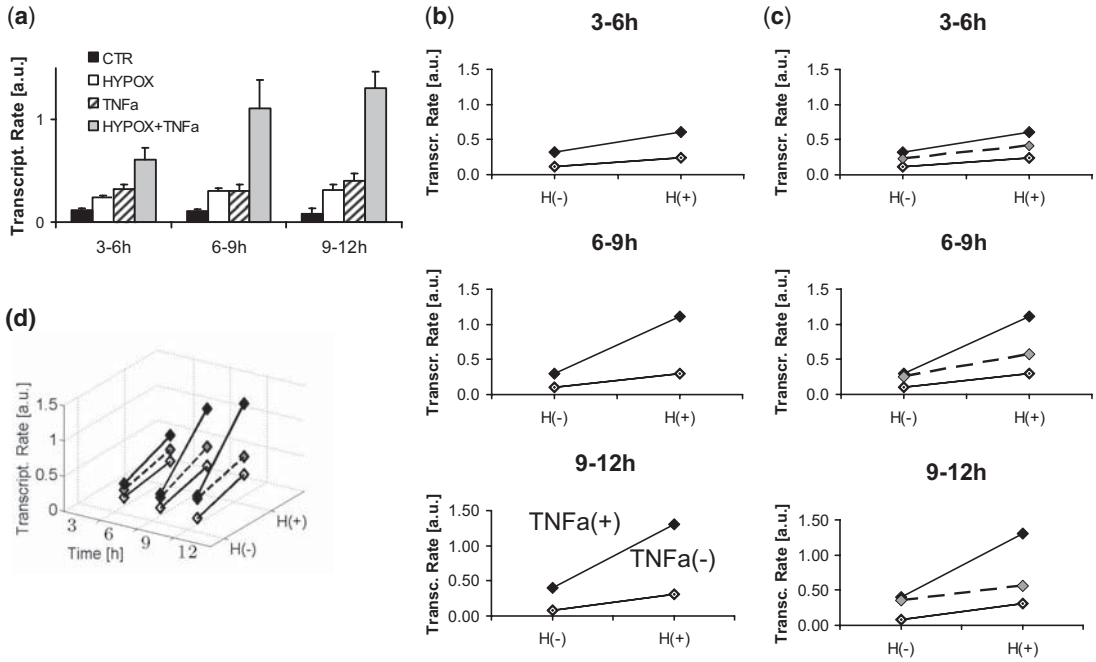

FIG. 2. Transcriptional activity of COX2 promoter induced by hypoxic cellular environment and stimulation with TNFα. (a) Transcriptional activity induced by four different stimulation conditions as observed in three consecutive epochs (error bars represent SEs). (b) Means plots representation of the data shown in panel (a). (c) Data as in A but additional data from weak TNFα stimulation trials are shown as well (dashed lines). (d) Three-dimensional graph interpreting the sub-plots of panel (c) in terms of the three-factor model. Notation: 'CTR'=Control, 'HYPOX'=hypoxia 'N'=normoxia; 'H'=hypoxia.

TABLE 2 *OLS Estimates of AR-1 parameters (means and SDs) for the transcriptional activity of the COX2 promoter reported in Bruning* et al. *(2012)*

	CTR	HYPOX	TNFa	HYPOX + TNFa
μ	0.03 (0.02)	0.08 (0.02)	0.10 (0.04)	0.30 (0.09)
a_1	−0.6 (0.4)	−0.2 (0.3)	−0.3 (0.3)	−0.4 (0.4)

low TNFα doses. Finally, Fig. 2(d) illustrates the three-dimensional character of the three-factor model applied to the data shown in Fig. 2(c) by presenting the sub-plots as a three-dimensional graph.

Table 2 present the results of the model-based analysis for the AR-1 defined by (2.2). The parameter μ captures the average across all time points of the transcriptional rates shown in Fig. 2(a). Simulation with either hypoxia treatment or with TNFα induced a stronger prompter activity as under the control condition, where the impact of the TNFα treatment was somewhat stronger. Combined stimulation induced an even stronger transcriptional activity. A two-way ANOVA on the parameter μ produced results consistent with the three-way ANOVA reported above. Both main effects were statistically significant ($p = 0.01$ for hypoxia and $p < 0.001$ for TNFα). Importantly, the two-way interaction between hypoxia treatment and TNFα stimulation was significant ($p < 0.01$). The parameter a_1 was smallest in the amount for

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the individual stimulations with either hypoxia or TNFα. In the control condition a_1 was largest in the amount. However, a two-way ANOVA on the parameter a_1 showed that only the interaction effect between hypoxia treatment and TNFα stimulation was statistically significant ($p < 0.05$). That is, the main effects of treatment with hypoxia or TNFα stimulation were not significant. Accordingly, under normoxia (i.e., in the absence of a hypoxia treatment) the TNFα stimulation decreased a_1 in the amount and made the dynamics more stable. In contrast, under hypoxia the TNFα stimulation increased a_1 in the amount such that the transcriptional machinery became less stable.

EXAMPLE 3.3 Time-sensitivity of interaction effects in memory consolidation

In two related animal studies, it has been reported that memory consolidation is affected by an interaction effect between beta-adrenoceptor agonists and antagonists acting on the alpha-1-adrenoceptor/glucocorticoid-receptor system (Ferry *et al*., 1999; Roozendaal *et al*., 2002). Rats were trained in an avoidance task involving electrical foot shocks. Memory consolidation was measured 48 h after completion of the training. To this end, retention latencies were determined, where higher latencies reflect stronger avoidance and are assumed to demonstrate a higher degree of memory consolidation. Memory consolidation was tested under several conditions. For our purposes, only a few of them will be considered explicitly. In particular, in both studies the effect of clenbuterol administered to the basolateral nucleus of the amygdala (BLA) was examined. Clenbuterol is known as a beta-adrenoceptor agonist that affects memory consolidation in a highly nonlinear fashion. At relative low doses, clenbuterol improves memory consolidation see Fig. 2, left, in Ferry *et al*. (1999) and Fig. 2(a), left, in Roozendaal *et al*. (2002). At higher concentrations, the agonist is less effective. Consequently, under baseline conditions, the dose–response to clenbuterol exhibits a peak at a relative low concentration.

In addition to studying the impact of a beta-adrenoceptor agonist clenbuterol, Ferry *et al*. (1999) and Roozendaal *et al*. (2002) examined the effects of certain antagonists acting on the alpha-1-adrenoceptor/glucocorticoid-receptor system in the postsynaptic sites of the BLA. Roozendaal *et al*. (2002) studied memory consolidation in rats when the glucocorticoid antagonist RU38486 was administered to the BLA 10 min before avoidance training started. Likewise, Ferry *et al*. (1999) examined memory consolidation when the alpha-1-adrenoceptor antagonist prazosin was delivered to the BLA at beginning of the avoidance training. In both studies, the effects of the antagonists were examined at low and high doses of the beta-adrenoceptor agonist clenbuterol. In both studies, statistically significant interactions between agonist and antagonist levels were found. Figure 3(a) and (b) schematically illustrates these interactions. At low antagonist concentrations, the aforementioned nonlinear dose–response of clenbuterol was observed. That is, retention latencies were high (reflecting good memory consolidation) at low levels of clenbuterol and were low at relatively high clenbuterol doses. However, at relative high antagonist concentrations, the peak of the dose–response to clenbuterol was shifted to higher concentrations. That is, at low clenbuterol doses retention latencies were low (memory consolidation was poor). In contrast, at higher clenbuterol doses latencies were high. In short, the antagonists altered the dose–responses to the beta-adrenoceptor agonist demonstrating that memory consolidation was subjected to an interaction effect between two stimuli.

However, there is evidence that this interaction effect depends on the time point at which the antagonist is delivered to the BLA. As mentioned above, in Ferry *et al*. (1999) and Roozendaal *et al*. (2002) the antagonists were delivered before training started (10 min earlier and immediately before start). Sandi & Rose (1994) conducted a learning experiment in which stimulation of glucocorticoid receptors significantly increased memory consolidation as tested in a retention test 24 h after training. However, this effect was only present when the stimulation was delivered within 1 h of the training period. Therefore, it

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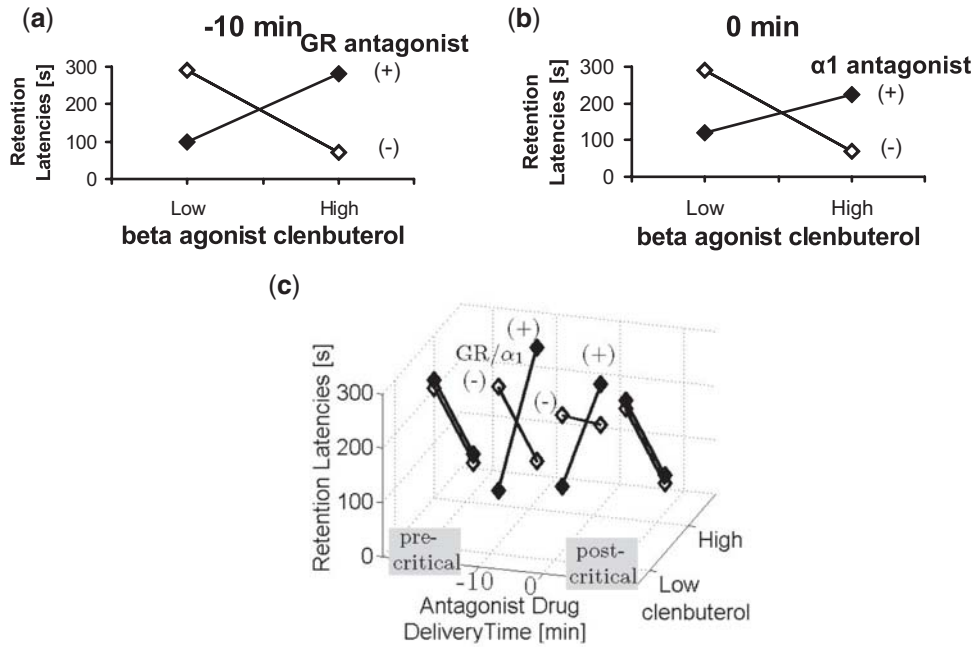

FIG. 3. Schematic presentation of retention latencies reported in animal studies by Roozendaal *et al.* (2002) (panel a) and Ferry *et al.* (1999) (panel b) involving an interaction effect. Panel (c) Hypothesized three-factor model postulating that the interaction effect depends on the antagonist drug administration time point. See text for details.

has been suggested that drug treatment via beta-adrenoceptor agonists and antagonists acting on the alpha-1-adrenoceptor/glucocorticoid-receptor system affects cognitive functioning that in turn reveals itself in variations in retention latencies (Roozendaal *et al.*, 2002). In line with this argument it is plausible to assume that there is a critical time point for the administration of the antagonist to be effective. Beyond that time point, the context between the administration of the antagonist and the avoidance training will be lost and memory consolidation will not be subjected to an interaction effect. Likewise, we speculate that when the antagonist is delivered to early, then memory consolidation will not involve an interaction effect. This argument is illustrated in Fig. 3(c). In the precritical and postcritical region, the agonist clenbuterol is assumed to induce the abovementioned baseline dose–response exhibiting a peak at low concentrations. Only in a certain time window, memory consolidation will be subjected to an interaction effect. Figure 3(c) illustrates a three-way interaction between agonist and antagonist doses and time. In view of the Examples 3.1 and 3.2, it is clear that the three-factor model would be a promising candidate model to describe such a three-way interaction quantitatively.

4. Concluding remarks

Examples 3.1 and 3.2 demonstrate that means plot analysis in combination with hypothesis testing on the basis of three-factor models is a useful tool to identify interaction effects and to determine variations of interaction effects over time and other subtleties related to the emergence of interaction effects. The time-evolution of ERK activation depends on the interplay of various regulatory signals (Borisov *et al.*, 2009).

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We demonstrated that the three-factor model can account for the temporal variations in the degree of the activation of ERK by insulin and EGF. We found a statistically significant (three-way) interaction between time and the stimulus–stimulus interaction effect, which is an important piece of information for developing a computational models of the ERK signalling network (see, e.g. Borisov *et al*. (2009)). Likewise, the ANOVA results for our second example on the transcriptional activity of the COX2 promoter may assist the computation modelling of the hypoxia-dependency of signalling pathways in HEK293 cells. In particular, according to our analysis based on the three-factor model, the hypoxic condition interacts with the TNFα-dependent pathway in a stable (i.e. time-invariant) manner, which can be regarded as a constraint that should be satisfied or explained by a computational model. However, it is important to note that in the two studies the cellular responses evolve on different time-scales. While the time-evolution of the interaction effect on ERK activation was examined on a time-scale of a few minutes, the interaction effect in the transcriptional activity of the COX2 promoter was studied on a comparatively long time-scale of several hours. That is, when we state in Section 3 that we showed the time-dependency or time-invariance of an interaction effect, then these statements are conditional to the time-scales on which the respective experimental data were recorded.

In a similar vein, in the third example (Example 3.3) the absolute time scale plays a crucial role. We argued that the time point of drug administration affects the degree to which learning and memory consolidation is regulated by an interaction effect. The three-factor model allows to address quantitatively such a time-dependency. In general, for drug treatment it is crucially important to know whether the impact of a drug delivered at a certain time point will be altered by an interaction effect involving another drug.

In Section 3, interaction effects given in terms of 'greater-than-additive' effects were considered. However, three-factor models can also be applied to analyse different types of cooperative effects, such as an inhibition of a cellular response subjected to an interaction effect. As mentioned in the introduction, Propst *et al*. (2010) found that ceftazidime interacts with gamma interferon (a cytokine) to inhibit the replication of *B. pseudomallei*. Such inhibitory interaction effects play important roles for the development of drugs for treating bacterial infections. In this context, three-factor models have the advantage that they can be used to monitor the dynamics of an inhibitory interaction effect. In doing so, variations across short-term and long-term periods in the degree of the inhibition by two putatively therapeutically relevant stimuli can be detected.

The proposed method can be generally used for quantifying time-varying interaction effects from the broader perspective of the three-factor ANOVA. That is, interaction effects in general can depend on a variety of factors. In particular, an interaction effect induced by two extra-cellular stimuli A and B may depend on the presence or absence of a third extra-cellular cue: a stimulus C. In the three-factor model the concentration levels of stimulus C tested in an experiment would be labelled by the index t. For example, for a fundamental $2 \times 2 \times 2$ experimental design transcriptional activity would be measured under all eight possible combinations for which the stimuli A, B, C would be either 'off' (no stimulation) or 'on' (fixed dose stimulation) and the indices j, k, t in the observable Y_{jkt} would assume the numbers 1 and 2. Similarly, the statistical three-factor model could be used to quantify how sensitive a particular interaction effect is to our choice of a certain cell line. For example, by conducting two separate two-factor ANOVAs we may find that an interaction effect produced by stimuli A and B might be present in a cell line X but not in a cell line Y. In this case, we may conduct a three-factor ANOVA in order to demonstrate more rigorously that it is indeed the cell line that affects the two-way interaction effect (e.g. to rule out the possibility that we cannot observe a significant interaction effect for Y because for some reasons the measurement noise is considerably higher for experiments with Y than for X). A significant three-way interaction would provide support for this hypothesis.

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Finally, let us point out the benefit of illustrating and interpreting a three-factor ANOVA by means of means plots as shown in Figs. 1(b–d), 2(b–d), and 3. Means plots help researchers to identify cellular response patterns, e.g. a main effect of a certain stimulus or an interaction effect produced by two stimuli. This becomes more obvious for more complex experimental designs that test the impacts of several extracellular stimuli for a variety of doses. For example, let us consider Fig. 2(c) again. Figure 2(c) presents the transcriptional activity measured under 18 different conditions. The interaction effect is clearly visible in all three subplots and is clearly pronounced between the two conditions with the high stimulation doses. A bar graph presenting all 18 conditions as bars lined up in a single dimension would probably not provide such a clear illustration of the data, even if we would organize the bar graph in three groups of six bars.

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