LONGITUDINAL STUDIES: REPEATED MEASUREMENTS AND TRENDS ON BIOMEDICAL DATA

Ph.D. Thesis

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GLOSSARY OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AdH	Adenohypophysis
ANOVA	Analysis of variance
BBG	Brilliant blue G
cAMP	3’5’-cyclic-adenosine-monophosphate
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
ELF	Extremely low frequency
EMF	Electromagnetic fields
GLM	General linear model
GZLM	Generalized linear model
ICD	International Classification of Diseases
KYNA	Kynurenic acid
LMM	Linear mixed model
NE	Norepinephrine
PRL	Prolactin
PRLoma	Adenohypophyseal prolactinoma
RBC	Red blood cells
REML	Restricted maximum likelihood
RM-ANOVA	Repeated measures ANOVA
RR	Relative risk
SE	Standard error

1 INTRODUCTION

Applied biostatistical analyses need mathematical research to find the best analysis model for the scientific questions, based on the specific data structure. Data exploration is fundamental for the description of distributions and to identify errors, missing data and outliers. However, beyond descriptive statistics, statistical models can highlight differences and/or relationships in the data based on more than one variable at the same time. Application of more complex analysis methods needs cautious consideration, because the focus is always on the specific scientific field, and statistics is a tool to find answers for the scientific questions. But this tool has to be used properly; thus, appropriate model selection and result interpretation are important.

Data collection over time is common in biomedical studies. Although analysis methods for longitudinal data have been available for a long time, they are often misapplied. Several data structures can be the input of the analysis and many types of models can be used, such as trend analysis, time series analysis, repeated measures models, survival analysis. In this thesis I focus on trend and repeated measures analyses.

These analysis methods often occur in biological and medical research, requiring an interdisciplinary approach today, in which the analysis has equal emphasis within the research team. Finding suitable methods for this, requires biostatistical research. To investigate appropriate risk measures and trends are important in epidemiological studies, rather than using only descriptive statistics. Paired aspects are crucial to examine in the case of repeated measurements, to differentiate within-subject and between-subject effects. Repeated measurement aspects are usually examined in time or space. Selecting the suitable covariance structure is relevant in the analysis of dimensional distances from the paired aspect in repeated measurements. Appropriate data management techniques are needed to handle extreme outliers in a dataset, in order to find the best suitable model. In the case of joint linear trajectories, proper analysis models are essential.

In my research, negative binomial and joinpoint regression methods were used to find risk estimates and segmented trends on frequencies in an *epidemiological study*. In other *neurological* and *neuroendocrine* studies, repeated measurements were analyzed by repeated measures analysis of variance (RM-ANOVA) or by marginal models. In an *environmental study*, marginal and piecewise mixed models were applied on repeated measures data. That is, in all studies, time was an important effect to analyze. In the first case, the response was frequency data, then the dependent variable was continuous in the other studies. Therefore,

it is important to deeply know the data we use for analysis, to find the best fitted model and to interpret the results precisely in detail referring to the original scientific questions.

In this thesis, six case studies of the above mentioned four areas (*epidemiological, neurological, neuroendocrine and environmental*) are presented.

1.1 Epidemiological study: Trends in suicide rates in Hungary

Suicide is an immense multifactorial problem throughout the world. Data have been reported from numerous countries (based on death registries), relating to various periods, with examinations of potential associations between suicide and aspects such as gender, age, ethnic origin, employment status, occupation, psychiatric disorders, physical characteristics, smoking, suicide methods and a history of self-harm or suicide attempts [1-5].

Värnik [5] revealed that the highest suicide rates worldwide between 1950 and 2009 were observed in Japan, Hungary and Lithuania. The overall suicide rates were reported to be highest of all in Hungary between 1965 and 1990 [5], third highest in Hungary among 17 countries between 2000 and 2004 [1], and eighth highest among 105 countries in time interval 1978-2009 according to the suicide data reported by the WHO [5]. Gender-specific analyses indicated that the observed suicide rates for males and females were highest among 47 countries in Hungary between 1980 and 1984, but then decreased to seventh and third highest, respectively, between 1995 and 1999 [2].

Suicide rates in Hungary have been investigated from many aspects in the past 50 years. Rihmer et al. [6] described the epidemiological and clinical perspectives in a narrative review relating to the period between 1961 and 2011. Studies have been published on the variation in annual suicide rates with gender and age [7], marital status, season, urban vs. rural living, regional distribution, alcohol and tobacco consumption, antidepressant prescription, unemployment rate, psychosocial factors, cultural, sociopolitical and economic features [8], and genetic and biological contributions (Finno-Ugrian suicide hypothesis) [6].

Trends and risk estimates of annual suicide rates overall or broken down by risk factors have rarely been investigated for the whole of a country [9] and not in a complex way in Hungary: only descriptive annual rates of suicide have been reported from Hungary, and publications were not found in our literature search on trends (especially segmented linear trends) and risk estimates of completed suicide by gender, age group or suicide method between 1963 and 2011.

1.2 Neurological studies: Repeated measurements in a rat model of migraine

Longitudinal analytical problems may appear in the neurological field, such as in the examination of pain in brain research, as repeated measurements are common in animal experimental studies. The analysis of within-subject effects are of importance in the case of data with repeated measurements [10]. Different substances have an effect on pain, such as the Brilliant Blue G (BBG), calcitonin gene-related peptide (CGRP) or kynurenic acid (KYNA). These effects can be investigated by multiple factors, such as during time in a behavioral test, or how these spatially spread within the brain under different circumstances.

Activation of the trigeminal system in the brain has a pivotal role in the pathomechanism of headaches [11]. BBG, as a P2X7 receptor antagonist, has proved effective in several models of pain, as have CGRP and KYNA, which show substantial effect in trigeminal nociceptive processing, thus in migraine-like headache [12, 13].

Repeated measurements in the rat had to be analyzed after electrical stimulation of the trigeminal ganglion to test the effect of BBG. Immunhistochemical parameters (the number of c-Fos immunoreactive cells and area covered by CGRP-immunoreactive fibres) were analyzed in mild and robust stimulation groups across different distances from the bregma in the brain. The immunhistochemical parameters were analyzed spatially, in consecutive sections from the bregma in the brain, and so were the nociceptive scores in time, to monitor behavior, comparing the effect of BBG to physiological saline in formalin and saline injection groups.

In another analytical study, the effects of two KYNA analogs were compared on repeated measurements of rats to examine behavior across time, and also on c-Fos immunoreactive cells spatially by different distance levels from the bregma in the brain, comparing left (contralateral) and right (ipsilateral) sides.

1.3 Neuroendocrine studies: Repeated measurements of hormone release under hypoionic conditions

Hypophyseal hormones, such as adrenocorticotrophic hormone (ACTH) and prolactin (PRL), play an important role when biological systems adapt to their environment [14]. The expressed ACTH is crucial for the functionality of the adrenal cortex and for the adaptation process of living organisms. The overexpression of this hormone, and the specific receptor functions are relevant factors in adrenal tumorigenesis [15]. PRL is a common mediator of the immune-neuroendocrine system, and its secretion can also be modified by stress [16].

To investigate the hormone secretion mechanisms of endocrine cells, is essential to understand disorders like adenohypophyseal prolactinoma (PRLoma) since this is the most

common (about 40% of) pituitary adenoma, which manifests itself in overexpression of PRL. PRLomas are more frequent in women compared to men in young adults, while this gender difference is not visible in the middle-aged population [17, 18].

The cellular hormone secretion is greatly determined by the extracellular ionic milieu, namely, the concentration of potassium and calcium. The potassium ion (K^+) plays an important role in cell exocytosis and hormone release through protein cascade activation, whose alteration can affect endocrine-related diseases [19] and cell proliferation [20]. The calcium ion (Ca^{2+}) is a messenger, which affects cellular functions [21].

Cell membrane functions and/or mechanisms had to be analyzed using appropriate repeated measures analysis methods in two studies: under hypokalaemic and hypocalcaemic conditions, as extracellular hypoionic conditions can affect endocrine disorders. In the analysis of repeated measurements, related data have to be taken into consideration [22]. In the case of extreme outliers, appropriate data management techniques need to be used to fit the analysis model well to our data [23]. Repeated measurements of ACTH and PRL hormone release were analyzed on cell cultures from rats, examining different K^+ or Ca^{2+} treatment groups in normal adenohypophysis (AdH) and PRLoma groups over time.

1.4 Environmental study: Repeated measurements in turkeys exposed to electromagnetic radiation

Spontaneous electromagnetic radiation (20-30 μT) has an effect on the natural selection of living organisms on Earth. Natural electromagnetic background radiation has been altered by technological inventions and innovations of modern civilization (extensive use of electric devices), and has resulted in increased electromagnetic fields (EMF).

There are several pieces of literature examining the potential biological effects of EMF (radiofrequency, microwave); however, very few of them deal with the issue of how extremely low frequency (ELF) radiation puts the welfare of humanity and/or the environment at any risk [24, 25]. ELF EMF are defined as frequencies between 0-300 Hz [26, 27].

Norepinephrine (NE) is a β-adrenergic catecholamine, which is a common mediator of behavior and physiological functions. Dysregulation of the NE systems can be reflected in panic disorders, post-traumatic stress disorders, special phobias, social anxiety and generalized anxiety disorders [28, 29]. NE can enhance the intracellular 3’5’-cyclic-adenosine-monophosphate (cAMP) level of the erythrocytes [30, 31]. In our environmental study, a turkey model was used to detect the differences at the level of NE-activated β-

adrenoceptor mechanisms and the biological effects of ELF EMF which represent commercially available electromagnetic devices.

Repeated measurements of cAMP levels from the blood had to be characterized by appropriate analysis models to compare ELF EMF treated group to the control over time considering inter-individual differences and intra-subject correlation. The reversibility of cell functions after ELF EMF exposures in turkeys needed to be explained by analyzing the rate of change in time [32, 33].

2 AIMS

The general objective of my research was to present known statistical methods that can be applied well in public health and biomedical studies to answer new biological questions. That is why we aimed to apply

- negative binomial and joinpoint regression methods to investigate trends and risk estimates in annual Hungarian suicide rates (*epidemiological study*)
- repeated measures ANOVA models to analyze the effects of different substances (BBG and KYNA analogs) in a rat model of migraine (*neurological studies*)
- repeated measures ANOVA and marginal models to study ACTH and PRL hormone release under hypoionic (low levels of potassium and calcium) conditions in adenohypophysis cell cultures (*neuroendocrine studies*)
- marginal and piecewise linear mixed models to examine the effects of extremely low frequency electromagnetic fields on turkeys (*environmental study*)

In connection with these studies, the following specific objectives were set:

Epidemiological study:

- Calculate risk estimates of suicide rates by gender, age group and suicide method in Hungary between 1963 and 2011
- Describe trends in annual Hungarian suicide rates overall, by gender and by age group
- Use proper reference population for the analysis of suicide rates
- Present detailed analyses applying negative binomial and joinpoint regression methods

Neurological studies:

- Analyze repeated measurements of trigeminal activation in the brain to investigate the effect of time and space considering related data in models of pain in the rat

Neuroendocrine studies:

- Investigate repeated measurements of ACTH and PRL hormone exocytosis over time at different, low extracellular K^+ and Ca^{2+} levels on normal adenohypophysis and adenohypophyseal prolactinoma cell populations using the appropriate statistical analysis methods, taking into account extreme outlier data and within-subject effect

Environmental study:

- Investigate repeated measurements in turkeys to explore the effect of ELF EMF on intracellular mechanisms to compare treated and untreated groups over time, considering inter-individual differences and intra-subject correlations
- Describe the pattern (rate of change) of the ELF EMF treated group over time
- Analyze trajectories of treated and untreated groups in the regeneration period

3 MATERIALS AND METHODS

Negative binomial and joinpoint regression analyses, commonly used risk measurements in epidemiology, and three approaches to analyze repeated measurements: RM-ANOVA, marginal and piecewise mixed models are introduced in the Materials and Methods section, as these advanced analyses were applied in this thesis. These data analysis models are available, but not all of them commonly used for biomedical data.

3.1 Regression models for counts

General linear models (GLMs) are a range of analysis methods for one continuous response and multiple linearly related explanatory variables, which could be discrete and continuous as well. Multiple linear regression, ANOVA and analysis of covariance are part of GLMs. Model residuals in these methods are assumed to be normally distributed [34]. Generalized linear models (GZLMs) are extensions of the GLMs with non-normal residual distributions. The response variable is related to the explanatory variables in the linear model through a link function, which is the simple identity function in the case of GLM and could be a variety of linear combination of the predictors in the case of GZLM (such as inverse, log, logit). GZLMs are a model family to analyze both continuous and categorical response variables. Count data is discrete numeric data. The most commonly used GZLMs for count data assume Poisson distribution for the response variable [35]. In a Poisson regression model, the log link function is applied.

3.1.1 Negative binomial regression

In the case of overdispersion – when the variance of the response exceeds its mean - negative binomial regression provides an alternative model of Poisson regression. Count data often show overdispersion, the reason for which may be the lack of some relevant explanatory variables, or dependency between observations. As the dispersion parameter in the model converges to zero, the negative binomial distribution converges to the Poisson distribution [34, 35].

3.1.2 Joinpoint regression

To detect changes in trends, joinpoint regression analysis is an appropriate method. This non-linear regression model is also known as multi-phase regression with continuity constraint, or piecewise-, segmented- or broken line regression [36, 37]. There are different techniques to analyze such piecewise data [37, 38], of which we applied the grid search

method [38] with the permutation test in an uncorrelated errors linear model to test the statistical significance of an apparent change in trend [36, 39].

3.2 Commonly used risk measurements in epidemiology

The primary measure of effect (risk) in a retrospective case-control study is the odds ratio, which shows how many times an event is more likely in the presence of a risk factor, than in the absence of it (for example: how does unemployment affect the odds of committing suicide compared to those who are employed). A similar measure to the odds ratio is the relative risk, which is commonly calculated in prospective follow-up studies. Specifically, it represents the ratio of probabilities of an event in the presence or absence of a risk factor, so it can describe, for example, how many times unemployment increases the probability (risk) of committing suicide. In the case of rare events, the odds ratio is approximately equal to the relative risk [40].

Incidence is the number of new cases of an event (i.e. disease) that develop over a certain amount of time. Incidence rate is the number of new cases of the event divided by the individual-time at risk during the observation period (i.e. 100,000 person-year at risk or 100,000 population per year). The relative risk is the ratio of incidences among exposed and unexposed individuals [40]. Incidence risk rates are estimated in Poisson regression models.

The rate ratio is the ratio of exposed and unexposed incidence rates, while the risk ratio is the ratio of incidence proportions. The rate ratio and risk ratio are often called relative risk measures [41]. In the case of rare events, the risk ratio is approximately equal to the odds ratio, hence it is approximately equal to the rate ratio [42]. ln many instances it is reasonable to assume that the rate ratio will be a good approximation to the risk ratio.

3.3 Analysis models of repeated measurements

Biological experiments often result in repeated measures data, when the outcome variable of interest is measured multiple times on the same experimental unit. Several statistical methods can be used to evaluate such data: three main approaches are GLM, marginal models (also known as population average models or covariance pattern models) and linear mixed models (LMMs, also known as linear mixed-effects models or mixed models) [22, 32, 43-45].

3.3.1 Repeated measures ANOVA

For repeated measures longitudinal data, one of the GLM models is called repeated measures analysis of variance (RM-ANOVA), where means could be compared across time

[46]. RM-ANOVA accounts for within-subject correlation and has a finite number of repeated measurements. Besides it has an assumption of sphericity, which requires equal variances for the difference of any pair of repeated measures; therefore, equal correlations among time (or space) levels for the response variable. Compound symmetry is a special case of sphericity of a covariance matrix, which could be used as the model assumption of repeated measures GLM. There are different methods to adjust degrees of freedom when compound symmetry does not hold (to correct the deviation from the sphericity), such as the Greenhouse-Geisser method, which decreases the degrees of freedom based on the extent to compound symmetry is violated, or the less conservative Huynh-Feldt correction [46, 47]. Littel and colleagues [22] prefer the Greenhouse-Geisser adjusted p-values in a RM-ANOVA instead of the multivariate tests such as Pillai's trace or Roy's greatest root. One of the multiple comparison options in RM-ANOVA is performing estimated marginal means, where a variety of p-value adjustments are available [46].

Repeated measures data can have two sources of variability: the between-subjects and the within-subjects variance [45]. The repeated measures GLM can have a between-subjects factor, which is the grouping variable for inter-individual effect (variation between subjects), and a within-subjects factor, which is represented by the repeated measurements for intra-individual effect (covariation within subjects).

3.3.2 Fixed and random effects: marginal model and general linear mixed model

In GLM and marginal models, only fixed effects are built in the model [47, 48]. An effect is fixed when the study investigates all possible levels of this effect, thus for example if a cell communication parameter is measured in 2 treatment groups, then the conclusion of this experiment is restricted only to these 2 treatments. This is true for a simple linear regression model, where the relationship is interpretable in the region of the observed values of the explanatory variable [48]. Based on sample data, fixed effects in a statistical model are used to make inferences about the population mean of the response [45]. An effect is random when its levels are randomly selected from the population of levels (usually the observed experimental unit – e.g. person, animal – is a random effect in a model). Thus, in the case of random effect, we can inference to a larger set of levels (population) than only our sample [47, 48]. Random effects are assumed to have probability distributions. Fixed and random effects are also parts of mixed model, thereby, mixed models are extensions of GLMs. The formulation of a general linear mixed model with fixed and random effects is shown in Table 1 using labeling conventions from Littell et al. [48].

Table 1 Formulation of the general linear mixed model

$$Y = X\beta + Zu + e$$

where $u \sim N(0, G)$ $e \sim N(0, R)$ $Cov[u,e] = 0$

Notations:
Y: vector of observations - response
X: fixed effects design matrix
β: vector of fixed effects parameters (coefficients)
Z: random effects design matrix
u: vector of random effects coefficients
G: covariance matrix of random effects u
e: vector of errors
R: covariance matrix of errors e

Table was constructed based on Littell et al. [48]

If random effects and the *Z* matrix is included in a model, that refers to a conditional model. In contrast, a model without a *Z* matrix, that captures covariance structure directly through the error matrix *e*, is called a marginal model. In marginal models, the response variable of interest is assumed to be normally distributed with mean $X\beta$ and variance $ZGZ'+R$ [48], thus variation is not modeled through random effects (formula in Table 1 without random effects *u*). The focus in the marginal model is the aggregate response to the population. One of the approaches available for marginal model inference is generalized estimating equations. It controls for variation among non-independent residuals (correlation between measurements taken from the same individual), but does not actually fit a random effect in the model, so variation is not investigated [45, 49].

The parameters of a general linear mixed model are β, G and R (Table 1). In a mixed model, the variability of the examined response is assumed to be partially attributable to the fixed effects and partly to the random effects [45]. Fixed effects define the expected values of the observations, and random effects define the variance and covariance of the observations [50]. When the effects in a statistical model are not significant, then in the case of fixed effects, it can be interpreted as no difference between the population means, and in the case of random effects, their variances are zero. Therefore, we can investigate whether the variance explained by the random effects in a model differs from zero. The mixed model allows the characterization of individual variation relative to the population mean [51]. Some variants of mixed models are also known as growth curve models [45], which estimates the inter-individual variability in intra-individual patterns of change over time [32], or, in other words, it estimates between-subject differences in within-subject change [52].

3.3.3 Data structures

There are two data structures for repeated measurements used in statistical software, the wide (multivariate mode) and the long (univariate mode, stacked data) structures. In the wide data structure, the multiple dependent variables of repeated measurements are in separate columns (one row per experimental unit) [22]. This is why if an individual is missing at any of the time points, that observation is dropped completely from the analysis [45]. There are different imputation techniques to complete missing values based on the pattern, but these should be used with caution [53]. However, missing values could be easily handled with the long data structure, because there is one row per experimental unit at each time, thus one subject is in multiple rows [22]. Because of this, if one or two measurements are missing from a subject's repeated measures, the others are still included in the analysis, so there is no need to delete the whole subject. Data in marginal and mixed models are often structured in a long format, while both the long and the wide formats are used for RM-ANOVA.

3.3.4 Estimation

Opposed to the GLM, which applies ordinary least squares [43], both marginal and mixed models are based on maximum likelihood or restricted maximum likelihood (REML) estimation. The REML sequentially (not simultaneously) estimates the fixed effects and then the variance components [32]. Mixed model gives unbiased estimates of model parameters assuming missing values are missing at random [46, 54]. RM-ANOVA cannot provide information about individual growth, while it can be described in the case of mixed model. The effect of time is treated as a classification variable in an RM-ANOVA model, while it could be used as a continuous variable in a marginal model.

The error (residual) sum of squares in a model can describe the amount of variation which is not explained by the model. This measure in the case of a simple ANOVA, describes the variance between individuals within a particular group (variation within a group), while in the case of RM-ANOVA, this explains the difference of variation within the individuals and between the groups [10]. A fixed-effects design (GLM) can be fitted in a mixed model, the results of which are the same as in GLM in the case of balanced data. Nevertheless, when we have random effects in a model, GLM compared to the LMM often provide the standard errors (SEs) biased from the parameter estimation. The LMM provides more accurate estimates, as errors can be modeled [47].

3.3.5 Covariance structures

To get valid inferences from a model, the suitable variance structure of the data needs to be used. Responses measured on the same individual are not independent, they are correlated [47]. Variances of repeated measures often change with time. The patterns of correlation and variation produce a covariance structure of repeated measures. The covariance structure refers to basically two aspects of the correlation: variances at individual times and correlation between measures at different times on the same individual [22]. Covariance structures reasonably used for repeated measurements are variance components, compound symmetry, Huynh-Feldt, first order autoregressive, toeplitz or unstructured.

In the variance components structure, the repeated observations on the same subject are uncorrelated and have equal variance at all time points. In a compound symmetry structure, repeated measures have the same variance at all time points and all pairs of measures on the same individual are correlated equally (constant, homogeneous), thus the only aspect of the covariance between repeated measures is due to individual contribution [22, 50, 55]. The Huynh-Feldt structure is more flexible than compound symmetry, it fits a few more parameters. In the first order autoregressive structure, the correlation between the repeated measures may be proportional to the distance between the repeated measures, so the farther in time we are, the lower the correlation is between the two time point measurements [47]. The toeplitz model is similar to the first order autoregressive model as neighboring pairs of within-subjects errors have the same correlation [46, 48]. The unstructured covariance structure makes no assumptions regarding equal variances or correlations, it is fully parametrized. Nevertheless, it requires estimation of a large number of variance and covariance parameters, which can lead to computational problems [22, 56]. Besides these, the heterogeneous versions of these structures are also meaningful, where the variances of the repeated measures may not be constrained to be equal [47].

3.3.6 Mixed models

Usually, the simplest mixed model, the random intercept (also known as the unconditional means) model is a good start to fit to the data, which controls for the variation between individuals. This partitions the total outcome variation into two parts: between and within individuals. In order to know how much variation is contributed to each random effect, the analysis can be started with the empty random intercept model, then the effects can be added into the model one-by-one. This starting model in the mixed models framework can be expanded with the time random effect to get the random slope (also known as

unconditional growth) model, which is a baseline model for change over time. Thereafter one or more predictors of growth can be included in the model [32].

After we have determined the grand mean of outcome across individuals and occasions, and the initial status and rate of change in total and by groups, these models can be modified in a variety of directions such as re-centering the within-subjects effect (time) or using polynomial or exponential functions. However, complications arise by involving these types of trajectories, because of the nonlinear nature of these parameters, which makes model estimation and also the interpretation more difficult. A flexible alternative is to apply a piecewise LMM, in which two or more linear trajectories are joined together to correspond to a nonlinear function [32, 52].

3.3.7 Model comparison

When more than one model can be applied, we have to choose from them. Nested models can be compared using the likelihood ratio test with the -2 log likelihood statistic based on the differences between model deviance [47, 52]. There are distinct goodness-of-fit statistics for not nested models, such as Akaike's information criterion and Bayesian information criterion which can be used to compare models to each other objectively. The smaller the value is, the better the fit of the model is [32, 46]. Akaike's information criterion and Bayesian information criterion are adjusted versions of log likelihood with a penalty based on the number of parameters estimated [22].

3.4 Specific study settings and analyses

3.4.1 Epidemiological study: Trends in suicide rates in Hungary

3.4.1.1 Suicide and population data

The annual Hungarian suicide data were collected from tables published in the Demographic Yearbooks issued by the Hungarian Central Statistical Office between 1963 and 2011. The frequencies of suicides by gender (male or female), for four age groups (7-14, 15-39, 40-59 and 60- years) and eight suicide methods (“Poison”, “Gas”, “Hanging”, “Drowning”, “Fire-arms and explosives”, “Cutting and stabbing instruments”, “Jumping from a high place” and “Non-specified”) were reported during the investigated period. The classification of suicide methods was based on the International Classification of Diseases (ICD; Tenth Revision: X60-X84, Y87.0 in 2010) [57]. Suicide codes of intentional self-harm

were categorized by using the 7^{th} – 10^{th} revisions of the ICD during the examined 49 years [57-59].

Although the ICD coding underwent change, data on all the above suicide methods were published during the study period. The category "Run-over" was defined only in the first six years, and was then aggregated into "Non-specified" cases from 1969. The ICD-10 codes [57, 59] were applied to classify suicide methods as: "Poison", "Gas" (X60-X69), "Hanging" (X70), "Drowning" (X71), "Fire-arms and explosives" (X72-X75), "Cutting and stabbing instruments" (X78), "Jumping from a high place" (X80) and "Non-specified" (X76-X77, X79, X81-X84, Y87.0).

The Hungarian population data were published for age in five-year intervals (0-4 years, 5-9 years, 10-14 years, etc.) and for gender for each year in the Demographic Yearbooks of the Hungarian Central Statistical Office between 1963 and 2011. The live birth data on the 5-9-year age group were used to determine the 7-9-year population, and the counts for the 7-9 and 10-14-year groups were summed to obtain the data on the 7-14-year group. Overall, therefore, the suicide and the population dataset (>6 years) were considered from the aspect of six variables: year, gender, age group, suicide method, suicide frequency and population frequency.

3.4.1.2 Statistical analysis

When events occur over time, it is usually more relevant to model the rate at which they occur than the number of the events [35]. The average annual Hungarian suicide rates per 100,000 population (>6 years) were calculated overall, by gender, age group and suicide method (ranges are given as minimum and maximum for annual suicide rates). Trends between 1963 and 2011 for suicide rates overall and in stratified analyses by factors such as gender (reference group: female), age group and suicide method, were investigated by using negative binomial regression models [35]. The negative binomial regression model for total annual data contained only one independent variable year, all other negative binomial regression models included explanatory variables year and gender.

The following effects were analyzed in the statistical model overall and by gender:

$$SuicideRate \sim Year$$

while the next model was run in each strata of age group (4 levels) and suicide method (8 levels):

$$SuicideRate \sim Year + Gender$$

where

SuicideRate: response variable of crude suicide rates calculated from suicide frequencies relative to population frequencies (> 6 years) multiplied by 100,000

Year: effect of year in the period 1963-2011 (49 years)
Gender: effect of gender (2 levels: male relative to female)

The relative risk (RR) for factor gender (male relative to female) and its 95% confidence interval (CI) with p-value were calculated. Trends were also described by using joinpoint regression analyses, determining turning points for the examined 49-year period [36].

Crude rates were expressed in joinpoint regression analyses as the number of suicide cases per 100,000 population at risk, with the year as an independent factor. The above presented first formula (*SuicideRate ~ Year*) was applied in the joinpoint regression models, overall, and for each strata of gender and age groups.

The assumption in the regression model was that the random errors followed Poisson distribution and the regression coefficients were estimated by weighted least squares to handle heteroscedasticity. Further, multiple tests were performed to select the number of joinpoints, using the Bonferroni correction for multiple testing. Monte Carlo sample of possible permutations of the data were applied, where the number of randomly permuted data sets for permutation tests was 4499 [36, 39]. CIs were calculated for the estimated joinpoints according to Lerman [38]. Annual changes in percentages were calculated for the fitted linear segments first getting the ratio of linearly fitted suicide rates at the end to the fitted suicide rates at the beginning of the segment then raised to the power of reciprocal of segment length in years, finally subtract one from this power and multiplied by 100.

We presented negative binomial regression models which had an acceptable goodness-of-fit chi-square test result. The type I error was reduced by using the Bonferroni correction for the p-values, multiplying them with the number of significance tests run in all negative binomial regression models and the number of models run separately in each strata in joinpoint regression models: total cases, by gender or by age groups [60]. When a p-value was greater than 1 after the multiplication in Bonferroni correction, it was reduced to 1.00.

3.4.2 Neurological studies: Repeated measurements in a rat model of migraine

3.4.2.1 Data analysis: effect of BBG administration

The experimental layout and details of data collection from the BBG administration neurological study is presented in Figure 1.

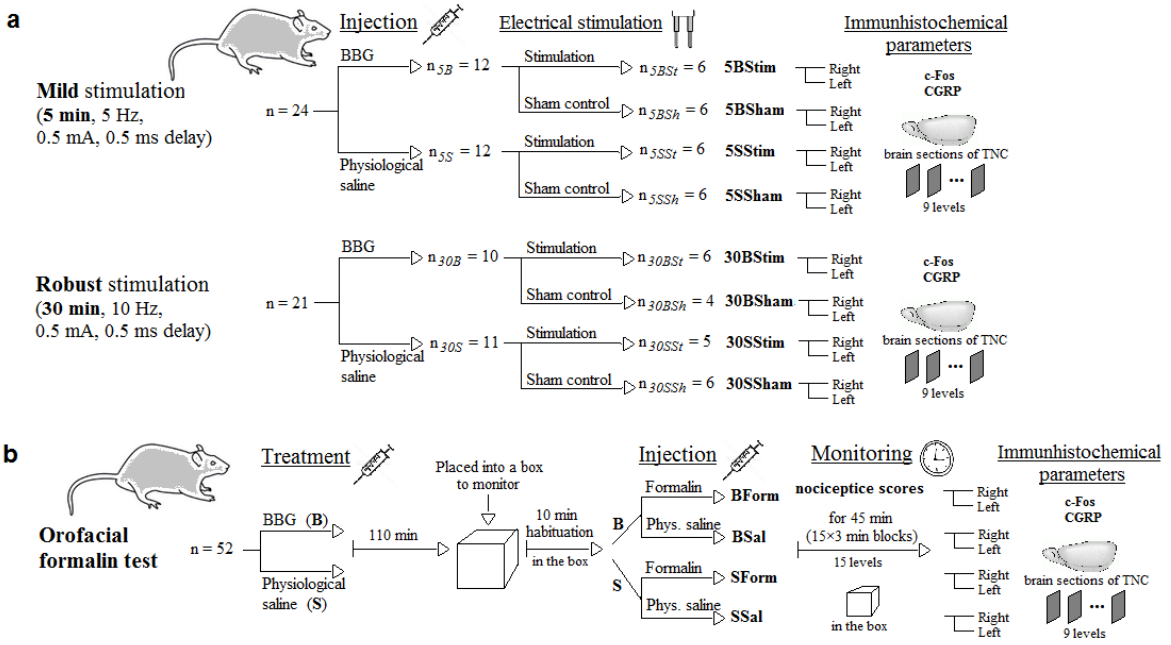


Figure 1 Experimental layout of the BBG administration neurological study. Immunhistochemical parameters (c-Fos, CGRP) were analyzed spatially as repeated measurements (9 levels) from the rat separately in mild and robust stimulation comparing the effect of BBG to physiological saline between stimulated and sham control animals (a). Nociceptive scores were analyzed over time as repeated measurements (15 levels) comparing formalin and saline injections between BBG and saline treatment groups from the orofacial formalin test (b). Repeated measurements of brain sections (9 levels) were investigated for c-Fos and CGRP response variables from the orofacial formalin test.
Notations: BBG: Brilliant blue G, c-Fos: number of c-Fos immunoreactive cells, CGRP: area covered by calcitonin gene-related peptide immunoreactive fibres, TNC: Trigeminal nucleus, nociceptive scores: time spent rubbing the injected (right) whisker pad measured in seconds

Number of c-Fos immunoreactive cells from different levels of the trigeminal nucleus, and separately the sums of the areas covered by CGRP-immunoreactive fibres were analyzed by two-way RM-ANOVA models. A stimulation group with 8 levels (BBG or saline treatments, for electrically stimulated or sham controls, for control and stimulated sides: BStim, BSham, SStim and SSham in both sides of the rat) was used as the between-subject factor (separately for mild – 5 Hz, 5 minutes – and robust – 10 Hz, 30 minutes – electrical stimulation) and brain sections (distances from the bregma) with 9 levels (from -13.89 mm to -18.21 mm in 0.54 mm increments) as the within-subject factor for the analyses. For the mild and robust stimulation groups the following models were run separately in the analysis:

$$ImmunhistochemicalParameter \sim Group + Section + Group \times Section$$

where

ImmunhistochemicalParameter: c-Fos or CGRP response variables
Group: merged grouping variable (between-subject factor) with 8 levels (effect of injection, electrical stimulation and side of the rat)
Section: within-subject factor of brain sections from the bregma with 9 levels
Group×*Section*: Interaction term

Nociceptive scores (time spent rubbing the injected (right) whisker pad measured in seconds) from the behavioral study were compared between 4 treatment groups (BBG or saline treatments before saline or formalin injections: BSal, SSal, BForm, SForm) during time (15 time blocks) by two-way RM-ANOVA. The following formula describes the variables used in the analysis model:

$$NociceptiveScore \sim Group + Time + Group \times Time$$

where

NociceptiveScore: response variable of time the rat spent rubbing the injected (right) whisker pad
Group: merged grouping variable (between-subject factor) with 4 levels (effect of treatment and injection)
Time: within-subject factor with 15 levels (3 minute blocks)
Group×*Time*: Interaction term

The immunhistochemical parameters (the number of c-Fos immunoreactive cells and the area covered by CGRP-immunoreactive fibres) of the orofacial formalin test were analyzed as dependent variables over time by two-way RM-ANOVA. The next formula explains the variables used in the analysis model:

$$ImmunhistochemicalParameter \sim Group + Section + Group \times Section$$

where

ImmunhistochemicalParameter: c-Fos or CGRP response variables
Group: merged grouping variable (between-subject factor) with 8 levels (effect of treatment, injection and side of the rat)
Section: within-subject factor of brain sections from the bregma with 9 levels
Group×*Section*: Interaction term

3.4.2.2 Comparison of KYNA analogs

The experimental layout and details of data collection from the KYNA analogs neurological study is presented in Figure 2. To compare the means of jaw rubbing counts from the orofacial formalin tests in the different treatment groups on rats during time (n = 13-15 animals in each group), two-way RM-ANOVA was run. Treatment with 6 groups (saline or formalin injections to two KYNA analog pretreatments compared to saline control) was used as between-subject factor and time with 15 time periods (3 minute long time blocks) as within-subject factor for the analysis.

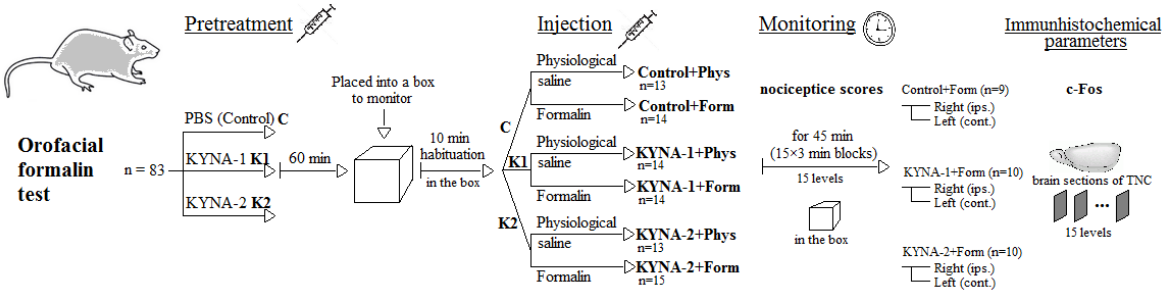


Figure 2 Experimental layout of the KYNA analogs neurological study. Nociceptive scores were analyzed over time as repeated measurements (15 levels of 3 minute long time blocks) comparing two KYNA analog groups to PBS control in formalin and saline injection subgroups from the orofacial formalin test in the rat. The number of c-Fos immunoreactive cells was analyzed spatially as repeated measurements (15 levels) comparing the effect of KYNA analogs to PBS control, taking into account right (ipsilateral) and left (contralateral) sides of the formalin injected rats.
Notations: PBS: phosphate-buffered saline, KYNA-1: kynurenic acid analog 1, KYNA-2: kynurenic acid analog 2, nociceptive scores: time spent rubbing the injected (right) fore- or hindpaw measured in seconds, ips: ipsilateral (right) side, cont: contralateral (left) side, c-Fos: number of c-Fos immunoreactive cells, TNC: trigeminal nucleus

The following formula explains the used variables in the analysis model:

$$NociceptiveScore \sim Group + Time + Group \times Time$$

where

NociceptiveScore: response variable of time the rat spent rubbing the injected (right) fore- or hindpaw
Group: merged grouping variable (between-subject factor) with 6 levels (effect of pretreatment and injection)
Time: within-subject factor with 15 levels (3 minute blocks)
Group×*Time*: Interaction term

The number of c-Fos immunoreactive cells from 15 adjacent measuring sites of the trigeminal nucleus were analyzed by three-way RM-ANOVA among sides (contralateral and ipsilateral) between pretreatment groups (control and two KYNA analogs) after formalin injection. Measuring sites and sides were used as within-subject factors, while pretreatment was the between-subject factor in the GLM.

$$cFos \sim Pretreatment + Section + Side + Pretreatment \times Section + Pretreatment \times Side + Section \times Side + Pretreatment \times Section \times Side$$

where

c-fos: number of c-Fos immunoreactive cells as response variable
Pretreatment: grouping variable of pretreatment (between-subject factor) with 3 levels (Control, KYNA-1, KYNA-2)
Section: within-subject factor of brain sections from the bregma with 15 levels
Side: within-subject factor of the right (ipsilateral) and left (contralateral) sides of the rat
Pretreatment×*Section, Pretreatment*×*Side, Section*×*Side*: 2-way interactions
Pretreatment×*Section*×*Side*: 3-way interaction

For both neurological studies, when Mauchly's test of sphericity [61] was significant, the Greenhouse-Geisser correction was performed in the RM-ANOVA models. Pairwise comparisons of group means were performed on the basis of estimated marginal means with the Sidak adjustment.

3.4.3 Neuroendocrine studies: Repeated measurements of hormone release under hypoionic conditions

3.4.3.1 Effect of hypokalaemia

The experimental setup is introduced in Figure 3.

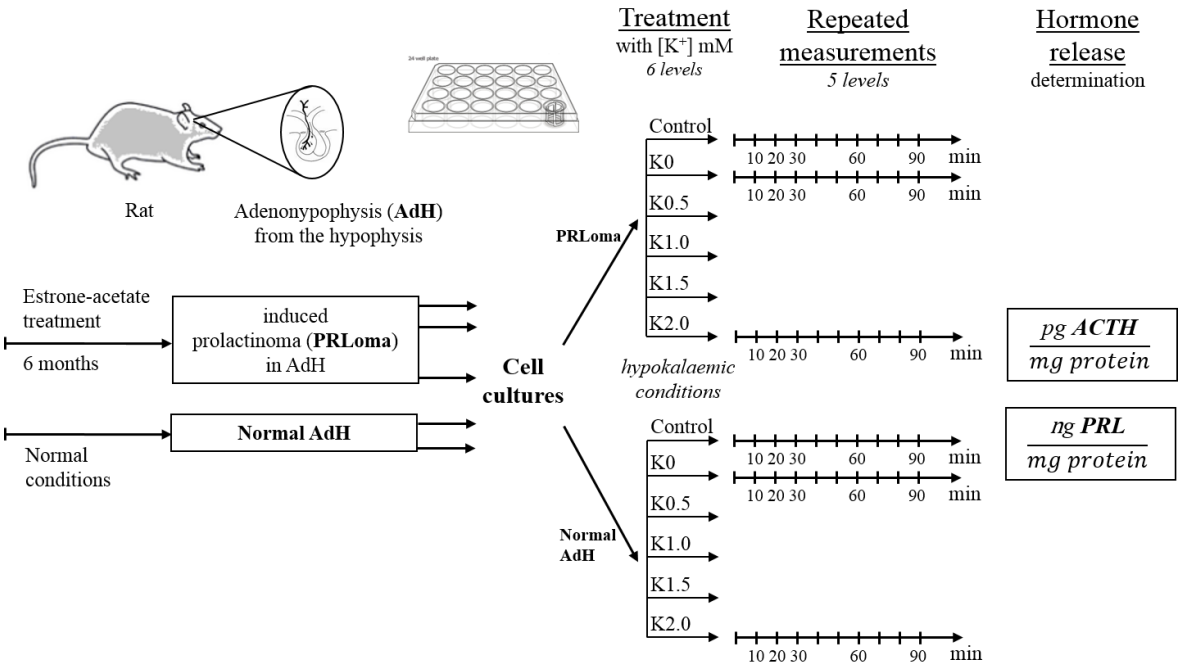


Figure 3 Experimental setup of the hypokalaemic neuroendocrine study. Adenohypophysis cell cultures were prepared from the hypophysis of rats in PRLoma and normal cell populations. ACTH and PRL hormone release were measured at different extracellular K^+ levels (6 concentration groups: control and 0, 0.5, 1.0, 1.5, 2.0 mM [K^+]) over time (5 time points: 10, 20, 30, 60 and 90 minutes after potassium treatment).
Notations: AdH: adenohypophysis, PRLoma: adenohypophyseal prolactinoma, [K^+]: potassium ion concentration, ACTH: adrenocorticotropic hormone, PRL: prolactin.

To compare the means of hormone release in adenohypophysis cell cultures between different concentrations of potassium ion treatment and control groups across time, two-way RM-ANOVA was used for each independent set of data: ACTH secretion of normal AdH or PRLoma, or PRL hormone secretion of normal AdH or PRLoma. Treatment with 6 levels ([K^+]: 0; 0.5; 1.0; 1.5; 2.0 mM and a control group; n=10 in each group) was considered as a between-subject factor and time with 5 time points (10, 20, 30, 60, 90 minutes) as a within-subject factor for the analysis. ACTH and PRL secretion of untreated AdH was compared with PRLoma group in hypokalaemia over time using RM-ANOVA.

The following effects were analyzed in the statistical model:

$$HormoneRelease \sim Treatment + Time + Treatment \times Time$$

where

HormoneRelease: response variable of determined hormone release measures from cell cultures of rats (separately for ACTH in normal AdH and PRLoma or PRL in normal AdH and PRLoma)
Treatment: Potassium ion concentration groups (6 levels) or separately 2 levels of normal AdH control and PRLoma K0 (in models for ACTH and PRL one by one)
Time: within-subject factor with 5 levels (5 time points)
Treatment×*Time*: Interaction term

When Mauchly's test of sphericity [61] was significant, the Greenhouse-Geisser correction was performed. Estimated marginal means were calculated with Sidak adjustment for multiple comparisons.

3.4.3.2 Effect of hypocalcaemia

Repeated measurements of ACTH and PRL hormone release in different treatment groups ($[Ca^{2+}]$: 0; 0.5; 1.0; 1.5 mM and a control group; n=12 in each group) on cell cultures in an 80 minute period (time points at 10, 20, 30, 60 and 90 minutes) were compared using marginal models [33, 45]. Disease with 2 groups (PRLoma and normal AdH) and treatment with 5 groups (calcium concentration levels) were used as between-subject fixed factors and time with 5 time points as within-subject fixed factor for the analysis. The reference group was the normal (healthy) control (no treatment) group at minute 10 (start) in the model. REML estimation and Kenward-Roger method for adjusting the degrees of freedom were applied.

The experimental setup is introduced in Figure 4.

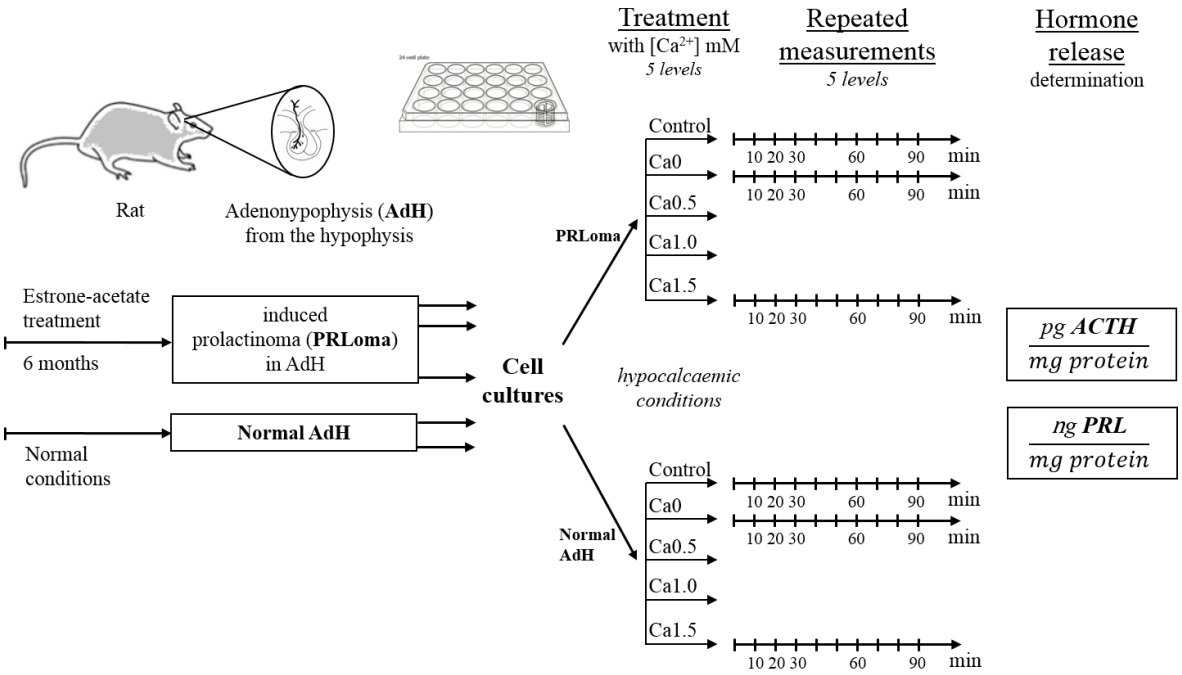


Figure 4 Experimental setup of the hypocalcaemic neuroendocrine study. Adenohypophysis cell cultures were prepared from the hypothalamus of rats in PRLoma and normal populations. ACTH and PRL hormone release were measured at different extracellular Ca^{2+} levels (5 concentration groups: control and 0, 0.5, 1.0, 1.5 mM [Ca^{2+}]) over time (5 time points: 10, 20, 30, 60 and 90 minutes after calcium treatment).
Notations: AdH: adenohypophysis, PRLoma: adenohypophyseal prolactinoma, [Ca^{2+}]: calcium ion concentration, ACTH: adrenocorticotropic hormone, PRL: prolactin.

The following effects were investigated in the applied marginal model:

$$HormoneRelease \sim Disease + Treatment + Time + Disease \times Treatment + Disease \times Time + Treatment \times Time + Disease \times Treatment \times Time$$

where

HormoneRelease: response variable of determined hormone release measures from cell cultures of rats (separately for ACTH or PRL)
Disease: between-subject effect with 2 levels (normal AdH and PRLoma)
Treatment: calcium ion concentration groups (5 levels)
Time: within-subject factor with 5 levels (5 time points)
Disease×*Treatment, Disease*×*Time, Treatment*×*Time*: 2-way interactions
Disease×*Treatment*×*Time*: 3-way interaction

In the case of ACTH, unstructured covariance matrix, for PRL data, the heterogeneous first order autoregressive covariance matrix resulted the best fit among different structures (variance components, compound symmetry, first order autoregressive, toeplitz, unstructured and their heterogeneous versions), based on Akaike's information criterion statistic [50]. Pairwise comparisons were estimated by least squares means using Sidak p-

value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions.

In case of extreme values, winsorization technique was applied by shifting the strongly outlying data toward the center to protect parameter estimation against the emergence of unexpectedly large errors [23, 62].

3.4.4 Environmental study: Repeated measurements in turkeys exposed to electromagnetic radiation

3.4.4.1 Experiment

The experiment was 9 weeks long: starting with a 1-week long adaptation period, followed by a 3-weeks long ELF-EMF treatment period and a 5-weeks long regeneration period at the end. The turkeys were exposed to intermittent (8 ms energy exposure – 2 ms energy free pause) ELF EMF treatment (50 Hz, 10 μT) for 20 minutes every 8 hours for the 3 weeks in the treatment period. NE-activated cAMP levels were determined from all blood samples taken weekly from the turkeys. All measurements performed 4-6 technical parallels.

3.4.4.2 Statistical analysis

Means were calculated from technical replicates for each subject (experimental unit: turkey), and used for the analyses of cAMP levels (as markers of β-adrenoceptor function). Descriptive statistics were calculated to identify distribution of cAMP levels by treatment groups and time points.

To estimate means in ELF EMF treated and untreated groups over time in the whole experimental period (weeks 1-9) considering between-subject differences and within-subject correlation, marginal model was applied [32, 45-47, 49, 50]. Differences of least squares means are calculated according to Sidak adjustment.

The following effects were investigated in the applied marginal model:

$$cAMP \sim Treatment + Time + Treatment \times Time$$

where

cAMP: response variable of NE-activated cAMP levels (nmol/ml RBC suspension)
Treatment: between-subject effect of ELF EMF treatment groups (2 levels: treated and untreated control)
Time: within-subject (repeated) effect with 9 levels (9 weeks)
Treatment×*Time*: interaction term

To characterize the reversible nature of NE-activated β-adrenoceptor function after ELF EMF treatment by the rate of change in time, piecewise LMM was used, which could

describe the linear trajectories of cAMP levels in the treatment and in the regeneration periods [32, 51, 52]. An intraclass correlation coefficient was calculated to describe variation [63].

The next effects were investigated in the applied piecewise linear mixed model:

$$cAMP \sim Treatment + TimeCentered + Treatment \times TimeCentered + Treatment \times TimeCentered \times Period$$

where

cAMP: response variable of NE-activated cAMP levels (nmol/ml RBC suspension)
Treatment: between-subject effect of ELF EMF treatment groups (2 levels: treated and untreated control)
TimeCentered: random effect of time (reference point was shifted into the center of the experiment, to week 4, when the treatment period ended)
Treatment×*TimeCentered*: interaction term of treatment and time
Treatment×*TimeCentered*×*Period*: 3-way interaction (Period is a binary variable with the value 0 in weeks 1-3, and value 1 in weeks 4-9. This variable separates the treatment period from the regeneration period.)

In marginal and mixed models, the REML estimation method was used with unstructured covariance structure. Kenward-Roger method was applied for adjusting degrees of freedom. Model residuals were checked for normality.

In all studies, tests were two-sided, and significance level was set at α=0.05. All negative binomial regression analyses were performed by using the statistical software package R (Version 3.0.2. R Foundation for Statistical Computing, Vienna, Austria). Joinpoint regressions were carried out with the freely available Joinpoint Regression Program (Version 4.0.4. Surveillance Research Program, National Cancer Institute, Bethesda, MD, USA). RM-ANOVA models were carried out using IBM SPSS Statistics (Version 21 IBM Corporation, Armonk, NY, USA) software. Marginal and mixed models were performed using SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA) [46].

4 RESULTS

Analyses results from the four investigated study areas are presented in the following subchapters.

4.1 Epidemiological study: Trends in suicide rates in Hungary

4.1.1 Suicide rates

Overall, 178,323 suicides were committed in Hungary during the period 1963-2011 (28.2% females and 71.8% males; Table 2). The average suicide rates per 100,000 population (>6 years) varied between 26.03 and 51.31 and the overall average suicide rate was 38.44 in the population older than 6 years. The average suicide rate in males was 57.75 (range: 41.93-75.19) and in females it was 20.78 (range: 11.11-32.12). The suicide rate was higher in males than in females overall, in each age group and in each suicide method. The average suicide rates increased with age (average suicide rates (range): 7-14 years: 1.13 (0.23-3.11), 15-39 years: 25.15 (13.17-35.81), 40-59 years: 52.56 (38.58-70.62), and 60- years: 67.09 (37.14-96.28)). Table 2 shows the distribution of the Hungarian suicide frequencies overall and in the different subgroups (broken down by gender, age group and suicide method).

The most frequent suicide methods in the investigated period were “Hanging” (56.9%) and “Poison” (23.7%), the others remaining under 5% (Table 2). The average suicide rates per 100,000 population (>6 years) for the various suicide methods during the 49 years were as follows: “Hanging”: 21.87, “Poison”: 9.11; “Jumping from a high place”: 1.83, “Non-specified”: 1.80, “Drowning”: 1.41, “Fire-arms and explosives”: 0.91, “Gas”: 0.80, “Cutting and stabbing instruments”: 0.72.

Table 2 Suicide frequencies, average suicide rates (SR) per 100,000 population (>6 years) and relative risk (95% confidence interval) of trends in suicide rates using negative binomial regression are summarized overall and by gender for various age groups and suicide methods in Hungary during the period 1963-2011.

		Descriptive statistics		Negative binomial regression	
Groups (N = no. of suicide cases)	**Gender**	**N** (1963-2011)	**Average SR** (per 100,000)	**Relative risk**[b] (95% confidence interval)	**p-value**[a]
Total annual data	-	178,323	38.44	0.9919 (0.9840 - 0.9997)[b]	< 0.05
Total annual data by gender	**Male**	128,058	57.75	2.8348 (2.6020 - 3.0884)	< 0.05
	Female	50,265	20.78		
Age groups					
7 – 14 (N = 639)	**Male**	502	1.73	3.4809 (2.2187 - 5.4613)	< 0.05
	Female	137	0.50		
15 – 39 (N = 46,181)	**Male**	36,559	39.43	3.8448 (3.1627 - 4.6740)	< 0.05
	Female	9,622	10.56		
40 – 59 (N = 68,589)	**Male**	51,532	82.70	3.3576 (2.8548 - 3.9488)	< 0.05
	Female	17,057	25.07		
60 - (N = 62,914)	**Male**	39,465	102.86	2.4899 (2.0527 - 3.0201)	< 0.05
	Female	23,449	42.61		
Suicide methods					
Poison (N = 42,338)	**Male**	21,698	9.75	1.1364 (0.8424 - 1.5330)	1.00
	Female	20,640	8.52		
Gas (N = 3,666)	**Male**	1,880	0.85	1.8509 (1.1357 - 3.0166)	< 0.05
	Female	1,786	0.75		
Hanging (N = 101,460)	**Male**	83,476	37.65	5.1135 (4.4196 - 5.9162)	< 0.05
	Female	17,984	7.44		
Drowning (N = 6,518)	**Male**	3,187	1.44	1.0535 (0.8757 - 1.2673)	1.00
	Female	3,331	1.38		
Fire-arms and explosives (N = 4,193)	**Male**	4,057	1.84	32.6679 (22.5979 - 47.2252)	< 0.05
	Female	136	0.06		
Cutting and stabbing instruments (N = 3,320)	**Male**	2,623	1.19	4.1136 (3.3926 - 4.9877)	< 0.05
	Female	697	0.29		
Jumping from a high place (N = 8,486)	**Male**	4,800	2.16	1.4029 (1.1114 - 1.7710)	< 0.05
	Female	3,686	1.52		
Non-specified (N = 8,342)	**Male**	6,337	2.87	3.4605 (3.0501 - 3.9262)	< 0.05
	Female	2,005	0.83		

[a]Bonferroni-corrected p-values are given

[b]Relative risk for the factor year is presented for the total annual data. In the other models (with explanatory variables year and gender), Table 2 focuses only on the factor gender (reference group: female) and relative risks for the year are not shown.

4.1.2 Regression models

RRs of gender difference were calculated to estimate the risk of committing suicide overall, by age groups and suicide methods during the 49 years (Table 2). Overall risk of suicide was nearly threefold higher among males than females, which was varied across age groups. Similarly, significantly higher risks were detected in the male groups as compared with the females in most suicide methods (more than twentyfold risk was the highest in the "Fire-arms and explosives" subgroup), except "Poison" and "Drowning" subgroups (Table 2).

The negative binomial regression model for total annual data revealed a decreasing annual trend in the suicide rates during the study period, which remained significant after the Bonferroni p-value correction (Table 2). The annual suicide rates data displayed segmented lines rather than a decreasing linear trend. Thus, the joinpoint regression analyses were applied to refine the annual trends. Overall, the joinpoint regression model fitted a segmented line indicating two main changes in the trend: a peak in 1982, followed by a significant decrease until 2006, and a basically constant period thereafter. From 1963 to 1982, there was a significantly increasing linear trend in the suicide data, and then a significant decline between 1982 and 2006, followed by a rather constant level from 2006 to 2011 (annual changes in percentages, slopes with p-values of each segment: 2.9%, 1.15 ($p<0.001$); -2.8%, -1.09 ($p<0.001$) and 0.2%, 0.06 ($p=0.84$) respectively; Figure 5).

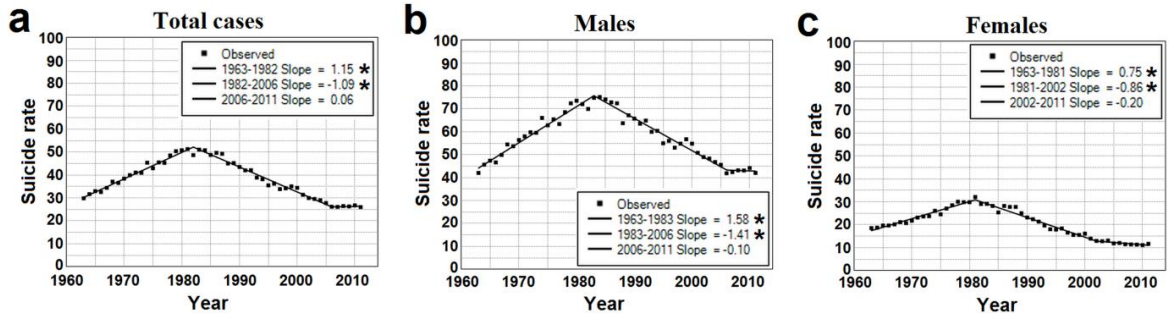


Figure 5 Results of fitted segmented lines of annual Hungarian suicide rates per 100,000 population (>6 years) using joinpoint regression models overall (a) and by gender (males (b), females (c)) created in Joinpoint Regression Program are given for the period 1963-2011. Legends on each diagram relative to the fitted joinpoint segments in yearly intervals and regression estimates (slopes). Asterisks are used to indicate significant slopes ($p<0.001$).

The gender-specific analyses depicted turning points in 1981 and 2002 for females and 1983 and 2006 for males, and changes in segmented linear trends (Table 3, Figure 5). The annual changes in percentages and slopes of the segments in the male cases for 1963-1983: 2.7%, 1.58 ($p<0.001$), for 1983-2006: -2.4%, -1.41 ($p<0.001$), and for 2006-2011: -0.2%, -0.10 ($p=1.00$), and in the female cases for 1963-1981 were: 3.2%, 0.75 ($p<0.001$), for 1981-2002: -4.1%, -0.86 ($p<0.001$) and for 2002-2011: -1.7%, -0.20 ($p=0.08$).

Table 3 Results of joinpoint regression models for Hungarian crude suicide rates per 100,000 population (>6 years) overall, by gender and by age group are presented for the period 1963-2011.

Suicide data (1963-2011) (N = no. of cases)	**No. of JPs**	**JP estimate** Year (95% confidence interval)[a]	**Model comparison**[b] (Bonferroni-corrected p-value)
Total cases	2	1982 (1980-1983)	JP1 vs. JP2 (0.0004)
(N = 178,323)		2006 (2001-2009)	JP2 vs. JP3 (0.1458)
Gender			
Males	2	1983 (1981-1984)	JP1 vs. JP2 (0.0160)
(N = 128,058)		2006 (1992-2009)	JP2 vs. JP3 (1.0000)
Females	2	1981 (1980-1983)	JP1 vs. JP2 (0.0009)
(N = 50,265)		2002 (1997-2005)	JP2 vs. JP3 (0.6693)
Age groups			
7 - 14	0	-	JP0 vs. JP1 (0.2827)
(N = 639)			
15 - 39	3	1975 (1971-1978)	JP2 vs. JP3 (0.0018)
(N = 46,181)		1986 (1983-1988)	JP3 vs. JP4 (0.4640)
		1996 (1993-2000)	
40 - 59	2	1984 (1982-1985)	JP1 vs. JP2 (0.0018)
(N = 68,589)		1995 (1990-1999)	JP2 vs. JP3 (1.0000)
60 -	2	1980 (1978-1982)	JP1 vs. JP2 (0.0018)
(N = 62,914)		2005 (2000-2007)	JP2 vs. JP3 (1.0000)

[a]Estimated turning points in years are given

[b]In model comparisons JPn represents a joinpoint model with n number of joinpoints

Notations: JP: joinpoint.

Significant joinpoint segmented line fits were found in each of the age groups except the 7-14-year old group. Detailed results of the joinpoint regression analyses are presented in Table 3. Additionally, Figure 6 depicts the regression estimations (slopes and significance level) of the joinpoint analyses in all age groups. Peaks were observed following significant increases in suicide trends in 1986, 1984 and 1980 in the age groups of 15-39, 40-59 and 60- years, respectively. The suicide rates declined in all the age groups 15 years and over, after the 1980s, this being more marked in those aged 60 years and over (annual change in percentage: -3.4%, slope -2.25; $p<0.001$) until 2005, but has remained constant since then.

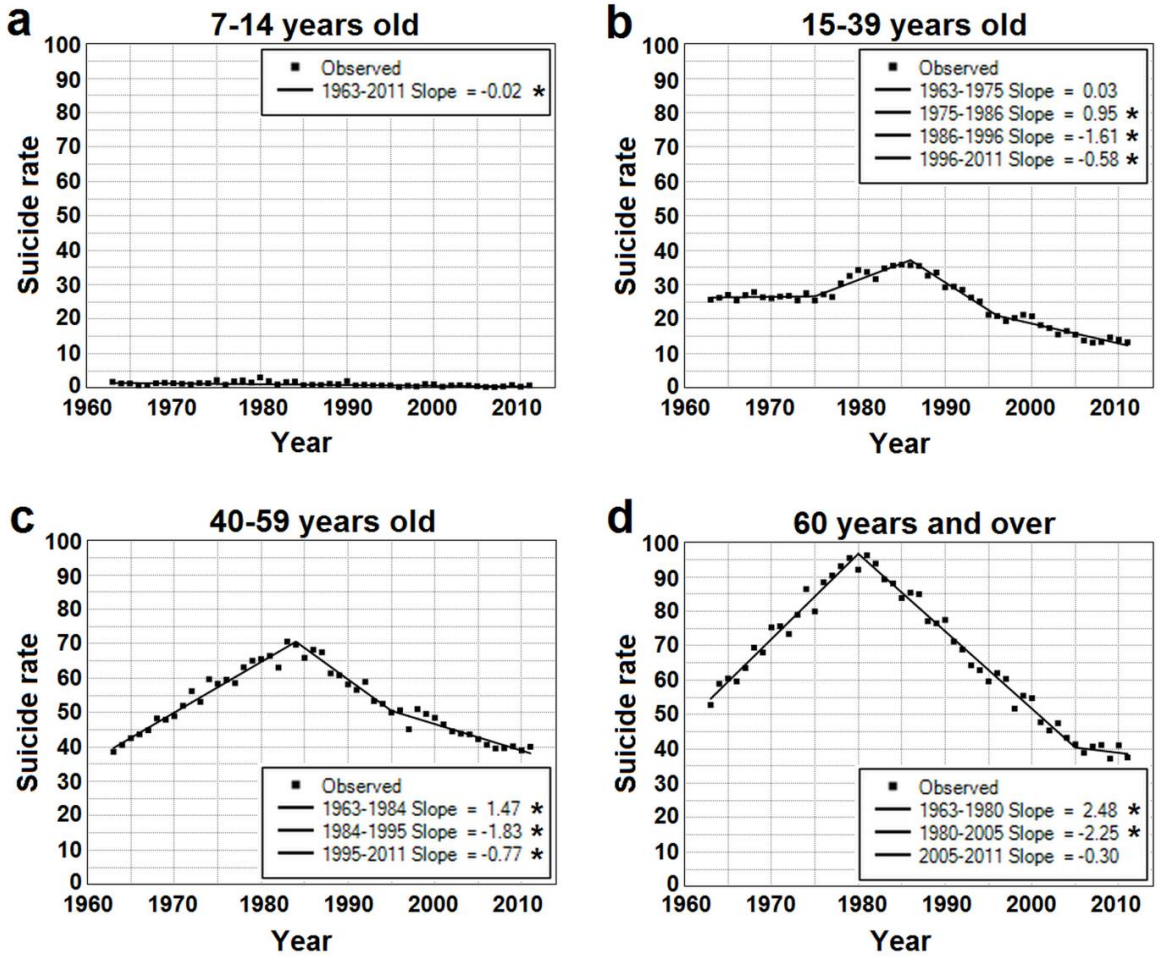


Figure 6 Results of fitted segmented lines of annual Hungarian suicide rates per 100,000 population (>6 years) using joinpoint regression models by age group (7-14 (a), 15-39 (b), 40-59 (c) and 60- years old (d)) created in Joinpoint Regression Program are given for the period 1963-2011. Legends on each diagram relate to the fitted joinpoint segments in yearly intervals and regression estimates (slopes). Asterisks are used to indicate significant slopes ($p<0.001$).

4.2 Neurological studies: Repeated measurements in a rat model of migraine

The used RM-ANOVA analysis models can describe the effect of BBG and electrical stimulation of the trigeminal ganglion in the first study, and also the effect of KYNA analogs in the second study in the modulation of trigeminal nociceptive processing considering the effect of time or space. We analyzed repeated measurements of c-Fos immunoreactive cell counts and area covered by CGRP-immunoreactive fibres in mild and robust stimulation groups, and also in the orofacial formalin test spatially from the bregma of the rat. The number of c-Fos immunoreactive cells were analyzed to highlight the comparison between two KYNA analogs at different distance levels from the bregma in contralateral (left) and ipsilateral (right) sides of the rat.

Nociceptive scores were analyzed in time to monitor behavior in the study of BBG administration of trigeminal activation, and also in the comparative study of two KYNA analogs.

In the mild stimulation, c-Fos did not show a difference between the saline stimulated control group (5SStim left) and any other control group (5BStim left, 5BSham left or 5SSham left). The number of c-Fos cells was significantly lower ($p=0.005$) in the BBG injected stimulated group (5BStim) compared to the saline injected stimulated group (5SStim) in the last (9^{th}) section of the brain from the bregma on the right sides. The right saline sham control group (5SSham) was significantly lower ($p<0.015$) from the right saline stimulated group (5SStim) in all examined brain sections. The model of CGRP did not result in any significant difference between the investigated groups of interest.

In the robust stimulation, c-Fos did not differ among the control groups (left sides: 30BStim, 30BSham or 30SSham vs. 30SStim). The right sides of the 30BSham and 30SSham were not different from the control (left side of 30SStim). Among the right sides, the 30SStim was significantly greater ($p<0.0001$) than the 30SSham, and 30BStim was significantly lower ($p<0.001$) than the 30SStim group. The model of CGRP did not result in any significant difference between the investigated groups of interest.

In the behavior study of the orofacial formalin test of BBG administration, the nociceptive scores were significantly higher ($p<0.05$) in the SForm group compared to the SSal group in time blocks 1, 5, 6 and 7. In the comparisons of BSal vs. SSal and BForm vs. SForm, there were no difference in any of the time blocks ($p>0.05$). The number of c-Fos cells on the right sides in the SForm and BForm groups in the orofacial formalin test significantly differ ($p<0.005$) from all other groups (but not from each other) in brain sections 4, 5 and 6, while in section 7, only the BForm right side group differs from the others (except SForm) significantly ($p<0.05$). The model of CGRP did not result in any significant difference between the investigated groups of interest in the orofacial formalin test.

In the behavior study of the orofacial formalin test of the comparison of KYNA analogs, the nociceptive scores were significantly higher ($p<0.05$) in the Control+Form group than in the Control+Phys group in time blocks 1 and 4-14. The KYNA-2+Form groups had a significantly lower ($p<0.01$) nociceptive score compared to the Control+Form group in time blocks 7, 9 and 10.

The number of c-Fos immunoreactive cells was found to be significantly higher ($p<0.05$) in the right (ipsilateral) side compared to the left (contralateral) side in the Control-

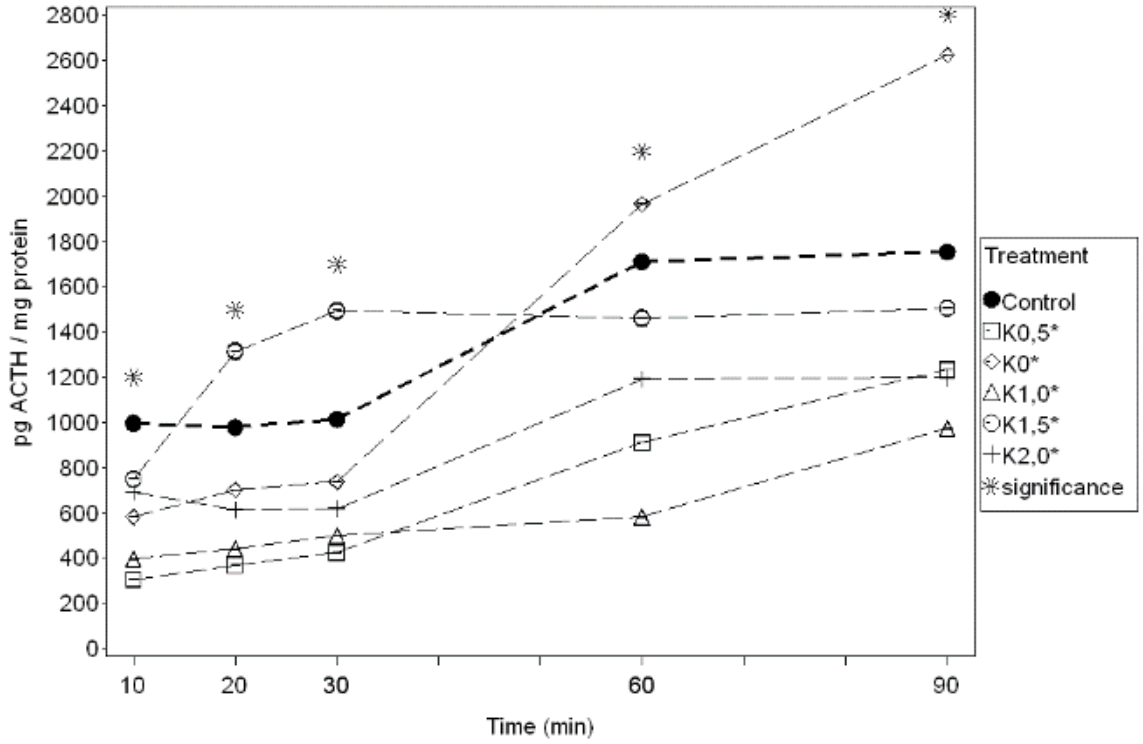
Form group in most sections (from 1st to 13th) of the brain. KYNA-1 analog significantly reduced ($p<0.05$) c-Fos than the control on the right side in sections 2 and 6 of the brain from the bregma. In the comparison of KYNA-2+Form and Control-Form on the right side, c-Fos was significantly lower in the KYNA-2 analog group in brain sections 2, 5 and 6.

4.3 Neuroendocrine studies: Repeated measurements of hormone release under hypoionic conditions

4.3.1 Effect of hypokalaemia

Applied RM-ANOVA models highlighted the effect of hypokalaemia on hormone exocytosis. Analyses results showed significant difference for all K^+ treatment groups compared to the control at all time points (Figures 7-9).

a



b

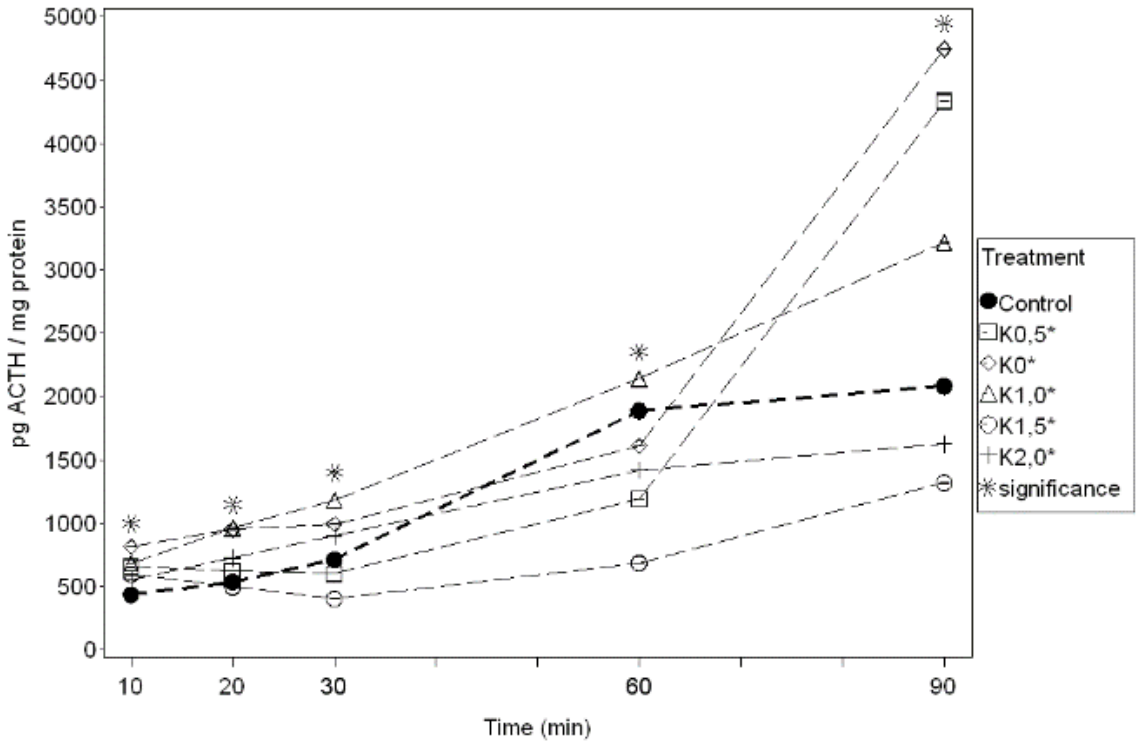
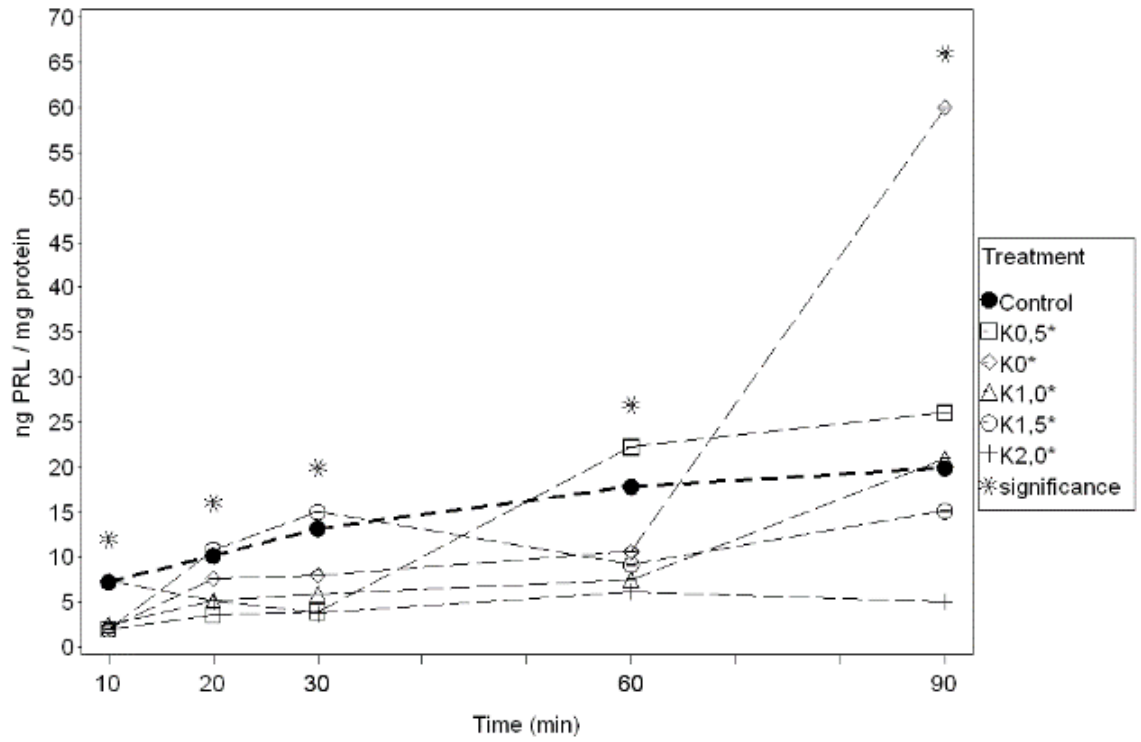


Figure 7 ACTH release (mean +/- SE) of normal (a) and PRLoma (b) AdH cell cultures by different K^+ treatments over time. Asterisks indicate statistical significance at α=0.05 level in the comparison of various treated groups and the control group.
Notations: ACTH: Adrenocorticotrophic hormone, SE: Standard error, PRLoma: Adenohypophyseal prolactinoma, AdH: Adenohypophysis.

a



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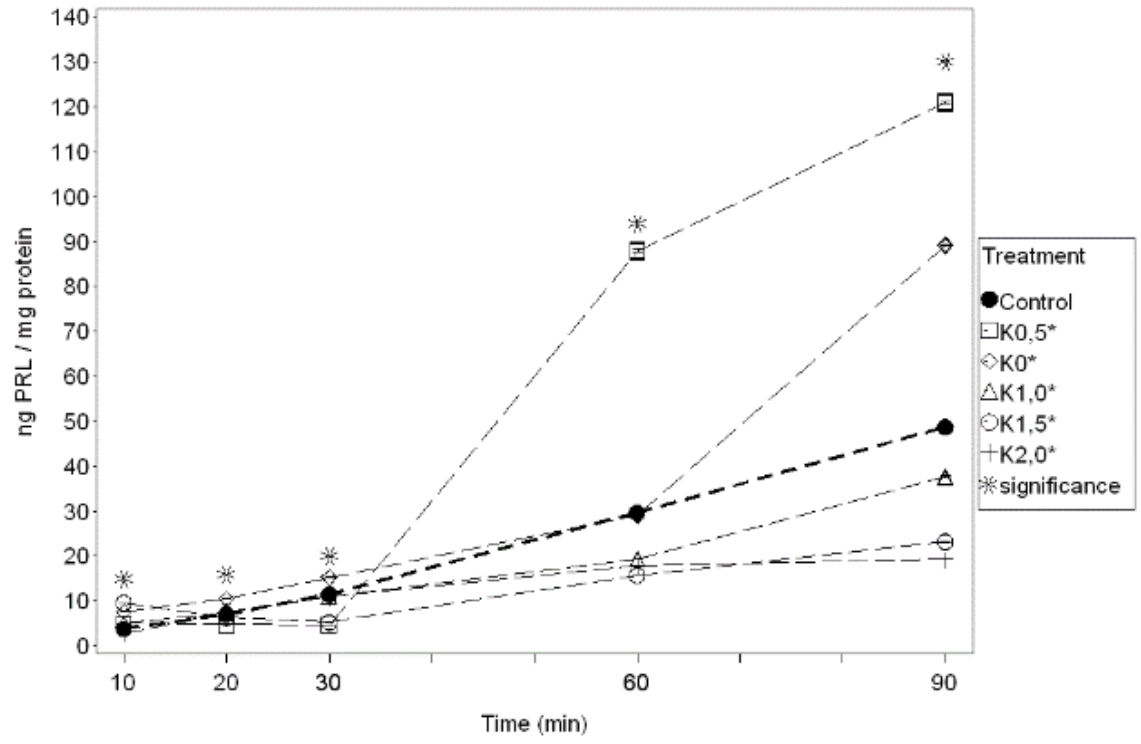
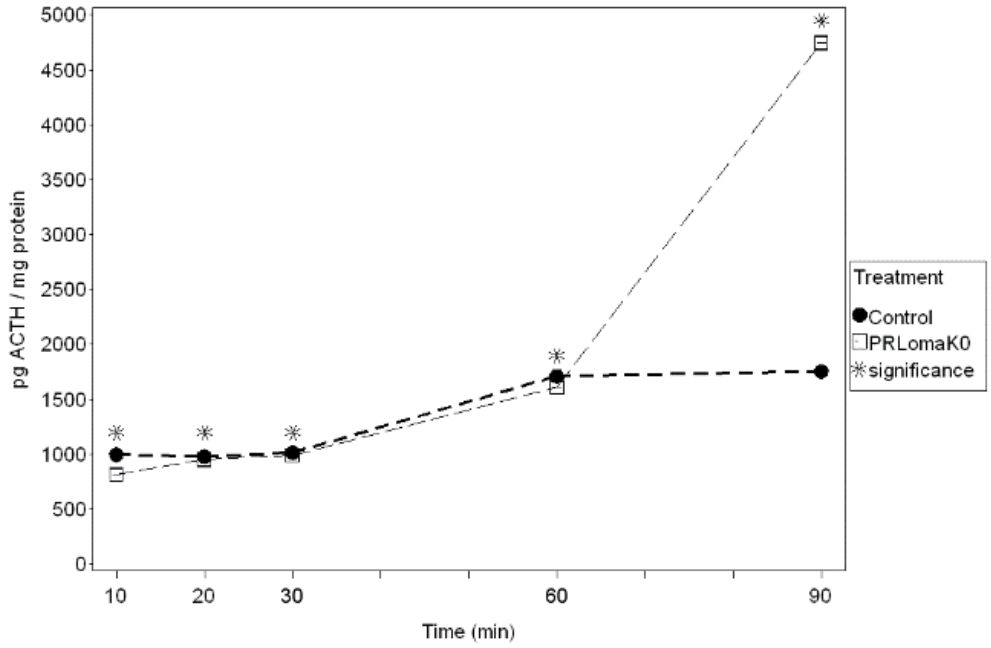


Figure 8 PRL hormone release (mean +/- SE) of normal (a) and PRLoma (b) AdH cell cultures by different K^+ treatments over time. Asterisks indicate statistical significance at α=0.05 level in the comparison of various treated groups and the control group.
Notations: PRL: Prolactin, SE: Standard error, PRLoma: Adenohypophyseal prolactinoma, AdH: Adenohypophysis.

a



b

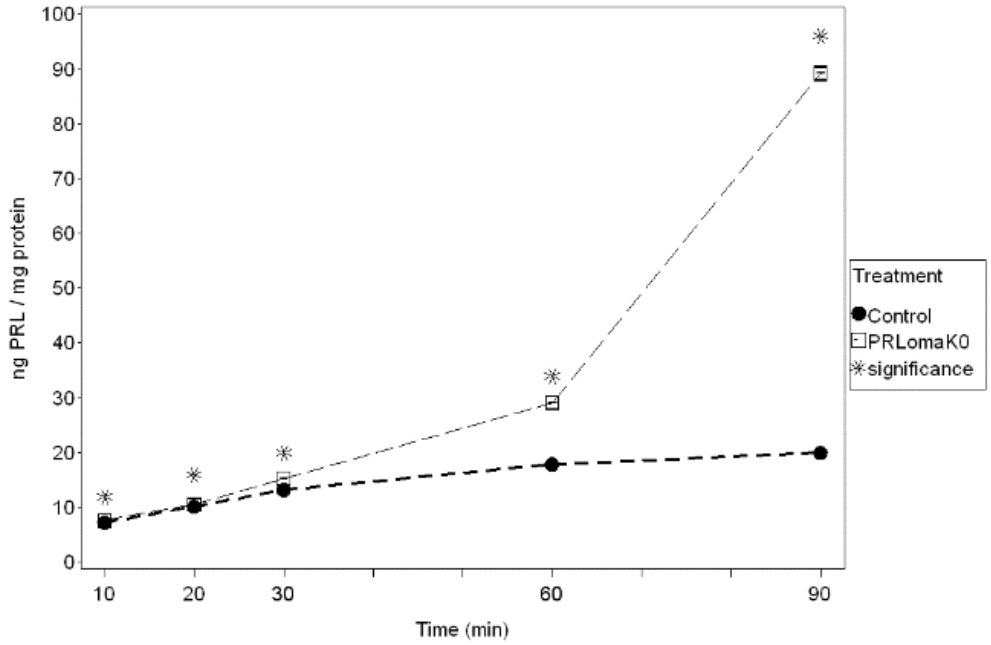


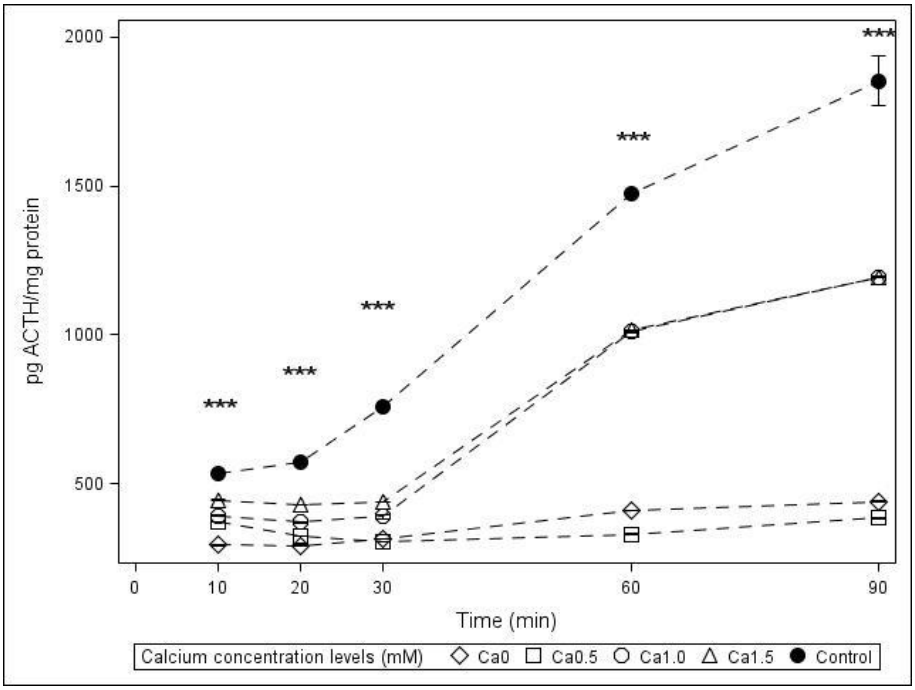
Figure 9 Normal control versus PRLoma K0 AdH cell cultures for ACTH (a) and PRL (b) hormone release (mean +/- SE) over time. Asterisks indicate statistical significance at α=0.05 level in the comparison of various treated groups and the control group.
Notations: PRLoma: Adenohypophyseal prolactinoma, AdH: Adenohypophysis, ACTH: Adrenocorticotrophic hormone, PRL: Prolactin, SE: Standard error.

All pairwise comparisons were significant in all RM-ANOVA models, even with Bonferroni p-value correction for the 6 models run: in the first 4 models control versus any K^+ treatment group for all time points, in the last 2 models normal control AdH vs. PRLoma K0 for all time points.

4.3.2 Effect of hypocalcaemia

The applied two marginal models fitted the data for both hormones, and model results interpreted the effect of low levels of Ca^{2+} on hormone release (Figures 10-11).

a



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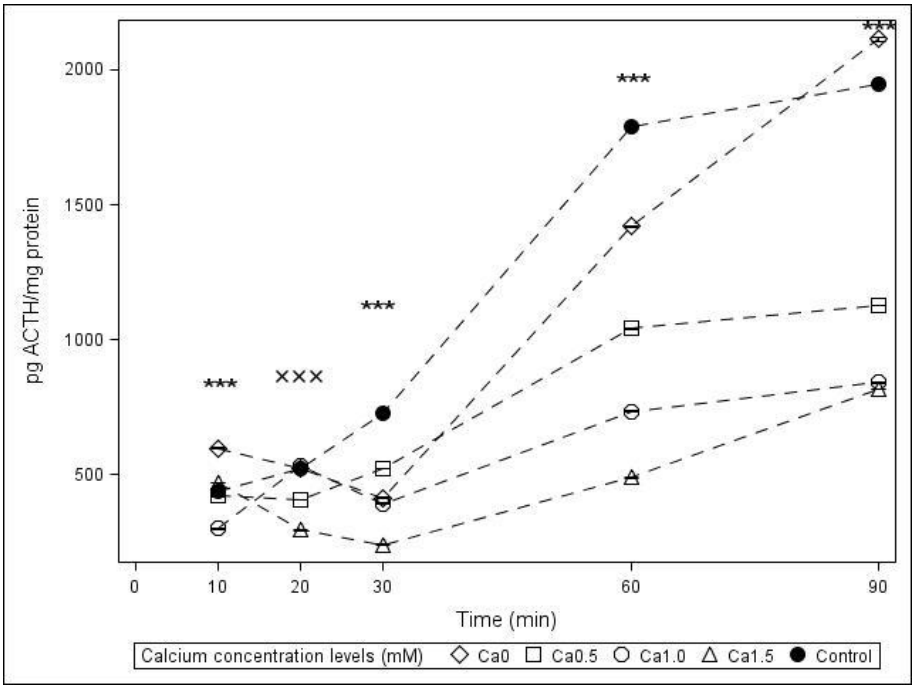
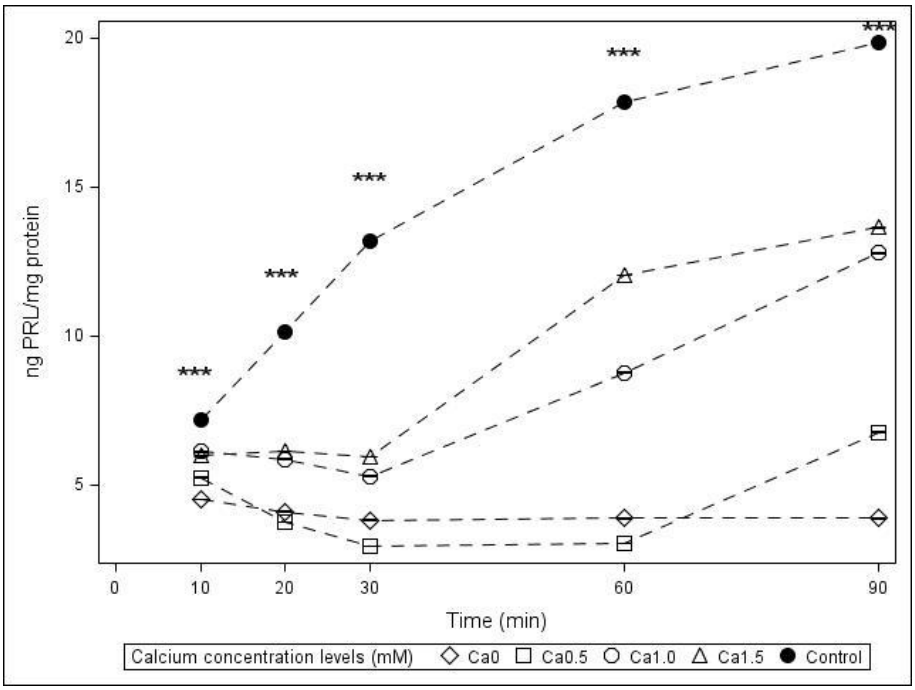


Figure 10 ACTH release (mean +/- SE) of normal (a) and PRLoma (b) AdH cell cultures by different Ca^{2+} treatments over time. *** indicates statistical significance at α=0.0001 level in the comparison of various treated groups versus the control group. ××× indicates no significant difference between 0 mM [Ca^{2+}] and the control.
Notations: ACTH: Adrenocorticotrophic hormone, SE: Standard error, PRLoma: Adenohypophyseal prolactinoma, AdH: Adenohypophysis.

a



b

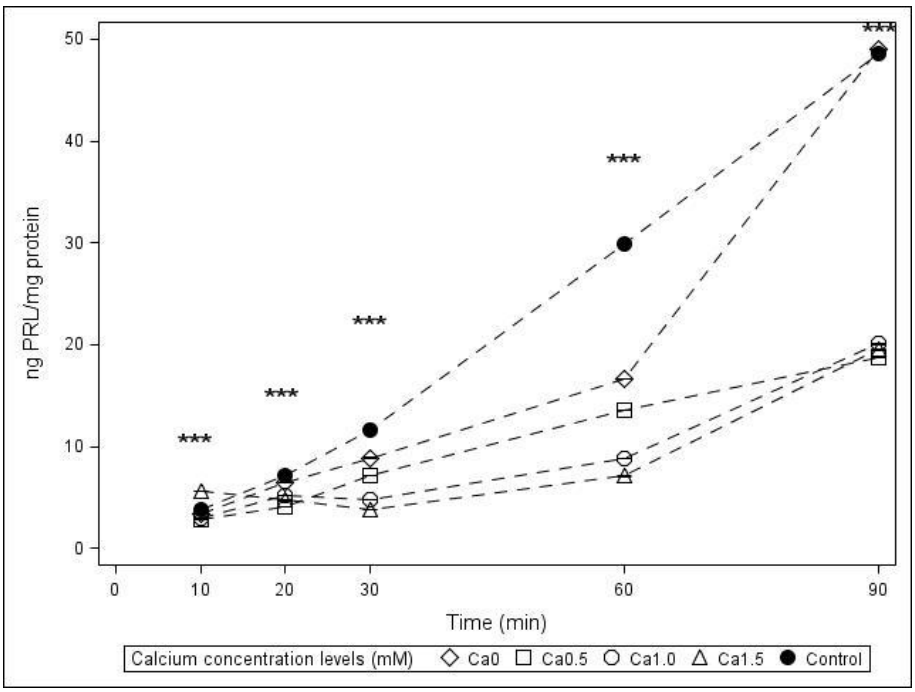


Figure 11 PRL hormone release (mean +/- SE) of normal (a) and PRLoma (b) AdH cell cultures by different Ca^{2+} treatments over time. *** indicates statistical significance at α=0.0001 level in the comparison of various treated groups versus the control group. Notations: PRL: Prolactin, SE: Standard error, PRLoma: Adenohypophyseal prolactinoma, AdH: Adenohypophysis.

Five cases were extreme values in the ACTH data, from all n=120 subjects structured in a long format dataset. Using winsorization or deleting these cases resulted in similar differences in ACTH release between the Ca^{2+} treatment groups and the control at all time points by disease (normal and PRLoma AdH cell cultures).

4.4 Environmental study: Repeated measurements in turkeys exposed to electromagnetic radiation

4.4.1 Characterization of ELF EMF treatment on NE-activated β-adrenoceptor functions in time

The effect of ELF EMF was significant over time compared to the control group during weeks 3-5 (differences: week 3: 4.04 with 95% CI of (0.66 – 7.41); week 4: 8.29 with 95% CI of (5.61 – 10.97); week 5: 5.66 with 95% CI of (2.08 – 9.24) measured in nmol cAMP/ml red blood cell (RBC) suspension; $p < 0.01$; Table 4 a). The values of the control group did not change (16.7 nmol cAMP/ml RBC suspension with the 95% CI of (15.3 – 18.2)) throughout the whole experiment (Figure 12; Table 4).

The cAMP level of subjects in the ELF EMF treated group had a decrease ($p < 0.001$) of 2.6 nmol/ml RBC suspension per week (95% CI: (-2.8 – -2.4)) in the treatment period, while there was an increase (1.5 nmol cAMP/ml RBC suspension per week; 95% CI: (1.2 – 1.9); $p < 0.001$) in the regeneration period (Figure 12; Table 4 b). At the intercept of the two fitted linear pieces (week 4), the mean cAMP level was 9.6 nmol/ml RBC suspension in the treated group, which was 7.1 nmol/ml RBC suspension (95% CI: (-8.6 – -5.5)) less than that of the control group (16.7 nmol/ml RBC suspension, Figure 12; Table 4 b). As 60% of the variation of cAMP levels comes from individual characteristics of turkeys, 40% is from within subject effects.

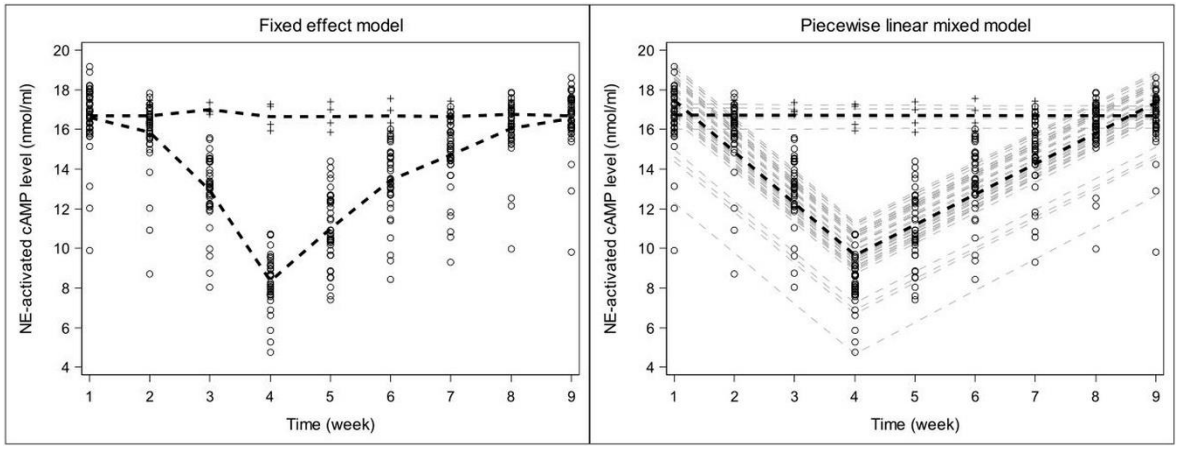


Figure 12 Fitted analysis models for the complete experiment: marginal model (left) and piecewise LMM (right). Dashed thick, black lines represent predicted means. Dashed dark grey lines show individual trajectories in the result of piecewise LMM.
Notations: + Control (n=4), ∘ Treated (n=40) subjects, cAMP: 3'5'-cyclic-adenosine-monophosphate, LMM: Linear mixed model, NE: Norepinephrine, nmol/ml: nmol cAMP/ml RBC suspension, RBC: Red blood cell.

Table 4 Estimations of the marginal model (a) and of the piecewise LMM (b) on the cAMP levels (NE-activated β-adrenoceptor function)

a

Marginal model (REML, UN, KR)		
Fixed effect	**F (DF)**	**p-value**
Group	14.1 (1, 42)	0.001
Time	31.8 (8, 35)	<0.001
Interaction (group × time)	31.2 (8, 35)	<0.001
Time	**LS Mean Diff (95% CI)**	**Adj p-value**
Week 1: Adaptation	0.07 (-3.44 – 3.58)	1.000
Week 2: Treated 1	0.83 (-2.74 – 4.41)	1.000
Week 3: Treated 2	4.04 (0.66 – 7.41)	0.005
Week 4: Treated 3	8.29 (5.61 – 10.97)	<0.001
Week 5: Regeneration 1	5.66 (2.08 – 9.24)	<0.001
Week 6: Regeneration 2	3.26 (-0.37 – 6.88)	0.150
Week 7: Regeneration 3	1.94 (-1.69 – 5.57)	0.998
Week 8: Regeneration 4	0.71 (-2.36 – 3.78)	1.000
Week 9: Regeneration 5	0.11 (-3.02 – 3.24)	1.000

b

Piecewise LMM (REML, UN, KR)			
Fixed effect	**Estimate (95% CI)**	**SE**	**p-value**
Mid-point status of control group (intercept at week 4)	16.71 (15.26 – 18.16)	0.72	<0.001
Group	-7.07 (-8.60 – -5.54)	0.76	<0.001
Time (reference: week 4)	-0.01 (-0.15 – 0.14)	0.07	0.941
Interaction (group × time) in weeks 1-4	-2.60 (-2.80 – -2.41)	0.10	<0.001
Interaction (group × time) in weeks 5-9	4.14 (3.95 – 4.34)	0.10	<0.001
Covariance parameter			
UN(1,1)	1.89	0.46	<0.001
UN(2,1)	-0.02	0.04	0.539
UN(2,2)	0.00	.	.
Residual	1.29	0.10	<0.001

Notations: LMM: Linear mixed model, cAMP: 3'5'-cyclic-adenosine-monophosphate, NE: Norepinephrine, REML: Restricted maximum likelihood estimation method, UN: Unstructured covariance structure, KR: Kenward-Roger method for adjusting degrees of freedom, F: F-statistic, DF: Degrees of freedom, LS Mean Diff: Least squares means differences (control-treated), CI: Confidence interval, SE: Standard error, Adj p-value: Sidak adjusted p-value, Group: separates the control (n=4) and ELF-EMF treated (n=40) animals in the complete experiment, Time: the 9-week long complete experimental period, UN(1,1): variance between individuals of mid-point status (intercept at week 4), UN(2,2): variance between individuals of the rate of change in time (slope), UN(2,1): covariance between individual variances in intercepts and individual rates of change (slopes), Residual covariance parameter: within-subject variance.

4.4.2 The reversible nature of the biological experimental model

The piecewise LMM revealed a decreased effectiveness of β-adrenoceptors, as the NE-activated cAMP level was getting lower and lower during the treatment with ELF EMF. After the end of the ELF EMF treatment, the β-adrenoceptor functions returned to the starting state in 5 weeks. As mentioned above, the recovery (increase) of β-adrenoceptor functions in the treated group was slower in the regeneration period (slope of cAMP: 1.5 nmol/ml RBC suspension) compared to the rate of decrease (slope of cAMP: -2.6 nmol/ml RBC suspension) in the treatment period (Figure 12; Table 4 b). The treated group did not differ from the control at the end of the regeneration period (weeks 6-9, $p \geq 0.15$; Table 4 a).

5 DISCUSSION

5.1 Main findings

The trends in suicide rates in our *epidemiological study* were described by using segmented linear models overall, by gender and by age group in Hungary between 1963 and 2011. Results revealed an overall peak in 1982 and remained constant after 2006. There was a significant, approximately 3% annual increase until 1982 (an increment of ~20 suicides per 100,000 persons) during two decades. This was followed by a significant decrease during the next about two decades, when suicide rate decreased approximately to the same level where it started in 1963, and with relative constancy after 2006 overall. Similar trends were observed in both genders. The different numbers of turning points reported by age group in the segmented linear trends revealed significant, but shifted peaks in the 1980s.

Risk estimates of suicide rates were calculated by gender, age group and suicide method, and highlighted a more than twice as great risk in males than in females overall. The risk of suicide in males was more than twofold in all age groups. A significant gender difference was found for most suicide methods, except "Poison" and "Drowning". The highest risk, more than twentyfold in males relative to females, was observed in the "Fire-arms and explosives" subgroup. The most frequent method was "Hanging", with a risk of more than fourfold in males.

In the *neurological studies* RM-ANOVA models characterized BBG administration and the effect of two KYNA analogs of trigeminal activation in the rat in the investigation of migraine through the animal models of trigeminal nociceptive processing. The number of c-Fos immunoreactive cells and separately the sums of the areas covered by CGRP-immunoreactive fibres were analyzed as repeated measurements spatially in different levels of the trigeminal nucleus in mild and robust electrical stimulation groups, and also in the orofacial formalin test to investigate the effect of BBG. KYNA analogs were compared based on the number of c-Fos immunoreactive cells as repeated measurements spatially at different distance levels from the bregma by paired aspects (contralateral and ipsilateral sides of the rat).

Effect of time was analyzed on nociceptive scores (number of seconds the rat spent rubbing the injected area in the orofacial formalin test) to compare BBG and saline treatments before saline and formalin injections and also in the comparison of two KYNA analogs.

In the *neuroendocrine studies*, effects of hypokalaemia and hypocalcaemia on ACTH and PRL hormone release could be described by appropriate repeated measures analysis methods. RM-ANOVA resulted in significant difference in both ACTH and PRL hormone release of normal and PRLoma AdH cell cultures for all K^+ treatment groups versus the control across time. Marginal models fitted the exocytosis data for both hormones in case of normal and PRLoma AdH cell cultures in the comparison of low levels of Ca^{2+} treatment groups versus the control over time. For the ACTH release dataset the winsorization technique resulted in similar differences as the deletion of the extreme outlier cases in the comparison of hypocalcaemic groups and the control for all time points in normal and PRLoma AdH cell cultures.

In our *environmental study*, marginal and mixed models confirmed the effect of *in vivo* 10 μT ELF EMF treatment on the NE-activated β-adrenoceptor function of physiological processes as cellular mechanisms. Additionally, fitting piecewise linear mixed model to the data, the treatment and regeneration periods could be characterized separately, which reflected a sharp linear reduction in NE-activated β-adrenoceptor function in the treatment period, and then a remarkable – likewise linear – growth in the regeneration period. The control group did not change throughout the whole experiment.

5.2 Strength and limitation

In the *epidemiological study*, instead of reporting only the suicide rates, we applied regression models to estimate the trends in Hungarian suicide rates between 1963 and 2011, including joinpoint regression, which involves the best fit of segmented lines connected at the "joinpoints" (turning points) through use of a Poisson model of variation [36]. As far as we know, this was the first utilization of joinpoint regression to investigate the pattern of trends in suicide rates in Hungary. In the joinpoint regression analysis, the slopes with annual changes in percentages of the segmented lines were used to determine the annual changes in suicide rates in Hungary, while other studies applying joinpoint regression for suicide data involved only the estimated annual percentage change [9]. We consider that the use of slopes is more appropriate in the case of linear fit and generally gives a complete picture of trends with the annual percent change.

The risk of suicide in Hungary was calculated by negative binomial regression, which can handle overdispersed data common in rare discrete events, such as suicide frequencies relative to the population [35, 64]. Although suicide rates in Hungary have often been investigated, detailed statistics have scarcely been reported. The negative binomial

regression method applied gives valuable risk estimates for the whole of the examined period, and joinpoint regression analyzes the pattern of the trends in detail.

The reference population was given only by gender and age group to calculate the suicide rates. We could investigate the risk of suicide by gender, age group and suicide method only from the data published in the Demographic Yearbooks of the Hungarian Central Statistical Office. However, the suicide methods were broken down in more detail than the violent and non-violent categories usually used in the literature.

In the case of the comparison of two KYNA analogs in the *neurological studies*, related aspects could be analyzed by using 3-way RM-ANOVA, where not only the effect of space (adjacent measuring sites of the trigeminal nucleus) could be studied as repeated measurements, but also the effect of both sides (left: contralateral and right: ipsilateral) of the rat as another related factor in the analysis model. Both factors (measuring sections and sides) were used as within-subject factors in the model of comparison of pretreatment groups (two KYNA analogs and control), where the response variable was the number of c-Fos immunoreactive cells. By fitting this model to the data, we could take into account when several measurements were taken from the same individual.

On the other hand, using more factors in an analysis model increases model complexity because of the statistical interaction which can be detected in 2- or more ways in the coexistence of the used factor variables affecting the investigated response. In the case of statistically significant interaction, the effects of factors cannot be separated for the dependent variable, so these terms must be interpreted together [65]. Besides statistical interactions the researcher also has to focus on biological interactions, which could be essential with no interaction in the analysis model being statistically significant [66, 67].

A limitation of RM-ANOVA would be the inefficient treatment of missing data [45]. In the neurological studies, there were no missing data, so we did not have to drop all repeated measurement data for any subject.

In the case of the analysis of the hypocalcaemic effect on hormone exocytosis in the *neuroendocrine studies*, applying the marginal model resulted in a better fit than the RM-ANOVA model, as unique covariance structure could be incorporated, rather than just the homogeneous structure for the observations on the same individual [50]. Furthermore, the use of winsorization reduced the effect of outliers on statistical analysis [62], at the same time, it did not give any other result than when we extracted the few extreme outlier values.

There were no missing values in our measured data of the *environmental study*, although mixed models can be used on longitudinal data with missing values [68]. The

difference in sample size (4 vs. 40) of the investigated groups, could cause biased estimates, though the mixed model is designed to handle unbalanced data sets [32]. Different statistical methods were applied which basically revealed the same result concerning the significant effect of ELF EMF over time.

Piecewise linear mixed model could explain the regeneration phenomenon by the linear increasing trajectory of the ELF EMF treated group, which was returned to the control level.

5.3 Comparison with other studies

According to the *epidemiological study*, Rihmer et al. [6] recently reported on suicide rates in Hungary in different associations (e.g. marital status, seasonal aspects, regional distribution, alcohol and tobacco consumption, antidepressant prescription, unemployment rate), but the relationships between the suicide rates and the above factors were not subjected in detailed analyses. The previous study used the entire Hungarian population as reference to calculate the suicide rates, whereas we focused only on the population older than 6 years, for whom suicide frequencies have been published. Nevertheless, our findings show similar patterns in suicide rates overall, by gender and by age groups to those reported previously in Hungary for the investigated period.

The Hungarian average suicide rate was over twofold compared with the overall world suicide rates found in 105 countries in 2008 [5]. In the previously mentioned study, a large difference in average suicide rate was also observed by gender in Hungary, as compared with worldwide suicide rate: about fourfold in males, and about twofold in females. The overall male/female suicide rates ratio of 4 was found to be the highest in Europe (48 countries) by the previous author, and greatly (more than twofold) exceeded the world ratio.

Studies often investigate suicide methods broken down into violent (ICD-10: X70-X84; e.g. “Hanging”, “Drowning”, “Fire-arms and explosives”, “Cutting and stabbing instruments” or “Jumping from a high place”) and non-violent (ICD-10: X60-X69; e.g. “Poison”, “Gas”) categories [69], rather than focusing on more detailed subgroups. Our results were consistent with the findings of previous studies that the risk of suicide is greater in males than in females, and males tend to use more violent methods [1, 6, 70-72]. Värnik et al. [4] reported RRs for males vs. females in more detailed suicide method subgroups in 16 European countries between 2000 and 2004/5, and found that suicide frequencies were higher in males than in females in all the suicide method subgroups. Although “Hanging” was the most frequent method in their analysis too, the RR of males for this subgroup was

about one-third of what we found for the 49-year period. Similarly to our results, the highest RR was found for the "Firearms" subgroup in the examined 16 countries between 2000 and 2004/5, as compared with our study between 1963 and 2011. Tamosiunas et al. examined similar suicide methods among 25-64-year-olds in Kaunas, Lithuania, from 1984 to 2003 [73]. "Hanging" was the most frequent suicide method in Lithuania during that period. Our findings on suicide frequencies by suicide methods in Hungary are consistent with those of Berecz et al. [74].

We also investigated age group as a risk factor in suicide in Hungary. Hawton and van Heeringen [1] reported in 2009 that suicide rates among elderly people have fallen in many countries, while those for younger age groups have risen. The situation in Hungary was slightly different as suicide rates were higher for older age groups.

Similarly to ourselves, Klieve et al. [75] analyzed the annual trends in suicide rates and determined RRs by using negative binomial regression in Australia. Morriss et al. [76] have also presented annual trends in suicide rates in Lancashire, United Kingdom, by applying negative binomial regression.

We additionally analyzed annual trends in suicide overall and by gender, age group and suicide method by utilizing negative binomial regression, but joinpoint regression analysis gave better fits of our data, as the trends were not exactly linear, but rather segmented linear. joinpoint regression was likewise the method of choice in Lithuania. Tamosiunas et al. [73] found significant increase by calculating estimated annual percent changes in joinpoint regression for men aged 25–64 years during 1984 to 2003. In contrast, we revealed a significant decrease in suicide rates during this period.

In our study we focused on completed suicide data; however, suicide attempt (also called parasuicide) is a well-known indicator for suicide [77]. A paper of Hungarian suicide attempters concluded that gender differences should be considered in developing efficient complex therapy and prevention strategies [71]. Other studies focused on suicidal elderly people with geriatric depression, whose attempts are more likely to lead to completed suicide than in younger age groups, and discussed treatment and psychotherapeutic interventions during which they require special attention as they have lower and delayed response [78, 79]. Besides the above mentioned studies, our analysis with sophisticated methodology may support the use of gender and age group as factors in developing intervention strategies in prevention of suicide.

Among 12 European countries, Hungary had high rates of attempted suicide by the use of pesticides and solvents in the period 1989-1993 [80]. It is consistent with the findings of

Berecz et al. [74] and with our results for the whole country having “Poison” as one of the most frequent suicide methods. Berecz et al. concluded that the decrease of suicide rates in Hungary between 1991 and 2001 may be due to the decreased access to highly toxic pesticides and other toxic substances. We agree with Michel et al. [80] on the importance of understanding and monitoring the influencing factors of suicide. Several intervention studies presented promising results in Hungary highlighting the importance of community-based interventions and the education of general practitioners [81, 82]. In contrast, Morriss et al. [76] found that a brief training program did not affect suicide rates in England in 1997. Balazs et al. [83] revealed that media reports on a suicide case in Hungary in 2011 were not in line with the recommendation of the international guidelines on media coverage of suicide, and emphasized the importance of using more preventive information rather than provocative aspects during the disclosure. The authors highlighted that Hungarian mass media coverage of suicide has rarely been investigated, although such analysis could provide useful information for preventive medicine.

In the *neurological studies*, we applied repeated measures analysis methods for time or space as within-subject effects in the examination of BBG administration or KYNA analogs in a rat model of migraine. The effect of BBG, as a P2X7 receptor antagonist, was also investigated in another study [84], where, similarly, ANOVA models were used for analysis, but not repeated measures analyses, considering within-subject effect. Goloncser and Sperlagh [84] also concluded the therapeutic potential of P2X7 antagonist for the treatment of headaches.

RM-ANOVA was also applied on repeated measurements, as in our case, in the investigation of the neuroprotective effect of KYNA analogs in Huntington’s disease on body weights with aging in a mouse study [85]. Besides, Zadori et al. [85] analyzed survival time as well by applying Mantel-Cox log rank test to compare the probability of survival between the KYNA analog treatment and the control group. This analysis method was not relevant in our study. In another rat study, ANOVA models were used with repeated measures to examine the effect of endogenous levels of KYNA on prepulse inhibition, as a model that reflects sensory gating deficits and is reduced in neuropsychiatric disorders [86]. Repeated measures were used for startle trial types in the behavioral experiments. Erhardt et al. [86] concluded that elevated levels of endogenous KYNA are associated with a disruption in prepulse inhibition and participate in the pathophysiology of schizophrenia.

Concerning the *neuroendocrine studies*, repeated measurements were also analyzed on pituitary adenomas in other studies. Among different types of pituitary adenomas, in a

recent study [87] You et al. revealed that ACTH-pituitary adenoma had a significant association with postoperative hypokalaemia. They also applied RM-ANOVA as we did, but they used it to characterize the change in mean serum potassium levels between ACTH and control groups over time. You et al. [87] used Student's t test for the comparison of ACTH and control groups for each of 4 investigation time points, while we applied the Sidak post hoc test for pairwise comparisons in our analyses. In their analysis, the response variable was the serum potassium level in the comparison of ACTH-pituitary adenoma group and the non-ACTH-pituitary (control) group over time, while in our case, the examined response was the amount of ACTH or PRL hormone release in the comparison of different concentrations of potassium treatment and control groups over time. In an earlier study, Toufexis and Walker [88] also used RM-ANOVA to analyze ACTH response in virgin and lactating female rats over time to compare the 2 groups: sham or 6-hydroxydopamine lesions over the paraventricular nucleus. Their data structure was similar to ours using between-subject and within-subject effects for the analysis. Their analyses supported that brainstem noradrenergic inputs to the paraventricular nucleus facilitate ACTH stress response in virgin rats, but not during lactation.

As in our hypocalcaemic neuroendocrine study, repeated measures analysis was also applied in another research to investigate the effect of hypocalcaemia. In this other study, another hormone, the parathyroid hormone was analyzed on repeated measurements by using RM-ANOVA to compare calcium intake groups in elderly women [89]. The increase of the serum parathyroid hormone contributes to age-related bone loss. McKane et al. [89] highlighted the effect of hypocalcaemia on the increased bone resorption in elderly women.

Concerning the *environmental study*, the effect of ELF EMF has already been investigated from many aspects, using repeated measurements to characterize the pattern of the examined indicator over time: breeding and migrating behavioral aspects of birds [90], hematological parameters in mice [91], melatonin levels in calves [92]; however, behavioral patterns defined by receptor functions using mixed models have been rarely considered. Repeated measures experiments have been used commonly in animal, plant, and human research for several decades, and computing methodologies have been available to analyze them effectively and efficiently in the last few decades [22].

Using the most appropriate data evaluation and statistical approaches, NE-activated β-adrenoceptor function patterns were explained before, during and after ELF EMF treatment by piecewise linear trajectories, revealing a significant decrease during the treatment, and a significant increase after the treatment. The level of the indicator

(intracellular cAMP) decreased and so represented loss of function under the effect of ELF EMF. However, the examined NE-dependent β-adrenoceptor attractor was converged to the starting condition in the regeneration period by the support of the *in vivo* homeostatic complex network. At the same time, data of the control system reflected no induced alteration. We confirm previous findings, that the decreased NE-activated β-adrenoceptor function has an important role in the formation of emotional disinterest and depression [93, 94]. In another study [95], piecewise linear model was also applied on poultry-related data, as it was in our case. In our study we fitted the piecewise LMM considering the treatment group effect, so we could separately describe piecewise linear effects in the treated group. In contrast, Naumova et al. [51] had only one group over time in their study.

Incidence rate ratio is estimated in a Poisson regression model. However, in the case of rare events, this is a good approximation of the relative risk. Relative risk is a measure, which can explain the risk of event occurrence under the influence of a risk factor [40]. In the case of overdispersion – which is often the case with frequency data –, the negative binomial regression provides an alternative model of Poisson regression [34, 35]. To explain changes in trend data such as incidence rates, the joinpoint regression model can be applied, in which segmented trends can be described [36].

In the case of repeated measurements, the choice of marginal versus mixed model depends on the aim of the study. If the purpose is to predict the mean, then the marginal model may be suitable. When the focus is on understanding individual characteristics, the mixed model could be used. According to Hamer and Simpson [47], mixed models are typically used for repeated measures data, because mostly a within-subjects factor, and so the generalization of the particular individual is of interest. SEs in GLM are usually not computed correctly, while mixed models make valid computations for hypothesis tests and SE estimates [22]. Ignoring the within-subject correlation and covariance issues on repeated measurements on a subject may result in inaccurate SE estimates and incorrect conclusions [22, 45]. While different numbers of levels in fixed effects and numerous types of covariance structures can be set in a model, degrees of freedom can change. The more parameters we fit in a model, the more degrees of freedom we use up, and the less powerful the tests are. Power can be higher in a model with random effects, as it uses less degrees of freedom than fixed effects. There are different SE estimates depending on the type of the covariance structure and even the best fit might not result in the smallest SEs [22]. As LMM is more complex and flexible than GLM, the potential for confusion and errors is higher[47].

Studies show that mixed models are characterized by higher statistical power than comparable traditional methods applied to the same data when the underlying assumptions (adequate sample size, continuous and normally distributed repeated measures) are not met [52, 96].

6 SUMMARY AND CONCLUSIONS

Longitudinal analyses often occur in biomedical research, which require an interdisciplinary approach today, where the biostatistical analysis is as important as the scientific research. Investigating appropriate risk measurements and trends is of essence in epidemiological studies, using merely descriptive statistics will not suffice. Examining paired aspects is crucial in the case of repeated measurements, to differentiate within-subject and between-subject effects. Repeated measurement aspects are usually examined in time or space. Selecting the suitable covariance structure is relevant in the analysis of dimensional distances from the paired aspect in repeated measurements. Appropriate data management techniques are needed to handle extreme outliers in a dataset, in order to find the best suitable model. In the case of joint linear trajectories, proper analysis models are essential.

The trends in annual Hungarian suicide rates per 100,000 population (>6 years) were analyzed by negative binomial and joinpoint regression methods to determine relative risks for gender differences overall, by age group or by suicide method, and to find peaks in the linear segmented trends by gender or age group between 1963 and 2011. The joinpoint regression models gave a more complete picture of Hungarian suicide trends than the negative binomial regression models. Our results furnish the first complete analysis for annual Hungarian suicide rates with the trends and risk estimates, such a complex analysis is not commonly used for suicide data (merely either trend analyses or risk estimates are given usually). We have presented detailed analyses rather than purely descriptive statistics for the suicide trends, as descriptive statistics provide only virtual trends, whereas both fitted regression models reveal more specific and precise information. Furthermore, the reference population selection by age (> 6 years) in the calculation of suicide rates helps to avoid biased estimates. The importance of the interpretation of analyses is getting into the focus nowadays as social changes are more common. Thus, we have reported internationally comparable data and descriptions of the patterns of suicide in Hungary. To identify subgroups at high risk, such detailed results relating to the trends may be of importance in helping to shape strategies designed to help prevent suicide.

The applied repeated measures analysis models described BBG administration and effect of two KYNA analogs in trigeminal nociceptive processing in the rat. Within-subject effect was considered as a spatial factor (brain sections from the bregma) to study the impact of BBG – on area covered by CGRP-immunoreactive fibres and c-Fos immunoreactive cell counts in mild and robust stimulation groups –, and also in the orofacial formalin test. In

other analysis models, the within-subject effect was the time, which was considered as a related factor – on nociceptive scores to compare BBG and saline treatments before saline and formalin injections – in the examination of behavior. In the case of the investigation of KYNA analogs, paired aspects were considered in two ways: spatially (from the bregma) – on the number of c-Fos immunoreactive cells to compare different pretreatment groups (KYNA analog groups and the control) –, and by the side of the rat (left: contralateral, right: ipsilateral). The fitted analysis model characterized the pattern of monitored nociceptive scores across time in the comparison of two KYNA analogs.

Repeated measures analysis methods fitted the ACTH and PRL hormone exocytotic data on normal and PRLoma AdH cell cultures considering within-subject effect, and characterized the effects of hypokalaemia and hypocalcaemia separately by comparing different K^+ and Ca^{2+} treatment groups to their control, over time. In the investigation of hypocalcaemia on hormone release, marginal models described the difference in treatment groups – low levels of Ca^{2+} versus the control –, over time. This analysis method presented us with the opportunity to analyze the covariance structure. Winsorization of dataset – in the comparison of ACTH release in different Ca^{2+} treatment groups to the control –, resulted in similar differences as did deletion of the extreme outlier cases from the normal and PRLoma AdH cell population.

The marginal model described the difference of mean cAMP levels – representing the level of NE-activated β-adrenoceptor mechanisms in turkeys – in ELF EMF treated and untreated groups over time. The marginal and piecewise linear mixed models fitted to the longitudinal data, proved the reversibility of the investigated biological system. Using the piecewise linear mixed model, the rate of change in β-adrenoceptor function over time was characterized, while considering inter-individual differences and suitable intra-subject correlation structure. Such sophisticated analysis methods used in biological investigations would be useful in environmental studies, may help prevent harmful environmental effects, and could affect public health and the economy.

Appropriate analytical methods that fit the data are crucial in result interpretation and drawing valid conclusions in biomedical studies, thus may have a role in preventive medicine.

NOVELTIES AND KEY MESSAGES

Epidemiological study: Trends in suicide rates in Hungary

1. Risk estimates of suicide rates were calculated using negative binomial regression models by gender, age group and suicide method in Hungary between 1963 and 2011. The risk of suicide in males was more than twofold of that in females overall and in all age groups. A significant gender difference was found for most suicide methods, except "Poison" and "Drowning". The highest risk, more than twentyfold, in males relative to females was observed in the "Fire-arms and explosives" subgroup. The most frequent method was "Hanging", with a risk of more than fourfold in males.
2. Segmented linear trends in annual Hungarian suicide rates were described using joinpoint regression models overall, by gender and by age group. Three linear segments were identified in suicide rates overall and by gender: a significant increase in the beginning which was followed by a significant decrease then a relative constancy period.
3. The reference population selection by age (> 6 years) in the calculation of suicide rates helps to avoid biased estimates.
4. Segmented trends and risk estimates were described using complex regression models for age-specific suicide frequencies in Hungary to present detailed analyses rather than purely descriptive statistics to reveal more specific and precise information. The practical application of negative binomial and joinpoint regression analyses was demonstrated on epidemiological data in preventive medicine.

Neurological studies: Repeated measurements in a rat model of migraine

5. Repeated measures analysis models were applied on trigeminal activation data over time and spatially from the bregma in the brain. These statistical models were able to investigate the effect of time and space considering related data in models of pain in the rat.

Neuroendocrine studies: Repeated measurements of hormone release under hypoionic conditions

6. ACTH and PRL hormone release were investigated over time at different, low extracellular K^+ and Ca^{2+} levels on normal and PRLoma AdH cell populations using repeated measures statistical models, taking into account within-subject effects. Significant alteration in hormone exocytosis was detected over time applying marginal models in hypocalcaemic treatment groups compared to the untreated group even with using winsorization or excluding the extreme values.

Environmental study: Repeated measurements in turkeys exposed to electromagnetic radiation

7. The effect of ELF EMF on repeated measurements of intracellular mechanisms was investigated using marginal and piecewise mixed models on turkeys, so behavioral patterns defined by receptor functions could be characterized to compare treated and control groups over time, considering inter-individual differences and intra-subject correlations.
8. Different statistical methods could reveal the significant effect of ELF EMF over time. Piecewise linear mixed model described the segmented pattern (rate of change) of the treated group during the experiment. A sharp linear reduction was found in NE-activated β-adrenoceptor function in the treated birds in the treatment period, and then a remarkable linear growth in the regeneration period (no ELF EMF exposure) based on the fitted statistical analysis model.
9. Based on the marginal and piecewise mixed models we could highlight that the examined NE-dependent β-adrenoceptor function of the ELF EMF treated group converged to the starting condition in the regeneration period while data of the control system reflected no induced alteration.

10. Appropriate statistical analysis methods need to be used to help answer the scientific question, thus these are crucial in result interpretation in public health and may improve the evaluation of longitudinal biomedical and environmental studies from preventive aspects.

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ORIGINAL PAPER

The use of regression methods for the investigation of trends in suicide rates in Hungary between 1963 and 2011

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Abstract
Purpose Suicide rates in Hungary have been analyzed from different aspects in recent decades. However, only descriptive rates have been reported. The aim of our epidemiological study was to characterize the pattern of annual rates of suicide in Hungary during the period 1963–2011 by applying advanced statistical methods.
Methods Annual suicide rates per 100,000 population (>6 years) for gender, age group and suicide method were determined from published frequency tables and reference population data obtained from the Hungarian Central Statistical Office. Trends and relative risks of suicide were investigated using negative binomial regression models overall and in stratified analyses (by gender, age group and suicide method). Joinpoint regression analyses were additionally applied to characterize trends and to find turning points during the period 1963–2011.
Results Overall, 178,323 suicides (50,265 females and 128,058 males) were committed in Hungary during the investigated period. The risk of suicide was higher among males than females overall, in all age groups and for most suicide methods. The annual suicide rate exhibited a significant peak in 1982 and remained basically constant after 2006. Different segmented patterns were observed for the suicide rates in the various age groups.
Conclusions Suicide rates revealed segmented linear pattern. This is the first detailed trend analysis with risk estimates obtained via joinpoint and negative binomial regression methods simultaneously for age-specific suicide frequencies in Hungary.

Keywords Hungarian suicide rate · Gender difference · Negative binomial regression · Relative risk · Joinpoint regression

Introduction

Suicide is a huge multifactorial and polycausal problem throughout the world. Data have been reported from numerous countries (based on death registries), relating to various periods, with examinations of potential associations between suicide and aspects such as gender, age, ethnic origin, employment status, occupation, psychiatric disorders, physical characteristics, smoking, suicide methods and a history of self-harm or suicide attempts [11, 14, 22, 29, 30]. The possible relationship of seasonality in suicide with characteristics, such as clinical, bioclimatic, sociodemographic or biological factors has also been investigated [9], as have the effects of deprivation indices reflecting the socioeconomic status [20], or educational impact for health professionals in contact with suicidal patients [17, 24, 27].

Värnik [30] reveals that the highest suicide rates (SRs) worldwide between 1950 and 2009 were observed in Japan, Hungary and Lithuania. The overall SR was reported to be highest of all in Hungary between 1965 and 1990 [30], third highest in Hungary among 17 countries between 2000 and 2004 [11], and eighth highest among 105 countries in

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1978–2009 according to the latest suicide data reported by the WHO [30]. Gender-specific analyses indicated that the observed SRs for males and females were highest from among 47 countries in Hungary in 1980–1984, but then decreased to seventh and third highest, respectively, in 1995–1999 [14].

SRs in Hungary have been investigated from many aspects in the past 50 years. Rihmer et al. [21] described the epidemiological and clinical perspectives in a narrative review relating to the period between 1961 and 2011. Studies have been published on the variation in annual SRs with gender and age [32], marital status, season, urban vs. rural living, regional distribution, alcohol and tobacco consumption, antidepressant prescription, unemployment rate, psychosocial factors, cultural, sociopolitical and economic features [2], and genetic (Finno-Ugrian suicide hypothesis) and biological contributions [21].

Trends and risk estimates of annual SRs overall or broken down by risk factors have rarely been investigated for the whole of a country [19] and not in a complex way in Hungary: only descriptive annual rates of suicide have been reported from Hungary, and there have been no publications on trends (especially segmented linear trends) and risk estimates of completed suicide by gender, age group or suicide method between 1963 and 2011.

The aim of our epidemiological study was to investigate the trends in the annual Hungarian SRs and simultaneously to estimate the risk of suicide overall and in the different age groups and with the different suicide methods by gender, involving the use of statistically internationally comparable risk measurements of the pattern of suicide in Hungary.

Methods

Suicide and population data

The annual Hungarian suicide data were collected from tables published in the Demographic Yearbooks issued by the Hungarian Central Statistical Office between 1963 and 2011. The frequencies of suicides by gender (male or female), for four age groups (7–14, 15–39, 40–59 and 60-years) and eight suicide methods ("Poison", "Gas", "Hanging", "Drowning", "Fire-arms and explosives", "Cutting and stabbing instruments", "Jumping from a high place" and "Non-specified") were reported during the investigated period. The classification of suicide methods was based on the International Classification of Diseases (ICD; Tenth Revision: X60–X84, Y87.0 in 2010) [33]. Suicide codes of intentional self-harm were categorized using the 7th–10th revisions of the ICD during the examined 49 years [8, 16, 33].

Although the ICD coding underwent change, data on all the above suicide methods were published during the study period. The category "Run-over" was defined only in the first 6 years, and was then aggregated into "Non-specified" cases from 1969. The ICD-10 codes [16, 33] were applied to classify suicide methods as: "Poison", "Gas" (X60–X69), "Hanging" (X70), "Drowning" (X71), "Fire-arms and explosives" (X72–X75), "Cutting and stabbing instruments" (X78), "Jumping from a high place" (X80) and "Non-specified" (X76–X77, X79, X81–X84, Y87.0).

The Hungarian population data were published for age in 5-year intervals (0–4, 5–9, 10–14 years, etc.) and for gender for each year in the Demographic Yearbooks of the Hungarian Central Statistical Office between 1963 and 2011. The live birth data on the 5–9-year age group were used to determine the 7–9-year population, and the counts for the 7–9 and 10–14-year groups were summed to obtain the data on the 7–14-year group. Overall therefore, the suicide and the population dataset (>6 years) were considered from the aspect of six variables: year, gender, age group, suicide method, suicide frequency and population frequency.

Statistical analysis

The average annual Hungarian SRs per 100,000 population (>6 years) were calculated overall, by gender, age group and suicide method (ranges are given as minimum and maximum for annual SRs). Trends between 1963 and 2011 for SRs overall and in stratified analyses by factors, such as gender (reference group: female), age group and suicide method were investigated using negative binomial (NB) regression models [1]. The NB regression model for total annual data contained only one independent variable year, all other NB regression models included explanatory variables year and gender. The relative risk (RR) and its 95 % confidence interval (95 % CI) with p value were calculated. Trends were also described using joinpoint (JP) regression analyses, determining turning points for the examined 49-year period [12].

Crude rates were expressed in JP regression analyses as the number of suicide cases per 100,000 population at risk, with the year as an independent factor. The assumption in the regression model was that the random errors followed Poisson distribution and the regression coefficients were estimated by weighted least squares. Further, multiple tests were performed to select the number of joinpoints [18]. Annual changes in percentages were calculated for the fitted linear segments first getting the ratio of linearly fitted SR at the end to the fitted SR at the beginning of the segment then raised to the power of reciprocal of segment length in years, finally subtract one from this power and multiplied by 100.

We presented NB regression models which had an acceptable goodness-of-fit Chi-square test result. All p values of <0.05 were considered to indicate statistical significance. The type I error was reduced on use of the Bonferroni correction for the p values multiplying them with the number of significance tests run in all NB regression models and the number of models run separately in each strata in JP regression models: total cases, by gender or by age groups [7]. When a p value was greater than 1 after the multiplication in Bonferroni correction, it was reduced to 1.00.

All NB regression analyses were performed using the statistical software packages Stata (Release 9. StataCorp LP, College Station, TX, USA) and R (Version 3.0.2. R Foundation for Statistical Computing, Vienna, Austria). JP regressions were carried out with the freely available Joinpoint Regression Program (Version 4.0.4. Surveillance Research Program, National Cancer Institute, Bethesda, MD, USA).

Results

Suicide rates

Overall, 178,323 suicides were committed in Hungary during the period 1963–2011 (28.2 % females and 71.8 % males; Table 1). The average SR varied between 26.03 and 51.31 and the overall average SR was 38.44 in the population older than 6 years. The average SR in males was

Table 1 Suicide frequencies, average suicide rates (SRs) per 100,000 population (>6 years) and relative risk (95 % confidence interval) of trends in suicide rates using negative binomial regression are summarized overall and by gender for various age groups and suicide methods in Hungary during the period 1963–2011

Groups (N no. of suicide cases)	Descriptive statistics			Negative binomial regression	
	Gender	N (1963–2011)	Average SR (per 100,000)	Relative risk (95 % confidence interval)	p value[a]
Total annual data	–	178,323	38.44	0.9919 (0.9840–0.9997)[b]	<0.05
Total annual data by gender	Male	128,058	57.75	2.8348 (2.6020–3.0884)	<0.05
	Female	50,265	20.78		
Age groups					
7–14 ($N = 639$)	Male	502	1.73	3.4809 (2.2187–5.4613)	<0.05
	Female	137	0.50		
15–39 ($N = 46,181$)	Male	36,559	39.43	3.8448 (3.1627–4.6740)	<0.05
	Female	9,622	10.56		
40–59 ($N = 68,589$)	Male	51,532	82.70	3.3576 (2.8548–3.9488)	<0.05
	Female	17,057	25.07		
60– ($N = 62,914$)	Male	39,465	102.86	2.4899 (2.0527–3.0201)	<0.05
	Female	23,449	42.61		
Suicide methods					
Poison ($N = 42,338$)	Male	21,698	9.75	1.1364 (0.8424–1.5330)	1.00
	Female	20,640	8.52		
Gas ($N = 3,666$)	Male	1,880	0.85	1.8509 (1.1357–3.0166)	<0.05
	Female	1,786	0.75		
Hanging ($N = 101,460$)	Male	83,476	37.65	5.1135 (4.4196–5.9162)	<0.05
	Female	17,984	7.44		
Drowning ($N = 6,518$)	Male	3,187	1.44	1.0535 (0.8757–1.2673)	1.00
	Female	3,331	1.38		
Fire-arms and explosives ($N = 4,193$)	Male	4,057	1.84	32.6679 (22.5979–47.2252)	<0.05
	Female	136	0.06		
Cutting and stabbing instruments ($N = 3,320$)	Male	2,623	1.19	4.1136 (3.3926–4.9877)	<0.05
	Female	697	0.29		
Jumping from a high place ($N = 8,486$)	Male	4,800	2.16	1.4029 (1.1114–1.7710)	<0.05
	Female	3,686	1.52		
Non-specified ($N = 8,342$)	Male	6,337	2.87	3.4605 (3.0501–3.9262)	<0.05
	Female	2,005	0.83		

[a] Bonferroni-corrected p values are given

[b] Relative risk for the factor year is presented for the total annual data. In other models (with explanatory variables year and gender), Table 1 focuses only on the factor gender (reference group: female) and relative risks for the year are not shown

57.75 (range 41.93–75.19) and in females was 20.78 (range 11.11–32.12). The SR was higher in males than in females overall, in each age group and in each suicide method. The average SR increased with age [average SR (range): 7–14 years: 1.13 (0.23–3.11), 15–39 years: 25.15 (13.17–35.81), 40–59 years: 52.56 (38.58–70.62), and 60-years: 67.09 (37.14–96.28)]. Table 1 shows the distribution of the Hungarian suicide frequencies overall and in the different subgroups (broken down by gender, age group and suicide method).

The most frequent suicide methods in the investigated period were "Hanging" (56.9 %) and "Poison" (23.7 %), the others remaining under 5 % (Table 1). The average SRs per 100,000 population (>6 years) for the various suicide methods during the 49 years were as follows: "Hanging": 21.87, "Poison": 9.11; "Jumping from a high place": 1.83, "Non-specified": 1.80, "Drowning": 1.41, "Fire-arms and explosives": 0.91, "Gas": 0.80, "Cutting and stabbing instruments": 0.72.

Regression models

Relative risks of gender difference were calculated to estimate the risk of committing suicide overall, by age groups and suicide methods during the 49 years (Table 1). Overall risk of suicide was near threefold higher among males than females, which was varied across age groups. Similarly, significantly higher risks were detected in the male groups as compared with the females in most suicide methods (more than 20-fold risk was the highest in the "Fire-arms and explosives" subgroup), except "Poison" and "Drowning" subgroups (Table 1).

The NB regression model for total annual data revealed decreasing annual trend in the SRs during the study period, which remained significant after the Bonferroni p value correction (Table 1). The annual SR data displayed segmented lines rather than a decreasing linear trend. Thus, the JP regression analyses were applied to refine the annual trends. Overall, the JP regression model fitted a segmented line indicating two main changes in the trend: a peak in 1982, followed by a significant decrease until 2006, and a basically constant period thereafter. From 1963 to 1982, there was a significantly increasing linear trend in the suicide data, and then a significant decline between 1982 and 2006, followed by a rather constant level from 2006 to 2011 (annual changes in percentages, slopes with p values of each segment: 2.9 %, 1.15 ($p < 0.001$); −2.8 %, −1.09 ($p < 0.001$) and 0.2 %, 0.06 ($p = 0.84$), respectively; Fig. 1).

The gender-specific analyses depicted turning points in 1981 and 2002 for females and 1983 and 2006 for males, and changes in segmented linear trends (Table 2; Fig. 1). The annual changes in percentages and slopes of the segments in the female cases for 1963–1981 were: 3.2 %, 0.75 ($p < 0.001$), for 1981–2002: −4.1 %, −0.86 ($p < 0.001$) and for 2002–2011: −1.7 %, −0.20 ($p = 0.08$), and in the male cases for 1963–1983 were: 2.7 %, 1.58 ($p < 0.001$), for 1983–2006: −2.4 %, −1.41 ($p < 0.001$), and for 2006–2011: −0.2 %, −0.10 ($p = 1.00$).

Significant JP segmented line fits were found in each of the age groups except the 7–14-year-old group. Detailed results of the JP regression analyses are presented in Table 2. Additionally, Fig. 2 depicts the regression estimations (slopes and significance level) of the JP analyses in all age groups. Peaks were observed following significant increases in suicide trends in 1986, 1984 and 1980 in the age groups of 15–39, 40–59 and 60-years, respectively. The SRs declined in all the age groups 15 years and over, after the 1980s, this being more marked in those aged 60 years and over (annual change in percentage −3.4 %, slope −2.25; $p < 0.001$) until 2005, but has remained constant since then.

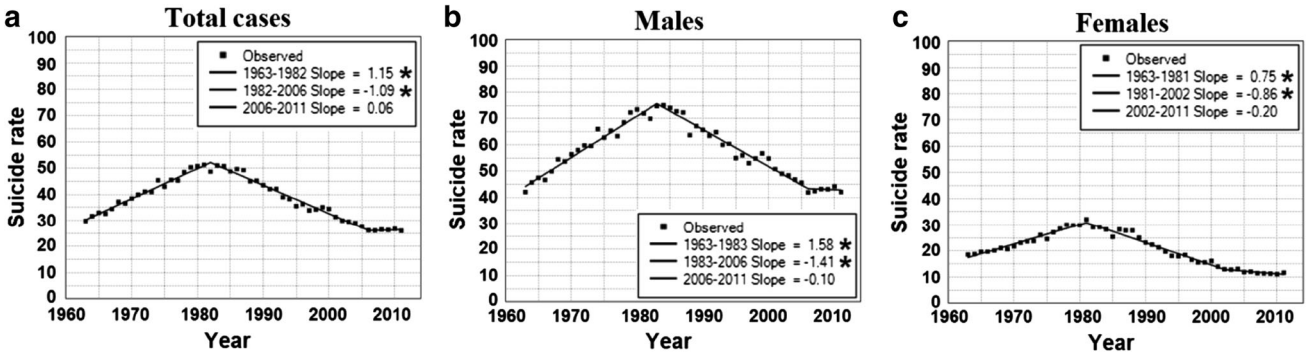

Fig. 1 Results of fitted segmented lines of annual Hungarian suicide rates per 100,000 population (>6 years) using joinpoint regression models overall (**a**) and by gender [males (**b**), females (**c**)] created in Joinpoint Regression Program are given for the period 1963–2011. Legends on each diagram relative to the fitted JP segments in year intervals and regression estimates (slopes). *Asterisks* are used to indicate significant slopes ($p < 0.001$)

Table 2 Results of joinpoint (JP) regression models for Hungarian crude suicide rates per 100,000 population (>6 years) overall, by gender and by age group are presented for the period 1963–2011

Suicide data (1963–2011) (*N* no. of cases)	No. of JPs	JP estimate year (95 % confidence interval)[a]	Model comparison[b] (Bonferroni-corrected *p* value)
Total cases ($N = 178,323$)	2	1982 (1980–1983)	JP1 vs. JP2 (0.0004)
		2006 (2001–2009)	JP2 vs. JP3 (0.1458)
Gender			
Males ($N = 128,058$)	2	1983 (1981–1984)	JP1 vs. JP2 (0.0160)
		2006 (1992–2009)	JP2 vs. JP3 (1.0000)
Females ($N = 50,265$)	2	1981 (1980–1983)	JP1 vs. JP2 (0.0009)
		2002 (1997–2005)	JP2 vs. JP3 (0.6693)
Age groups			
7–14 ($N = 639$)	0	–	JP0 vs. JP1 (0.2827)
15–39 ($N = 46,181$)	3	1975 (1971–1978)	JP2 vs. JP3 (0.0018)
		1986 (1983–1988)	JP3 vs. JP4 (0.4640)
		1996 (1993–2000)	
40–59 ($N = 68,589$)	2	1984 (1982–1985)	JP1 vs. JP2 (0.0018)
		1995 (1990–1999)	JP2 vs. JP3 (1.0000)
60– ($N = 62,914$)	2	1980 (1978–1982)	JP1 vs. JP2 (0.0018)
		2005 (2000–2007)	JP2 vs. JP3 (1.0000)

[a] Estimated turning points in years are given

[b] In model comparisons, JP*n* represents a JP model with *n* number of joinpoints

Discussion

Main findings

The trends in SRs in our study described using segmented linear models overall, by gender and by age group in Hungary between 1963 and 2011. Results revealed an overall peak in 1982 and remained constant after 2006. There was a significant, approximately 3 % annual increase until 1982 (an increment of $\sim$20 suicides per 100,000 persons) during two decades. This was followed by a significant decrease during the next about two decades, when SRs decreased approximately to the same level where they started in 1963, and with relative constancy after 2006 overall. Similar trends were observed in both genders. The different numbers of turning points reported by age group in the segmented linear trends revealed significant, but shifted peaks in the 1980s.

Risk estimates of SRs were calculated by gender, age group and suicide method, and highlighted a more than twice as great a risk in males than in females overall. The risk of suicide in males was more than twofold in all age groups. A significant gender difference was found for most suicide methods, except "Poison" and "Drowning". The highest risk, more than 20-fold, in males relative to females was observed in the "Fire-arms and explosives" subgroup.

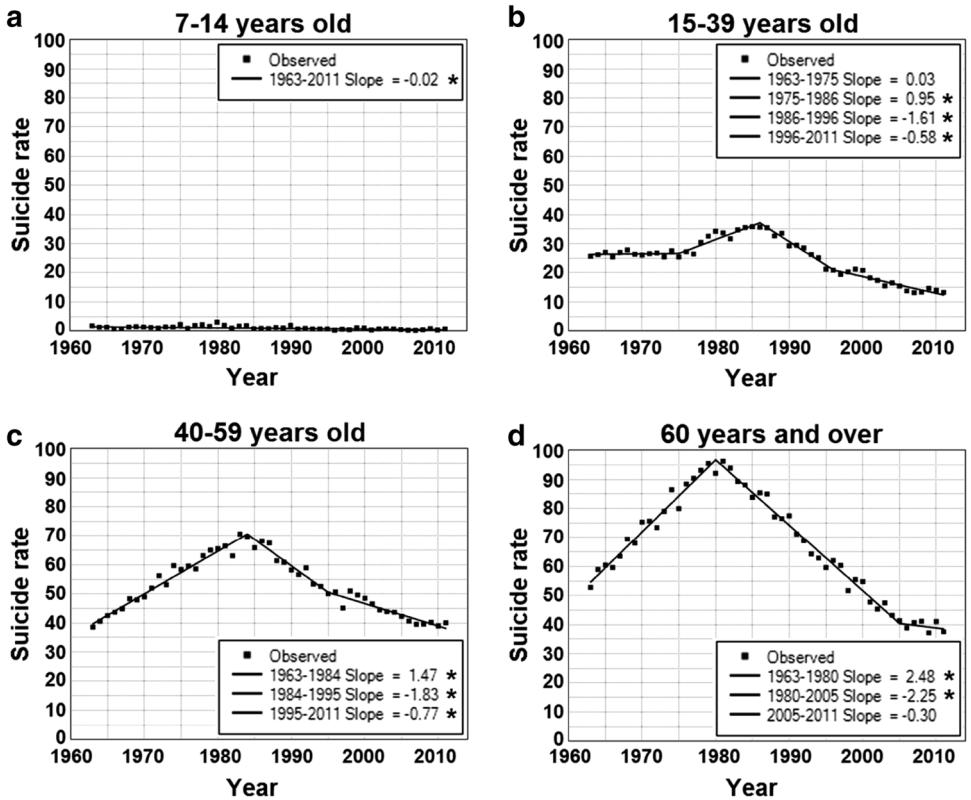

Fig. 2 Results of fitted segmented lines of annual Hungarian suicide rates per 100,000 population (>6 years) using joinpoint regression models by age group of 7–14 (**a**), 15–39 (**b**), 40–59 (**c**) and 60-years old (**d**) created in Joinpoint Regression Program are given for the period 1963–2011. Legends on each diagram relate to the fitted JP segments in year intervals and regression estimates (slopes). *Asterisks* are used to indicate significant slopes ($p < 0.001$)

The most frequent method was "Hanging", with a risk of more than fourfold in males.

Strength and limitation

Instead of reporting only the SRs, we applied regression models to estimate the trends in Hungarian SRs between 1963 and 2011, including JP regression, which involves the best fit of segmented lines connected at the "joinpoints" (turning points) through use of a Poisson model of variation [12]. As far as we know, this was the first utilization of JP regression to investigate the pattern of trends in SRs in Hungary. In the JP regression analysis, the slopes with annual changes in percentages of the segmented lines were used to determine the annual changes in SR in Hungary, while other studies applying JP regression for suicide data involved only the estimated annual percentage change [19]. We consider that the use of slopes is more appropriate in case of linear fit and generally gives a complete picture of trends with the annual percent change.

The risk of suicide in Hungary was calculated by NB regression, which can handle overdispersed data common in rare discrete events, such as suicide frequencies relative to the population [1, 6]. Although SRs in Hungary have often been investigated, detailed statistics have scarcely been reported. The NB regression method applied gives valuable risk estimates for the whole of the examined period, and JP regression analyses the pattern of the trends in detail.

The reference population was given only by gender and age group to calculate the SRs. We could investigate the risk of suicide by gender, age group and suicide method only from the data published in the Demographic Yearbooks of the Hungarian Central Statistical Office. However, the suicide methods were broken down in more detail than the violent and non-violent categories usually used in the literature.

Comparison with other studies

Rihmer et al. [21] recently reported on SRs in Hungary in different associations (e.g. marital status, seasonal aspects, regional distribution, alcohol and tobacco consumption, antidepressant prescription, unemployment rate, etc.), but the relationships between the SRs and the above factors were not subjected to detailed analyses. The previous study used the entire Hungarian population as reference to calculate the SRs, whereas we focused only on the population older than 6 years, for whom suicide frequencies have been published. Nevertheless, our findings show similar patterns in SRs overall, by gender and by age groups to those reported previously in Hungary for the investigated period.

The Hungarian average SR was over twofold compared with the overall world SR found in 105 countries in 2008 [30]. In the previously mentioned study, a large difference in average SR was also observed by gender in Hungary, as compared with worldwide: about fourfold in males, and about twofold in females. The overall male/female SR ratio of four proved to be the highest in Europe (48 countries) by the previous author, and greatly (more than twofold) exceeded the world ratio.

Studies often investigate suicide methods broken down into violent (ICD-10: X70–X84; e.g. "Hanging", "Drowning", "Fire-arms and explosives", "Cutting and stabbing instruments" or "Jumping from a high place") and non-violent (ICD-10: X60–X69; e.g. "Poison", "Gas") categories [9], rather than focusing on more detailed subgroups. Our results were consistent with the findings of previous studies that the risk of suicide is greater in males than in females, and males tend to use more violent methods [5, 10, 11, 21, 23]. Värnik et al. [29] reported RRs for males vs. females in more detailed suicide method subgroups in 16 European countries between 2000 and 2004/5, and found that suicide frequencies were higher in males than in females in all the suicide method subgroups. Although "Hanging" was the most frequent method in their analysis too, the RR of males for this subgroup was about one-third of what we found for the 49-year period. Similarly to our results, the highest RR was found for the "Fire-arms" subgroup in the examined 16 countries between 2000 and 2004/5, as compared with our study between 1963 and 2011. Tamosiunas et al. [28] examined similar suicide methods among 25–64-year olds in Kaunas, Lithuania, from 1984 to 2003. "Hanging" was the most frequent suicide method in Lithuania during that period. Our findings on suicide frequencies by suicide methods in Hungary are consistent with those of Berecz et al. [4].

We also investigated age group as a risk factor in suicide in Hungary. Hawton and van Heeringen [11] reported that SRs among elderly people have fallen in many countries, while those for younger age groups have risen. The situation in Hungary was slightly different as SRs were higher for older age groups.

Similarly to ourselves, Klieve et al. [13] analyzed the annual trends in SRs and determined RRs using NB regression in Australia. Morriss et al. [17] have also presented annual trends in SRs in Lancashire, United Kingdom, by applying NB regression.

We additionally analyzed annual trends in suicide overall and by gender, age group and suicide method by utilizing NB regression, but JP regression analysis gave better fits of our data, as the trends were not exactly linear, but rather segmented linear. JP regression was likewise the method of choice in Lithuania. Tamosiunas et al. [28]

found significant increase by calculating estimated annual percent changes in JP regression for men aged 25–64 years during 1984–2003. In contrary, we revealed a significant decrease in SRs during this period.

In our study, we focused on completed suicide data; however, suicide attempt (also called parasuicide) is a well-known indicator for suicide [31]. A paper of Hungarian suicide attempters concluded that gender differences should be considered in developing efficient complex therapy and prevention strategies [10]. Other studies focused on suicidal elderly people with geriatric depression, whose attempts are more likely to lead to completed suicide than in younger age groups, and discussed treatment and psychotherapeutic interventions during which they require special attention as they have lower and delayed response [25, 26]. Besides the above-mentioned studies our analysis with sophisticated methodology may support the use of gender and age group as factors in developing intervention strategies in prevention of suicide.

Among 12 European countries Hungary had high rates of attempted suicide in the use of pesticides and solvents in the period 1989–1993 [15]. It is consistent with the findings of Berecz et al. [4] and with our results for the whole country having "Poison" one of the most frequent suicide methods. Berecz et al. concluded that the decrease of SRs in Hungary between 1991 and 2001 could be due to the decreased access to highly toxic pesticides and other toxic substances. We agree with Michel et al. on the importance of understanding and monitoring the influencing factors of suicide. Several intervention studies presented promising results in Hungary highlighting the importance of community-based interventions and the education of general practitioners (GPs) [24, 27]. In contrast, Morriss et al. [17] found that a brief training program did not affect SR in England in 1997. Balazs et al. [3] revealed that media reports on a suicide case in Hungary in 2011 were not in line with the recommendation of the international guidelines on media coverage of suicide, and emphasized the importance of using more preventive information rather than provocative aspects during the disclosure. The authors highlighted that Hungarian mass media coverage of suicide has rarely been investigated, although such analysis could provide useful information for preventive medicine.

Conclusions

The trends in annual Hungarian SRs per 100,000 population (>6 years) were analyzed by NB and JP regression methods to determine RRs for gender differences overall, by age group or by suicide method, and to find peaks in the linear segmented trends by gender or age group between 1963 and 2011. The JP regression models gave a more complete picture of Hungarian suicide trends than the NB regression models. Our results furnish the first complete analysis for annual Hungarian SRs with the trends and risk estimates, such a complex analysis not being commonly used for suicide data (merely either trend analyses or risk estimates are usually given). We have presented detailed analyses rather than purely descriptive statistics for the suicide trends, as descriptive statistics provides only virtual trends, whereas both fitted regression models reveal more specific and precise information.

Further, the reference population selection by age (>6 years) in calculation of SRs helps to avoid biased estimates. The importance of analyses' interpretation is getting into the focus recently as social changes are more common. Thus, we have reported internationally comparable data and descriptions of the patterns of suicide in Hungary. To identify subgroups at high risk, such detailed results relating to the trends may be of importance in helping to shape strategies designed to help prevent suicide.

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Conflict of interest The authors declare that they have no conflict of interest.

Informed consent The article does not contain clinical studies or patient data.

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Diverse effects of Brilliant Blue G administration in models of trigeminal activation in the rat

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Abstract Activation of the trigeminal system plays an important role in the pathomechanism of headaches. A better understanding of trigeminal pain processing is expected to provide information helping to unravel the background of these diseases. ATP, a key modulator of nociceptive processing, acts on ligand-gated P2X receptors. Antagonists of the P2X7 receptors, such as Brilliant Blue G (BBG), have proved effective in several models of pain. We have investigated the effects of BBG after electrical stimulation of the trigeminal ganglion and in the orofacial formalin test in the rat. The right trigeminal ganglion of male rats was stimulated either with 5 Hz, 0.5 mA pulses for 5 min (mild procedure) or with 10 Hz, 0.5 mA pulses for 30 min (robust procedure), preceded by 50 mg/kg i.v. BBG. The animals were processed for c-Fos and calcitonin gene-related peptide (CGRP) immunohistochemistry. In the orofacial formalin test, 50 μL of 1.5 % formalin was injected into the right whisker pad of awake rats, following the pre-treatment with BBG. Behaviour was monitored for 45 min, and c-Fos and CGRP immunohistochemistry was performed. BBG attenuated the increase in c-Fos-positive cells in the caudal trigeminal nucleus (TNC) after robust stimulation, but not after mild stimulation. No alterations in CGRP levels were found with either methodology. BBG did not mitigate either the behaviour or the increase in c-Fos-positive cells in the TNC during the orofacial formalin test. These results indicate that P2X7 receptors may have a role in the modulation of nociception in the trigeminal system.

Keywords Trigeminal system · P2X7 receptor · Trigeminal ganglion stimulation · Formalin test

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Introduction

Primary headaches are very common, but underdiagnosed and undertreated neurological conditions. Even following the correct diagnosis, the available therapeutic options often do not provide complete resolution of the pain, and recurrence of the headache after treatment is also a common complaint. Research is, therefore, currently focused on gaining an understanding of the causes of different headache disorders and developing new therapeutic options.

A common mechanism in primary headaches involves activation and sensitization of the trigeminal system, but the exact mechanism of these phenomena remains to be discovered (Tajti et al. 2011).

Recent results suggest that the purine molecule ATP has an important role in the regulation of nociceptive transmission (Burnstock 2013). Molecules targeting the specific receptors (Rs) for ATP (P2X and P2Y-Rs) have been proven to be effective in modulating different pain

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conditions, e.g., neuropathic and inflammatory pain (Ando et al. 2010). Among the ligand-gated P2X-Rs, the P2X7 receptor (P2X7-R) has been intensively studied in different pain states. The P2X7-R is a non-selective cation channel, unique among the P2X-Rs by virtue of its long C terminal domain, and its ability to open a pore permeable to molecules up to 900 Da (Surprenant et al. 1996). Experiments with knock-out mice have revealed that the absence of the P2X7-R leads to the disappearance of mechanical and thermal hypersensitivity in models of neuropathic and inflammatory pain, whereas normal nociceptive processing is retained (Chessell et al. 2005). P2X7-R antagonists have been examined in similar models, and the results underline the importance of the P2X7-R in chronic pain conditions (Honore et al. 2006; McGaraughty et al. 2007). Results from an acute inflammatory pain model suggest that the P2X7-R may participate in the development of central sensitization (Itoh et al. 2011), a common feature in the trigeminal system during migraine attacks manifested by the presence of allodynia (Burstein et al. 2000).

However, little information is available as concerns the role of P2X7-Rs in the trigeminal system. In a model of orofacial pain, the chronic constriction injury model, inhibition of the P2X7-R led to a decrease in tactile allodynia through a p38 MAPK-dependent mechanism (Ito et al. 2013). Goloncser and Sperlagh recently reported that blockade of P2X7-Rs in mice by Brilliant Blue G (BBG) [a selective, non-competitive P2X7-R antagonist, with good blood–brain barrier permeability (Jiang et al. 2000)] reduced thermal hyperalgesia after systemic nitroglycerin administration (Goloncser and Sperlagh 2014). These results lend support to the theory that P2X7-Rs may play a crucial part in the development of headache disorders.

Calcitonin gene-related peptide (CGRP) has an essential role in trigeminal nociceptive processing. CGRP infusion causes migraine-like headache in migraineurs (Hansen et al. 2010; Lassen et al. 2002), and the levels of CGRP are higher in migraine patients than in healthy controls (Friberg et al. 1994).

Electrical stimulation of the trigeminal ganglion, an animal model of trigeminal activation, causes plasma protein extravasation (Markowitz et al. 1987), which can be attenuated by drugs effective in migraine therapy (Limmroth et al. 2001). The stimulation results in alterations in the dura mater (Knyihar-Csillik et al. 1995), in the trigeminal ganglion and in the caudal part of the spinal trigeminal nucleus (TNC) (Knyihar-Csillik et al. 1998). In previous investigations, various stimulation frequencies, intensities and durations were applied. Results related to peptide release and c-Fos expression in the TNC after the procedure suggest that both higher frequency and higher intensity lead to an increased stimulation of the trigeminal ganglion cells (Samsam et al. 1999; Takemura et al. 2000). Diverse periods of stimulation caused different morphological alterations in the CGRP-immunoreactive nerve terminals of the dura mater, suggesting the release of CGRP after prolonged stimulation (Knyihar-Csillik et al. 1995). Based on these previous results, we decided to perform the electrical stimulation in two different setups: a mild and a robust one.

Formalin applied to the whisker pad of rats (another model of trigeminal activation) causes a biphasic behavioural effect, the first phase being caused by the direct activation of Aδ and C fibres, while the second phase reflects the process of inflammation (Porro and Cavazzuti 1993). Activation of the trigeminal system has been demonstrated by c-Fos immunohistochemistry to be present at the level of the TNC after the injection of formalin (Wang et al. 1994). Both phenomena are suitable for assessments of the influence of substances on inflammation.

The aim of the present study was to examine the effects of a P2X7-R antagonist, BBG, in the electrical stimulation and orofacial formalin models of acute trigeminal activation.

Materials and methods

Ethical approval

The procedures used in this study followed the guidelines of the 8th Edition of the Guide for the Care and Use of Laboratory Animals and the Use of Animals in Research of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). The experiments were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV/352/2012). Male Sprague–Dawley rats were housed under standard laboratory conditions, on a 12-h light–dark cycle, with tap water and rat chow available ad libitum. The suffering of the animals and the number of animals used were kept at a minimum.

Mild stimulation procedure

Twenty-four animals (250–300 g) were used. Half of the animals received an intravenous (i.v.) injection of 50 mg/kg BBG, while the other half were injected with the vehicle of BBG, physiological saline. Two h after the BBG or saline injection, the animals were deeply anesthetised with chloral hydrate (400 mg/kg) and the head was secured in a stereotaxic apparatus (Stoelting Co., Wood Dale, USA). A hole, approximately 3 mm in diameter, was drilled into the

right side of the skull with a dental drill, and a concentric bipolar electrode (FHC Inc., Bowdoin, USA, CBBRE75) was lowered to the right trigeminal ganglion. Half of the animals from the saline-treated group (5SStim) and half of the animals from the BBG group (5BStim) were electrically stimulated for 5 min with 5 Hz, 0.5 mA, 0.5 ms delay twin pulses generated by an Electrostimulator ST3 (Medicor Hungary). The other animals from both groups were used as sham animals: the electrode was lowered to the right trigeminal ganglion for 5 min, but no stimulation was performed (5SSham and 5BSham groups). After both procedures, the animals were returned to their home cages and maintained under deep anaesthesia covered by a warming blanket for 2 h.

Robust stimulation procedure

Twenty-one animals (250–300 g) were used; the treatment and surgical procedures were identical to the previous ones, except that the stimulation parameters of 10 Hz, 0.5 mA, 0.5 ms delay twin pulses were applied for 30 min, and the animals were maintained under deep anaesthesia, covered by a warming blanket, in their home cages for 4 h from the beginning of the stimulation. In both the mild and the robust paradigm, the jaw of the animal was twitching during the electrical stimulation, indicating the correct placement of the electrode.

Overview of the robust stimulation groups:

Saline + 30-min sham: 30SSham ($n = 6$).
Saline + 30-min stimulation: 30SStim ($n = 5$).
BBG + 30-min sham: 30BSham ($n = 4$).
BBG + 30-min stimulation: 30BStim ($n = 6$).

Orofacial formalin test

Behavioural tests

Rats ($n = 52$, 200–240 g) were injected i.v. either with 50 mg/kg BBG or with physiological saline. One hour and fifty minutes later, the animals were placed in a 30 × 30 × 30 cm box, with mirrored walls for the monitoring of behavioural activity. After 10 min of habituation, the animals were taken out of the box and under minimal restraint were injected subcutaneously with 50 µL of either physiological saline (SSal and BSal groups) or 1.5 % formalin (SForm and BForm groups) to the right whisker pad. After the injection, they were returned immediately to the box and their behaviour was monitored for 45 min under video surveillance. The injection of formalin causes a behavioural response, which consists in rubbing and scratching of the injected whisker pad with the ipsilateral fore- or hindpaw. The rate of this behaviour correlates with the pain sensation caused by formalin (Clavelou et al. 1989). The 45-min period was divided into 15 × 3-min blocks, and the total time spent rubbing the injected whisker pad, measured in seconds, was taken as the nociceptive score in the given block. The normal grooming activity of the saline-treated animals was measured as control. After the monitoring period, the animals were returned into their home cages and maintained under standard laboratory conditions until perfusion, which was performed under deep chloral hydrate anaesthesia 4 h after the whisker pad injections.

Immunohistochemistry

Animals were perfused with 0.1 M phosphate-buffered saline (PBS), followed by 4 % paraformaldehyde in 0.1 M phosphate buffer. The whole brain and the upper cervical spinal cord were removed and postfixed overnight in the same fixative. The correct placement of the electrode was checked during autopsy, the electrode was in the ganglion in all cases. Cryoprotection was performed, using gradient sucrose solutions up to 30 %. Sections from the TNC were prepared from the block, ranging from 1 mm rostral to 4 mm caudal from the obex, and the ventral part of the control (left) side of the blocks was marked by a small incision to enable side discrimination on the sections. 30 µm thick transverse sections were cut and serially collected in 18 wells containing PBS with 0.1 % sodium azide, the overall distance therefore being 540 µm between consecutive sections. Free-floating sections were immersed in 0.3 % H_2O_2 in PBS to block endogenous peroxidase activity. After several washes in PBS containing 1 % Triton X-100 (PBS-T), they were incubated for 1 h in PBS-T containing 10 % normal goat serum. The sections were then incubated overnight at room temperature in the primary antibody for c-Fos (1:2000, Santa Cruz Biotechnology, sc-52) or CGRP (1:20,000, Sigma-Aldrich C8198). The immunohistochemical reaction was visualised by using the Vectastain Elite avidin–biotin kit (PK6101; Vector Laboratories,) with 3,3′-diaminobenzidine as chromogen (Sigma-Aldrich) intensified with nickel ammonium sulphate (Scharlau Chemie). The specificity of the immune reactions was verified by omitting the primary antisera.

Sections were mounted onto glass slides, air-dried and coverslipped with DPX mounting medium (Scharlau Chemie). On the basis of anatomical observations, sections from the same rostro-caudal level were compared during the statistical evaluation.

An observer blinded to the treatment procedures used a Nikon Optiphot-2 light microscope under a 20× objective to count cells immunopositive for c-Fos. The whole area of the laminae I-II in each section of the TNC was evaluated and counted.

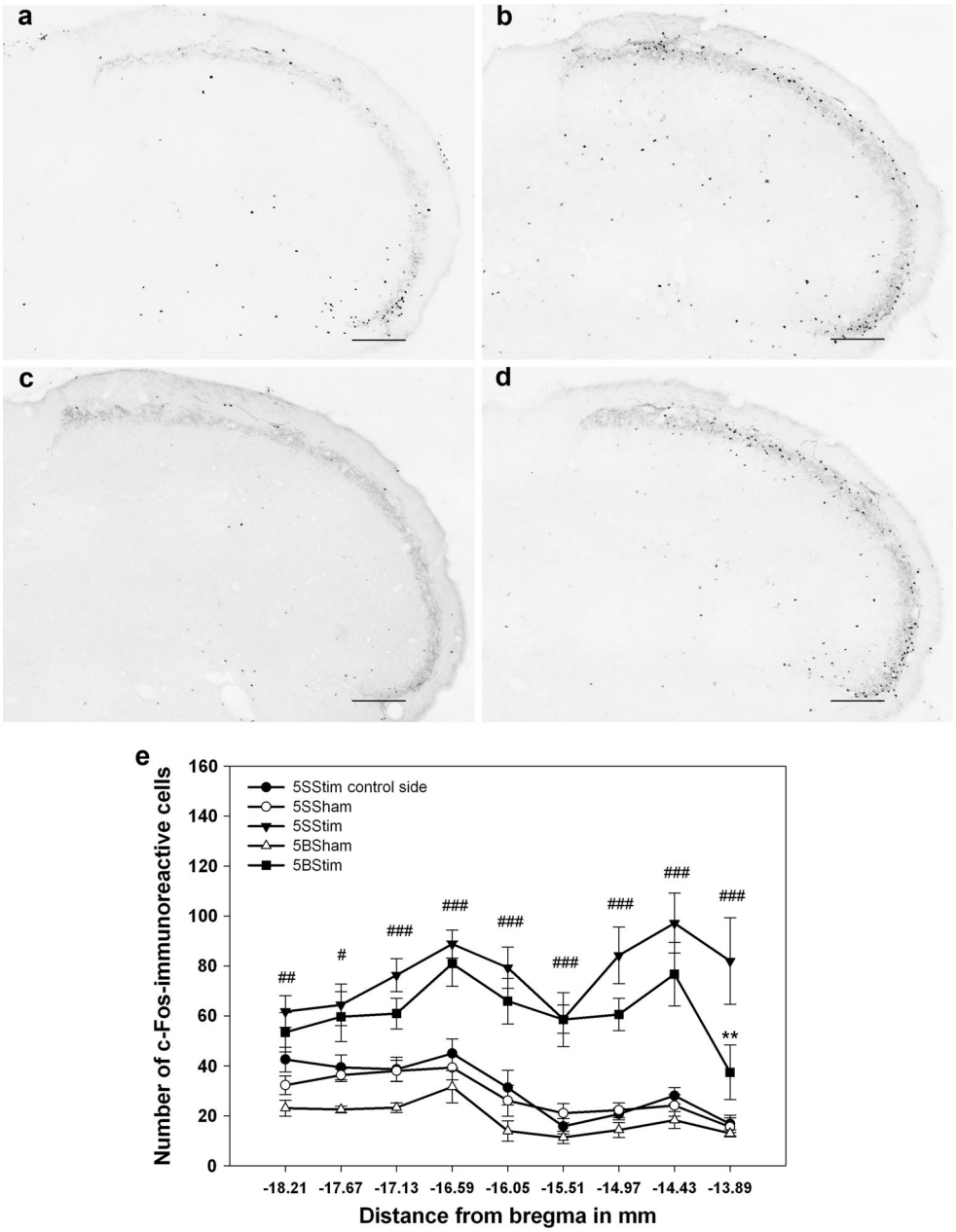

Fig. 1 Summary of the results from the mild stimulation paradigm regarding c-Fos immunostaining. Representative photos from the *right sides* of the four treatment groups after c-Fos immunohistochemistry, taken at 16.05 mm caudally from bregma: **a** 5SSham, **b** 5SStim, **c** 5BSham, **d** 5BStim. *Scale bar* 200 μm. Diagram showing the number of c-Fos-immunoreactive cells across the different levels of the TNC after mild electrical stimulation of the trigeminal ganglion (group mean ± SEM) (**e**). There was no significant difference between the control (*left*) sides (data not shown). For clarity, only the control side of the saline-treated stimulated group is presented. The *hashmarks* indicate significance in the comparison of the *right sides* of the 5SSham and 5SStim groups at different levels of the TNC ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). BBG treatment showed an attenuating tendency, though it proved to be significant merely at the level of −13.89 mm ($^{**}p < 0.01$, 5SStim–5BStim)

CGRP-stained sections were photographed with a Zeiss AxioCam MRc Rev.3 digital camera attached to a Zeiss AxioImager M2 microscope. Digital images were taken with a 20× objective in TNC laminae I–II, and the area covered by CGRP-immunoreactive fibres was measured through the use of ImageProPlus 6.2 software (Media Cybernetics Inc.) by an observer blinded to the treatment procedures.

Statistical analysis

The cell count results were aligned according to the rostrocaudal location of the section as mentioned above. Data from different levels of the TNC were handled separately, and analysed by two-way repeated measures ANOVA. The group was used as the between-subject factor and the levels (−13.89, −14.43, −14.97, −15.51, −16.05, −16.59, −17.13, −17.67, −18.21 mm from bregma) as the within-subject factor for the analysis.

When Mauchly's test of sphericity proved to be significant, the Greenhouse-Geisser correction was performed. Pairwise comparisons of group means were performed on the basis of estimated marginal means with Sidak adjustment for multiple comparisons.

The sums of the areas covered by CGRP-immunoreactive fibres were compared between groups according to the

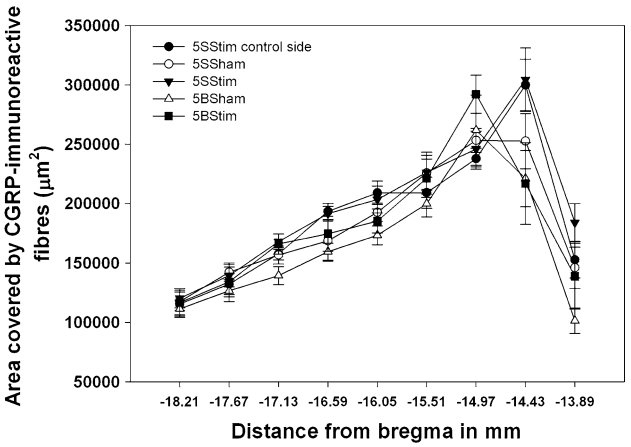

Fig. 2 Diagram showing the area covered by CGRP-immunoreactive fibres at the different levels of the TNC in the different treatment groups after mild electrical stimulation of the trigeminal ganglion (group mean ± SEM). There was no significant difference between either the control or the stimulated sides or the different groups. For clarity, only the control side of the saline-treated stimulated group is presented

different levels, two-way repeated measures ANOVA being used as detailed above.

Nociceptive scores from the behavioural study were compared block by block through two-way repeated measures ANOVA. Groups were used as between-subject factor and blocks (1–15) as within-subject factor for the analysis. Other statistical parameters were identical to those mentioned above.

Statistical analyses were carried out with IBM SPSS Statistics, version 20 (IBM Corporation) software. All tests were two-sided, and $p < 0.05$ was considered to be statistically significant. Graphs were prepared by using SigmaPlot 12.0 (Systat Software Inc.). Data are reported as mean ± SEM.

Results

Mild stimulation procedure

As a significant interaction was found between the two investigated factors (levels and groups, $p < 0.01$) for the number of c-Fos-immunoreactive cells in the mild stimulation paradigm, both effects could not be reported independently, whereas the group differences could be examined separately across different levels on the basis of the estimated marginal means for multiple comparisons.

Lowering of the electrode to the trigeminal ganglion for 5 min without stimulation (5SSham group) did not cause any significant change in the number of c-Fos-immunoreactive cells in the TNC relative to the control side. The comparisons of the cell numbers from the control (left) sides for each of the four treatment groups did not reveal any significant changes (data not shown), and therefore only the data for the stimulated (right) sides of the groups are presented in Fig. 1a–d. Electrical stimulation of the ganglion caused a significant increase in the number of c-Fos-immunoreactive (IR) cells along the whole extent of the examined region of the TNC (Fig. 1a, b, e). BBG exhibited a significant effect ($p < 0.01$) compared with the saline-treated stimulated animals only at the level of −13.89 mm from bregma (Fig. 1b, c, e).

A significant interaction was found between the groups and levels, when the area values from the CGRP measurements were examined. However, the comparisons of the groups and stimulated-control sides at different levels did not reveal any significant alteration (Fig. 2).

Robust stimulation procedure

A significant interaction was not found between the investigated factors of levels and groups for the number of c-Fos IR cells in the robust stimulation paradigm. The levels had a significant effect ($p < 0.001$), and there was also a significant difference between the groups ($p < 0.001$). Pairwise comparisons revealed that there was no significant difference between the control sides of the four treatment groups (data not shown). Lowering of the electrode without stimulation did not cause significant changes in the number of c-Fos IR cells in either sham-treated group as compared with the control (Fig. 3e, 30SSham and 30BSham with the 30SStim control side). Robust stimulation caused a marked increase in the number of c-Fos IR cells in the saline-treated animals (Fig. 3a, b, e, 30SStim compared with 30SSham, $^{\#}p < 0.001$). BBG had a significant attenuating effect on this increase (Fig. 3b, d, e, 30SStim compared with 30BStim).

CGRP expression was not altered by stimulation or BBG administration in any of the animal groups (Fig. 4).

Orofacial formalin test

A significant interaction was found between time and groups ($p < 0.05$) during the analysis of the behavioural data from the orofacial formalin test. The pairwise comparison revealed that the nociceptive scores of the saline-injected groups (SSal and BSal) did not differ from each other at any time point (Fig. 5). The injection of formalin caused a significant increase in the nociceptive scores in blocks 1 and 5–7 (Fig. 5, SForm compared with SSal). After the injection of formalin into the whisker pad, the BBG-treated animals demonstrated significantly increased nociceptive scores in blocks 1, 6 and 8 as compared with the control (Fig. 5, $^{\$}p < 0.05$). The BBG-treated animals spent less time rubbing their formalin-injected side in both

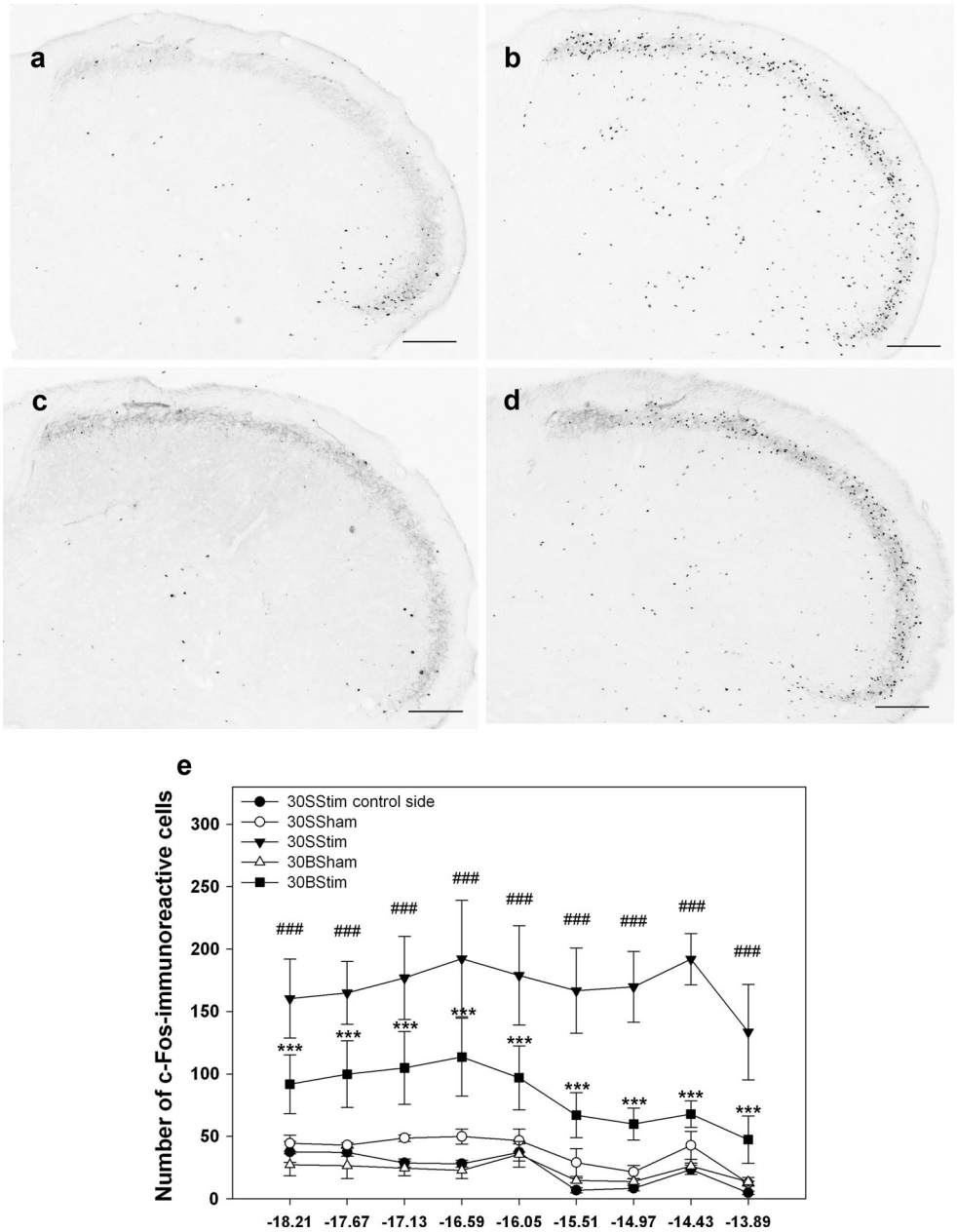

Fig. 3 Summary of the results from the robust stimulation paradigm regarding c-Fos immunostaining. Representative photos from the *right sides* of the four treatment groups after c-Fos immunohistochemistry, taken at 16.05 mm caudally from bregma: **a** 30SSham, **b** 30SStim, **c** 30BSham, **d** 30BStim. *Scale bar* 200 µm. Diagram showing the number of c-Fos-immunoreactive cells at different levels along the rostro-caudal axis in the TNC (group mean ± SEM) in the robust stimulation paradigm (**e**). There was no significant difference between the control sides (data not shown). For clarity, only the control side of the saline-treated stimulated group is presented. Electrical stimulation of the right trigeminal ganglion caused a significant increase in the number of c-Fos-positive cells as compared with the right side of the sham group at all levels examined ($^{###}p < 0.001$ 30SSham–30SStim). BBG treatment had a significant decreasing effect on the cell counts ($^{***}p < 0.001$ 30SStim–30BStim)

blocks 1 and 5–7, but this difference was not significant compared with the SForm group. In block 8, BBG-treated animals spent significantly more time rubbing their whisker pad as did the animals in the formalin group (Fig. 5). In our experiments, the second phase of the formalin test subsided more quickly as expected in the SForm group, while it was more prolonged in the BForm group, and this difference may account for the significant effect of BBG in block 8.

A significant interaction was observed between the levels and groups ($p < 0.001$) for the number of c-Fos IR cells in the orofacial formalin test. There was no significant difference when either the control (left) sides or the control and saline-injected sides were compared at any level (Fig. 6e). The injection of formalin increased the number of c-Fos-IR cells significantly at the levels −16.59 to −15.51 mm, mainly in the central part of the TNC as compared with the saline-injected side in the SSal group. In the BForm group, a similar pattern was observed, except that the difference involved one additional level (−14.97 mm, Fig. 6e). There was no significant difference between the SForm and BForm groups at any level (Fig. 6b, d).

No group difference was found in any of the measured parameters as regards the CGRP immunoreactivity (Fig. 7).

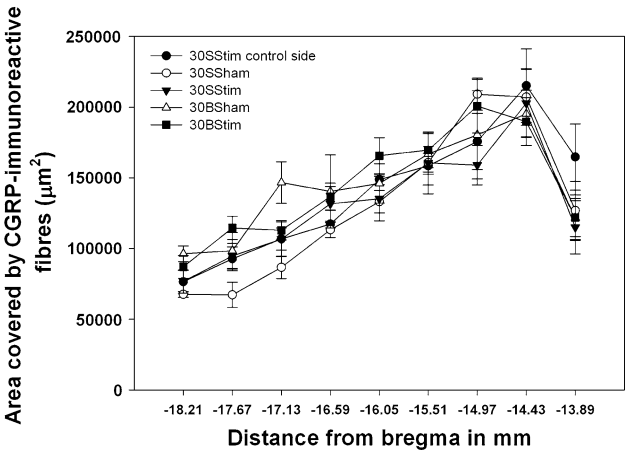

Fig. 4 Diagram showing the area covered by CGRP-immunoreactive fibres at the different levels of the TNC in the different treatment groups after robust electrical stimulation of the trigeminal ganglion (group mean ± SEM). There was no significant difference between either the control or the stimulated sides or the different groups. For clarity, only the control side of the saline-treated stimulated group is presented

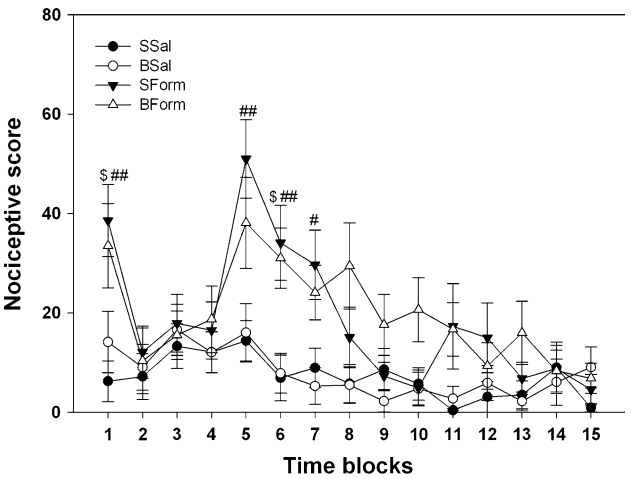

Fig. 5 Diagram showing the mean nociceptive scores in different time blocks from the four treatment groups (group mean ± SEM, $n = 13$) in the orofacial formalin test. The injection of formalin increased the nociceptive score in blocks 1 and 5–7 as compared with the saline-injected animals ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$). BBG treatment did not affect the nociceptive scores after saline injection, and did not modify the normal behaviour. The nociceptive scores of the BBG and saline-treated formalin-injected groups did not differ significantly in any block

Discussion

Numerous stimulation parameters and stimulation times have been applied in previous experiments, and we therefore decided to make use of two stimulation procedures, a short, mild stimulation and a longer, robust stimulation, in order to examine the possible effects of P2X7-R antagonism on trigeminal activation. As P2X7-R blockade was previously found to be effective in inflammatory conditions, we additionally examined the effects of BBG in a model of orofacial inflammation, the orofacial formalin test.

Mild, short electrical stimulation of the trigeminal ganglion has been reported to lead to activation of the trigeminal system (Takemura et al. 2000), and this was supported by our results. The activation may be direct, stemming from depolarization of the central terminals of the primary trigeminal afferents, or it may be indirect, resulting from the release of peripheral mediators (histamine, bradykinin, substance P or CGRP).

We observed a similar pattern in the robust stimulation paradigm, the number of c-Fos IR cells increasing profoundly, indicating the activation of the trigeminal system.

The main difference seen between the two paradigms was in the number of cells activated after stimulation. Following the robust stimulation procedure more c-Fos IR cells were found in the TNC, suggesting a higher degree of activation than in the mild stimulation procedure. This higher degree of activation may be attributed to the higher frequency applied in the robust paradigm, which can lead to the more rapid firing of the primary trigeminal neurons. An increased firing rate may cause increased levels of transmitter release at both central and peripheral terminals, resulting in a higher degree of activation at the TNC level (Samsam et al. 1999). It is also plausible that the longer stimulation interval leads to more primary trigeminal cells being activated in the TG, and hence in the TNC. We assume that in our experimental setting both the increased frequency and the increased stimulation interval contributed to the higher activation level in the TNC.

Pre-treatment with the P2X7-R antagonist BBG was effective only in the robust stimulation paradigm, resulting in a decrease of the activity, reflected by the c-Fos expression. P2X7-Rs can be found in the trigeminal system, both in the ganglion (Teixeira et al. 2010) and in the TNC (D'Amico et al. 2010), therefore BBG may modulate peripheral and central processes. BBG could modulate the nociceptive processing by interfering with the peripheral neurogenic inflammation, or by modulating non-synaptic communication within the ganglion (Matsuka et al. 2001). At the central level, BBG could affect P2X7-Rs on central presynaptic terminals and modulate glutamate release (D'Amico et al. 2010), and thereby influence nociceptive transmission. Presumably, after the robust stimulation, where the more pronounced peripheral activation and more severe inflammation also involve P2X7-Rs, the blocking effect of BBG manifests, while in the mild paradigm due to the minor changes the effect of BBG does not emerge.

Neither the mild nor the robust stimulation procedure caused alterations in the levels of CGRP. It was earlier found that electrical stimulation of the trigeminal ganglion

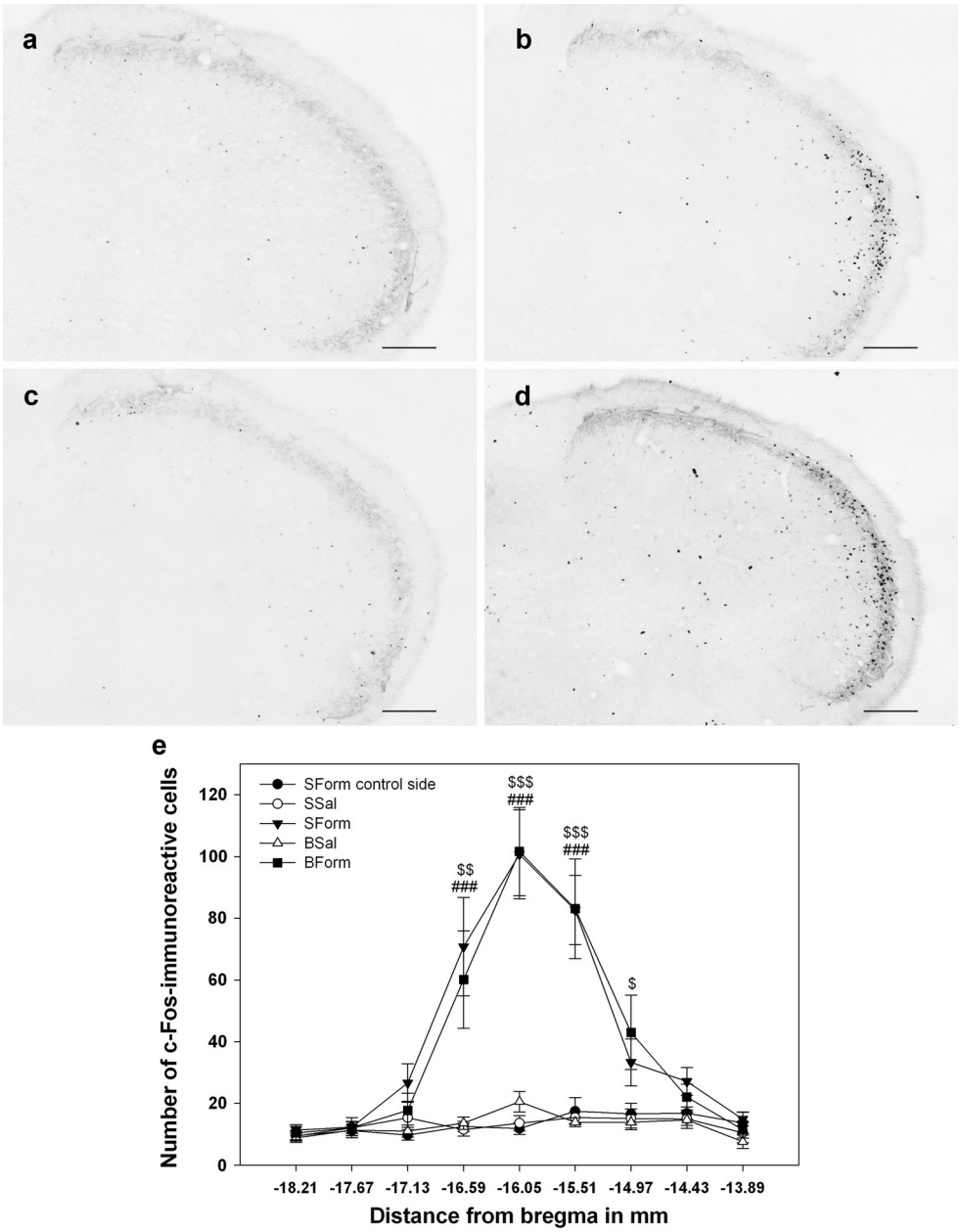

Fig. 6 Summary of the results from the orofacial formalin test regarding c-Fos immunostaining. Representative photos from the right (injected) sides of the four treatment groups after c-Fos immunohistochemistry taken at 16.05 mm caudally from bregma: **a** SSal, **b** SForm, **c** BSal, **d** BForm. *Scale bar* 200 µm. Diagram showing the mean number of c-Fos immunoreactive cells along the rostro-caudal axis in the TNC in the orofacial formalin test (group mean ± SEM) (**e**). Injection of saline did not cause any increase in the number of c-Fos-positive cells (SSal and BSal groups). The effect of formalin was visible only on the injected side; the control sides were similar to those in the saline-injected controls. For clarity, only the control side of the SForm animals is presented. Formalin caused an increase in c-Fos cell number on the ipsilateral side as compared with the saline-injected side of the controls (SForm compared with SSal), significant at the levels −16.59 to −15.51 mm ($^{\#\#\#}p < 0.001$). In the BBG-treated rats, formalin had a similar activity-increasing effect, significant at the levels −16.59 to −14.97 mm ($^{\$}p < 0.05$; $^{\$\$}p < 0.01$; $^{\$\$\$}p < 0.001$). The groups SForm and BForm did not differ significantly from each other

with parameters similar to our robust stimulation, led to the depletion of CGRP from the medial one-third of the central terminals of the trigeminal afferents (Knyihar-Csillik et al. 1998). However, those examinations were conducted immediately after stimulation of the trigeminal ganglion, whereas in our experiments a 2 or a 4 h survival time was included for better observability of the activity changes (c-Fos). These periods might be sufficient for the depleted CGRP to be resynthesised and for the changes in CGRP immunoreactivity seen immediately after stimulation to normalise. BBG treatment did not modify the levels of CGRP in either the sham or the stimulated group.

The injection of formalin into the whisker pad causes a biphasic behavioural effect (Clavelou et al. 1989), as also seen in our experiments. The first short and intense phase is thought to be caused by the immediate activation of the Aδ and C fibres and may be referred as acute pain. The second phase is less intense, but prolonged, and probably caused by sensitization of the trigeminal system due to the inflammatory processes occurring at the periphery. BBG did not exhibit any effect in the first phase of the formalin response. When formalin was applied to the hind paw and was combined with a selective P2X7-R antagonist, A-438079, in previous work, protective effect was exerted only in the second phase of the formalin test (McGaraughty et al. 2007). Furthermore, BBG was earlier shown to be hyperalgesic in the modulation of acute nociception in the hot-plate test (Ando et al. 2010). These results suggest that

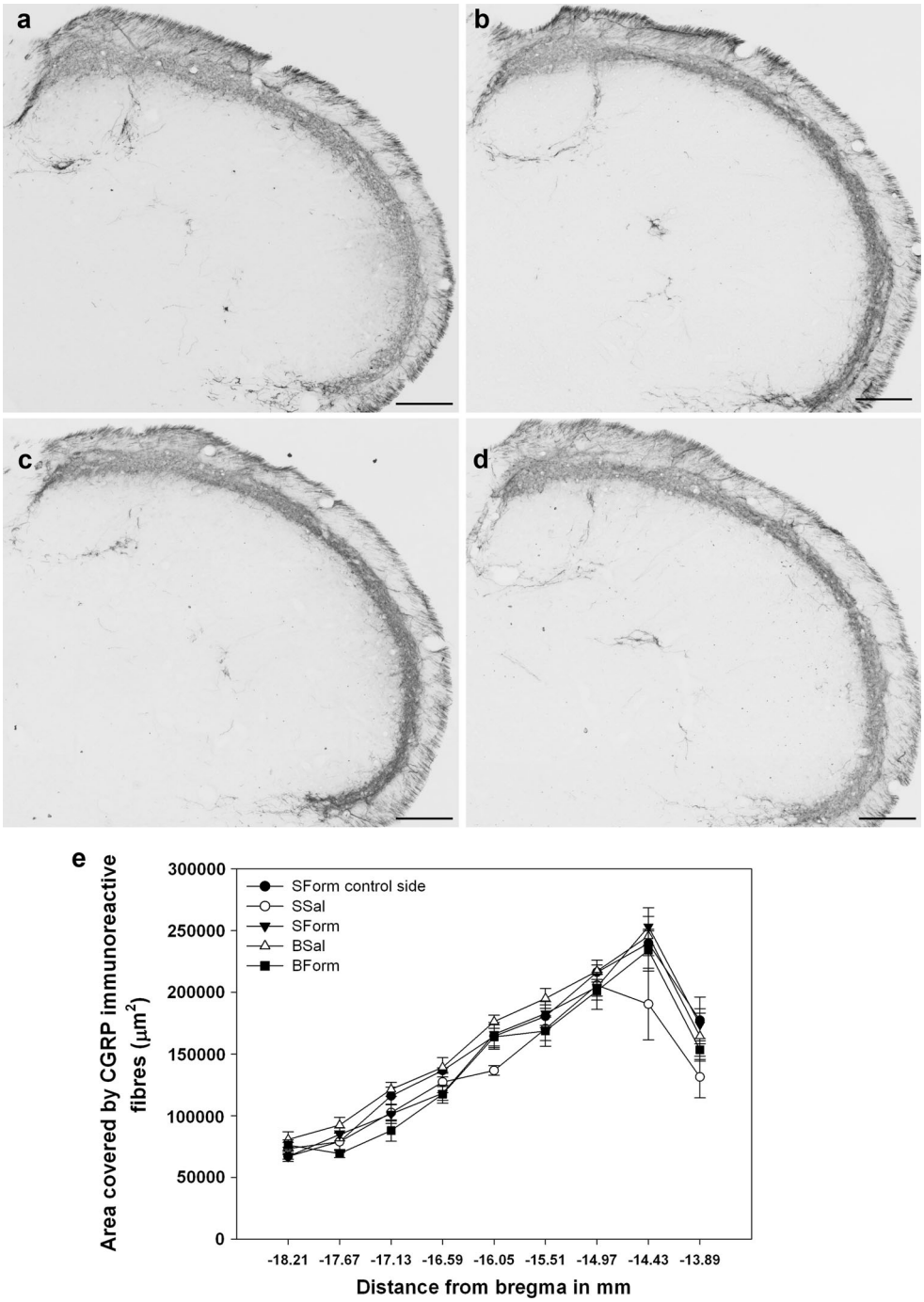

Fig. 7 Summary of the results after CGRP immunostaining in the orofacial formalin test. Representative photos of CGRP immunohistochemistry on the *right sides* of the TNC from the four treatment groups taken at 14.97 mm caudally from bregma **a** 5SSham, **b** 5SStim, **c** 5BSham, **d** 5BStim. *Scale bar* 200 µm. Diagram showing the area covered by CGRP-immunoreactive fibres at the different levels of the TNC in the different treatment groups in the orofacial formalin test (group mean ± SEM) (**e**). There was no significant difference between either the control or injected sides or the different groups. For clarity, only the control side of the saline-treated formalin-injected group is presented

BBG and blockade of the P2X7-Rs may not be effective against acute nociception.

In the second phase of the formalin test, BBG did not demonstrate any obvious effect. At the beginning of the second phase (in blocks 5–7), the nociceptive scores revealed a decreasing tendency, while in the later blocks the opposite could be observed. Since another P2X7-R antagonist was effective when formalin was applied at the hind paws, our results suggest that the role of the P2X7-Rs in the sensory system is not uniform.

Four h after formalin injection, c-Fos immunohistochemistry revealed that the TNC displays clear activation. The pattern of activation corresponds to the somatotopic projection pattern of the injected area. BBG had no effect on the activation of the trigeminal system after formalin. Our results in the orofacial formalin test are somewhat surprising, considering that other antagonists of the P2X7-Rs (Borsani et al. 2010; Honore et al. 2006; McGaraughty et al. 2007) and even BBG (Ando et al. 2010) have proven effective in numerous inflammatory models. However,

none of these experiments related to the trigeminal system, and our results are the first regarding the effects of blockade of the P2X7-Rs by BBG in this area after inflammation caused by formalin.

The levels of CGRP were not altered 4 h after formalin injection, and following treatment with BBG. Alterations in CGRP usually occur immediately during or after the applied stimulus and cease within a matter of hours (Buzzi et al. 1991; Greco et al. 2008), and our results agree with this. However, in the nitroglycerin model, changes in CGRP immunoreactivity were seen 4 h after nitroglycerin administration, suggesting that the alterations in CGRP levels can be long-term. The effect of P2X7-R antagonism on the expression of CGRP in the formalin test should be further elucidated with regard to the time scale.

BBG in the micromolar range was previously shown to inhibit voltage-dependent sodium channels in vitro (Jo and Bean 2011), and it might, therefore, be possible that this feature of BBG contributes to its effects in our experiments. This is rather unlikely, considering that we applied BBG in a single dose, which has been shown not to reach micromolar levels even after continuous administration in mice (Diaz-Hernandez et al. 2012).

In conclusion, our results suggest that P2X7-Rs have a role in the modulation of trigeminal nociceptive processing. Further investigations of the relations of the trigeminal system and P2X7-R signalling may provide important details concerning trigeminal nociceptive processing and the pathomechanism of headaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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III.

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A comparative assessment of two kynurenic acid analogs in the formalin model of trigeminal activation: a behavioral, immunohistochemical and pharmacokinetic study

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Abstract Kynurenic acid (KYNA) has well-established protective properties against glutamatergic neurotransmission, which plays an essential role in the activation and sensitization process during some primary headache disorders. The goal of this study was to compare the effects of two KYNA analogs, *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KA-1) and N-(2-N-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KA-2), in the orofacial formalin test of trigeminal pain. Following pretreatment with KA-1 or KA-2, rats were injected with subcutaneous formalin solution in the right whisker pad. Thereafter, the rubbing activity and c-Fos immunoreactivity changes in the spinal trigeminal nucleus pars caudalis (TNC) were investigated. To obtain pharmacokinetic data, KA-1, KA-2 and KYNA concentrations were measured following KA-1 or KA-2 injection. Behavioral tests demonstrated that KA-2 induced larger amelioration of formalin-evoked alterations as compared with KA-1 and the assessment of c-Fos immunoreactivity in the TNC yielded similar results. Although KA-1 treatment resulted in approximately four times larger area under the curve values in the serum relative to KA-2, the latter resulted in a higher KYNA elevation than in the case of KA-1. With regard to TNC, the concentration of KA-1 was under the limit of detection, while that of KA-2 was quite small and there was no major difference in the approximately tenfold KYNA elevations. These findings indicate that the differences between the beneficial effects of KA-1 and KA-2 may be explained by the markedly higher peripheral KYNA levels following KA-2 pretreatment. Targeting the peripheral component of trigeminal pain processing would provide an option for drug design which might prove beneficial in headache conditions.

Keywords Kynurenic acid analog · Rat · Trigeminal pain · Formalin test · Pharmacokinetics

Introduction

The kynurenine pathway (KP) of the tryptophan (TRP) metabolism is extensively studied, mostly because of the well-established endogenous protective properties of kynurenic acid (KYNA) against the excitotoxic effect of other KP metabolites, such as quinolinic acid and 3-hydroxy-L-kynurenine (Vécsei et al. 2013; Zádori et al. 2011b). Thus, KYNA has become a molecule of interest for central nervous system (CNS) drug development for several disorders (Schwarcz 2004). The application of KYNA in in vivo preclinical studies would be difficult due to its chemical and pharmacokinetic properties. There are problems with its solubility in higher doses, it cannot pass the blood–brain barrier in an acceptable quantity and it has

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a rapid clearance from the CNS and in the periphery, mediated by organic anion transporters (Bahn et al. 2005; Fukui et al. 1991). Several analogs, derivatives and prodrugs have been synthesized with the aim of mitigating these disadvantages and of improving the utility of the molecule in preclinical studies (Fülöp et al. 2009). Among our newly synthesized KYNA amides, two lead compounds have been identified (Patent number #P0900281/PCT/HU2010/00050).

With regard to in vitro electrophysiology studies, *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KA-1, Fig. 1a) has been demonstrated to be an even more effective inhibitor of hippocampal excitatory synaptic transmission than KYNA (Nagy et al. 2011). Accordingly, in an in vivo model of trigeminal activation, KA-1 displayed better efficacy than that of its parent compound, KYNA (Knyihár-Csillik et al. 2008). Following this comparative study, KA-1 was tested in several experimental setups and proved to have beneficial effects (Gellért et al. 2012, 2011; Knyihár-Csillik et al. 2008; Marosi et al. 2010; Vámos et al. 2010, 2009; Zádori et al. 2011c). In contrast with the electrophysiological findings with KA-1, *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KA-2, Fig. 1b) did not decrease, but rather slightly increased the amplitudes of field excitatory postsynaptic potentials (fEPSPs) (Nagy et al. 2011). However, in a recent study, pretreatment with KA-2 was also able to attenuate the effects of nitroglycerine (NTG) in an experimental model of migraine (Fejes-Szabó et al. 2014).

The modes of action of these two compounds are still not fully understood. The two main possibilities are the following: (1) the intact structure is necessary for the mechanism of action, which would mimic some effects of the parent compound, KYNA; (2) the KYNA amides serve as prodrugs and dissociate into KYNA, which can exert its

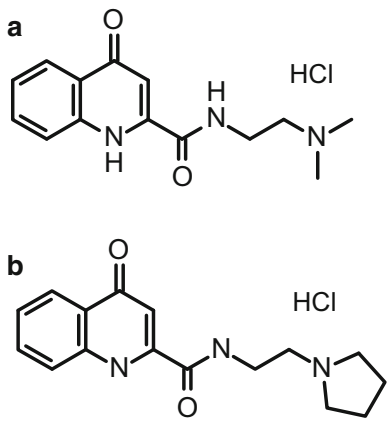

Fig. 1 The chemical structure of *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KA-1, **a**) and *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KA-2, **b**)

pharmacological effects. With regard to the clarification of this issue, only one pharmacokinetic study is available to date (Zádori et al. 2011a). In that study, KYNA and KA-1 levels were measured with high-performance liquid chromatography (HPLC) in C57B/6 mouse serum following the intraperitoneal administration of KA-1. The time-course profile of KA-1 exhibited a steep increase in concentration followed by a rapid decrease in the first hour. Although the concentration of KYNA also increased from the basal serum level following KA-1 administration, this increase was considerably less than that in the case of KA-1. It may therefore be concluded that only a small proportion of KA-1 is metabolized to KYNA. These findings and the results of electrophysiology studies led to the assumption that it is less likely that KA-1 acts as a prodrug. Similar pharmacokinetic studies relating to KA-2 have not yet been made, but in view of the results of the electrophysiology studies, it seems to have a different mode of action.

The trigeminal system is responsible for most of the pain processing originating from the area of the head (Carpenter and Sutin 1983), and its activation therefore plays an important role in the pathomechanism of several neurological disorders, including primary headaches and trigeminal pain syndromes. These disorders, including migraine cause an enormous burden to the society (Olesen et al. 2012) underlining the need of new treatment options with a possible different mechanism of action. On the basis of its antagonistic influence on *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Pereira et al. 2002), it is assumed that KYNA has a modifying effect on nociception (Näsström et al. 1992; Párdutz et al. 2012), and thus might possibly be a future candidate in headache treatment.

One aim of this study was to investigate the effects of KA-1 and KA-2 in the orofacial formalin test to quantify the nociception in the trigeminal region of the rat (Clavelou et al. 1995; Raboisson and Dallel 2004). A further aim was to perform a comparative pharmacokinetic study so as to further clarify the possible modes of action of these two KYNA amides.

Materials and methods

Animals

During the experiments, adult male Sprague–Dawley rats weighing 200–250 g were used. The animals were housed under standard laboratory conditions (in an air-conditioned, humidity-controlled and ventilated room) and were allowed free access to drinking water and regular rat chow on a 12 h–12 h dark–light cycle. The procedures used in

this study followed the guidelines of the International Association for the Study of Pain and the directive of the European Economic Community (86/609/ECC). They were approved by the Committee of Animal Research at the University of Szeged (I-74-12/2012) and the Scientific Ethics Committee for Animal Research of the Protection of Animals Advisory Board (XXIV./352/2012.).

Materials

The new KYNA amides (patent number #P0900281/PCT/HU2010/00050), KA-1 and KA-2, were synthesized in the Department of Pharmaceutical Chemistry, University of Szeged. The reference compounds [KYNA and 3-nitro-L-tyrosine (3-NLT)], zinc acetate dihydrate, chloral hydrate and 3,3′-diaminobenzidine were purchased from Sigma-Aldrich (Saint Louis, MO, USA), acetonitrile, H_2O_2, nickel ammonium sulfate and perchloric acid (PCA) were purchased from Scharlau (Barcelona, Spain), acetic acid and Triton X-100 were purchased from VWR International (Radnar, PA, USA), and paraformaldehyde was purchased from Merck (Darmstadt, Germany). The HPLC–MS grade acetonitrile and acetic acid were obtained from VWR International (Radnar, PA, USA).

Behavioral testing

To produce and quantify nociception in the trigeminal region of the rat, we used the orofacial formalin test.

The animals were divided into three groups ($n = 27$–28 per group) and received an intraperitoneal (i.p.) injection of phosphate-buffered saline (PBS, 0.1 M, pH 7.4) in the Control group or an i.p. injection of KA-1 (1 mmol/kg body weight; pH 7.4) in the KA-1 group or an i.p. injection of KA-2 (1 mmol/kg body weight; pH 7.4) in the KA-2 group. One hour after pretreatment, the animals were divided further into two subgroups ($n = 13$–15 per subgroup), half of the rats receiving a subcutaneous (s.c.) injection of 50 µl physiological saline without formalin (control, KA-1 and KA-2), while the other half of the rats were injected s.c. with 50 µl 1.5 % formalin solution containing 0.55 % formaldehyde (control–formalin, KA-1–formalin and KA-2–formalin), administered via a 26-gauge needle into the right whisker pad.

The testing procedures were performed during the light phase (between 8 a.m. and 2 p.m.) in a quiet room. The test box was a 30 × 30 × 30 cm glass terrarium with mirrored walls. For the offline analysis of rubbing activity directed to the whisker pad, the behavior of the individually tested rats was recorded with a video camera (Logitech HD Webcam C615) situated 1 m above the terrarium. One hour after pretreatment and following a 10-min habituation period in the test box, the whisker pads of the rats were injected s.c. with physiological saline or formalin and the animals were immediately replaced in the chamber for 45-min. The rats did not receive any food or water during the observation period. The test box was cleaned and decontaminated after each animal. An observer blind to the experimental procedures analyzed the recorded videos. The 45-min recording period was divided into 15 × 3-min blocks, and we distinguished two phases following formalin injection [Phase 1 (time block 1, i.e., 0–3 min) and Phase 2 (time blocks 5–15, i.e., 12–45 min)] according to the previously published methods (Clavelou et al. 1995; Raboisson and Dallel 2004), and the total time (number of seconds) spent in rubbing/scraping the injected area with the ipsilateral fore- or hindpaw was measured in each block and defined as the nociceptive score for that block. Earlier literature findings led us to use the grooming activity of animals in the control subgroup as control (Clavelou et al. 1995).

Immunohistochemistry

Four hours after the formalin injection, the rats were perfused transcardially with 100 ml PBS, followed by 500 ml 4 % paraformaldehyde in phosphate buffer under deep chloral hydrate (0.4 g/kg body weight) anesthesia. The medullary segment containing the spinal trigeminal nucleus pars caudalis (TNC) between +1 and −5 mm from the obex was removed, postfixed overnight for immunohistochemistry in the same fixative and cryoprotected (10 % sucrose for 2 h, 20 % sucrose until the blocks sank, and 30 % sucrose overnight). Before sectioning, each segment was marked with a small incision on the ventral and left (contralateral) side of the tissue block, allowing side discrimination during the quantification process. 30-µm transverse cryostat sections were cut through the rostrocaudal axis from the beginning of the TNC and were serially collected in wells containing cold PBS. Each well contained every tenth section at 0.3-mm intervals along the rostrocaudal axis (15 levels = sections). The free-floating sections were rinsed in PBS and immersed in 0.3 % H_2O_2 in PBS for 30 min to suppress endogenous peroxidase activity. After several rinses in PBS containing 1 % Triton X-100 (PBST), sections were incubated at room temperature overnight in PBST containing rabbit anti-rat c-Fos polyclonal antibody (Santa Cruz Biotechnology, sc-52) at a dilution of 1:2000. The immunohistochemical reaction was visualized using Vectastain Elite avidin–biotin kits (Vector Laboratories, PK6101). Briefly, the sections were incubated at room temperature for 2 h in PBST containing goat anti-rabbit biotinylated secondary antibody. After several rinses in PBST, and incubation at room temperature for 2 h in PBST containing avidin and biotinylated horseradish peroxidase, the sections were stained with 3,3′-

diaminobenzidine intensified with nickel ammonium sulfate. The specificity of the immune reactions was checked by omitting the primary antiserum.

The immunoreactive (IR) cells in the superficial layer of the TNC were counted by an observer blind to the experimental procedures under the 10× objective of a Nikon Optiphot-2 (Nikon Instruments, Melville, NY, USA) light microscope in every tenth transverse section in each animal. Before the counting, the location of each section along the rostrocaudal axis and the location of the TNC on each medullary section were determined by means of The Rat Brain in Stereotaxic Coordinates Atlas (Paxinos and Watson 2007). The c-Fos neurons with obvious specific nuclear staining were taken into consideration and were counted in the TNC both ipsilaterally and contralaterally to the formalin injection.

HPLC measurements

Sample preparation

At set time points (15, 30, 60, 120 and 300 min) following the i.p. injection with the KYNA amides (1 mmol/kg), the rats were deeply anesthetized with i.p. injection of chloral hydrate (0.4 g/kg, Sigma-Aldrich). Blood samples were collected from vena cava caudalis and centrifuged at 13709 RCF (relative centrifugal force) for 10 min at 4 °C. The supernatants, i.e., the serum samples, were collected and centrifuged at 13709 RCF for 10 min at 4 °C again and the supernatants were stored at −80 °C until use. After the collection of blood samples, the animals were transcardially perfused with 100 ml 0.1 M PBS for 5 min. The CNS samples containing the medullary segment of the TNC were then removed and stored at −80 °C until measurements. The animals in the Control group underwent a similar procedure with one measurement point at 300 min.

For the measurement of KYNA concentration, the CNS samples were cut in half, weighed and then sonicated for 1.5 min in an ice-cooled solution (250 µl) comprising PCA (2.5 % w/w), 3-NLT (10 or 2 µM) and distilled water in an Eppendorf tube. The content of the Eppendorf tube was centrifuged at 13709 RCF for 10 min at 4 °C and the supernatant was measured. Before analysis, the serum samples were thawed and, after a brief vortex, the serum sample was 'shot' onto a precipitation solvent (containing PCA with 3-NLT as internal standard, with resulting concentrations of 2.5 w/w% and 2 µM, respectively). The samples were subsequently centrifuged at 13709 RCF for 10 min at 4 °C, and the supernatants were collected for measurement.

For the analysis of KYNA amides, the other half of the CNS samples were weighed and then sonicated in ice-cold (250 µl) distilled water for 1.5 min and centrifuged at 13709 RCF for 10 min at 4 °C. From the supernatant, 100 µl was transferred to an Eppendorf tube containing 750 µl HPLC gradient grade acetonitrile and 150 µl distilled water. After a brief vortex, the samples were centrifuged at 13709 RCF for 10 min at 4 °C and 900 µl of supernatant was evaporated in a vacuum centrifuge. After thawing and brief stirring with a vortex, 200 µl of serum sample was transferred to an Eppendorf tube containing 700 µl HPLC gradient grade acetonitrile and 100 µl distilled water. After a brief vortex, the samples were centrifuged at 13709 RCF for 10 min at 4 °C and 900 µl of supernatant was evaporated in a vacuum centrifuge. The evaporated CNS and serum samples were stored at 4 °C until use.

Chromatographic conditions

The KYNA concentrations of the CNS samples were quantified on the basis of a slight modification of a literature method (Hervé et al. 1996) as described in detail with method validation in our previous article (Fejes-Szabó et al. 2014). For the measurement of concentration of the above-mentioned metabolite from serum samples we applied the same method with a slight modification. Furthermore, we applied the method validation procedures to the serum samples too. Briefly, we used an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with fluorescence and a UV detector; the former was applied for the determination of KYNA and the latter for the determination of the internal standard (3-NLT). Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm × 4.6 mm I.D. (Phenomenex Inc., Torrance, CA, USA) after passage through a SecurityGuard pre-column C18, 4 × 3.0 mm I.D., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA) with a mobile phase composition of 0.2 M zinc acetate/ACN = 95/5 (v/v), the pH of which was adjusted to 6.2 with acetic acid, applying isocratic elution. The flow rate was 1.5 ml/min and the injection volume was 20 µl for serum, and 50 µl for CNS samples. The fluorescence detector was set at excitation and emission wavelengths of 344 and 398 nm. The UV detector was set at a wavelength of 365 nm.

For the determination of KYNA amides, a Thermo LCQFleet ion trap mass spectrometer was used equipped with an ESI ion source combined with a Dionex Ultimate 3000 HPLC system. The ionization parameters were as follows: heater temperature: 500 °C, sheath gas flow rate: 60, auxiliary gas flow rate: 20, spray voltage: 4 kV, capillary temperature: 400 °C. Chromatographic separations were performed on a Kinetex C18 column, 100 mm × 4.6 mm, 2.6 µm particle size (Phenomenex Inc., Torrance, CA, USA) after passage through a SecurityGuard pre-

column C18, 4 × 3.0 mm, 5 µm particle size (Phenomenex Inc., Torrance, CA, USA), with a mobile phase composition of 0.05 % aqueous CH_3COOH/ACN = 90/10 (v/v), applying isocratic elution. The flow rate and the injection volume were 1 ml/min and 50 µl, respectively.

Calibration curve and linearity

Calibrants were prepared at six different concentration levels, from 1 to 100 nM, 0.5 to 5 µM and 0.01 to 100 µM for KYNA, 3-NLT and the KYNA amides, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R software (R Development Core Team 2002). Very good linearity was observed throughout the investigated concentration ranges for KYNA, 3-NLT and the KYNA amides when either fluorescence, UV or MS detection was applied.

Selectivity

The selectivity of the method was checked by comparing the chromatograms of KYNA, KYNA amides and the internal standard for a blank serum and CNS sample and those for a spiked sample. All compounds could be detected in their own selected chromatograms without any significant interference.

LOD and LLOQ

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined via the signal-to-noise ratio with a threshold of 3 and 10, respectively, according to the ICH guidelines (ICH 1995). The LOD and LLOQ for KYNA in the serum samples were 1 and

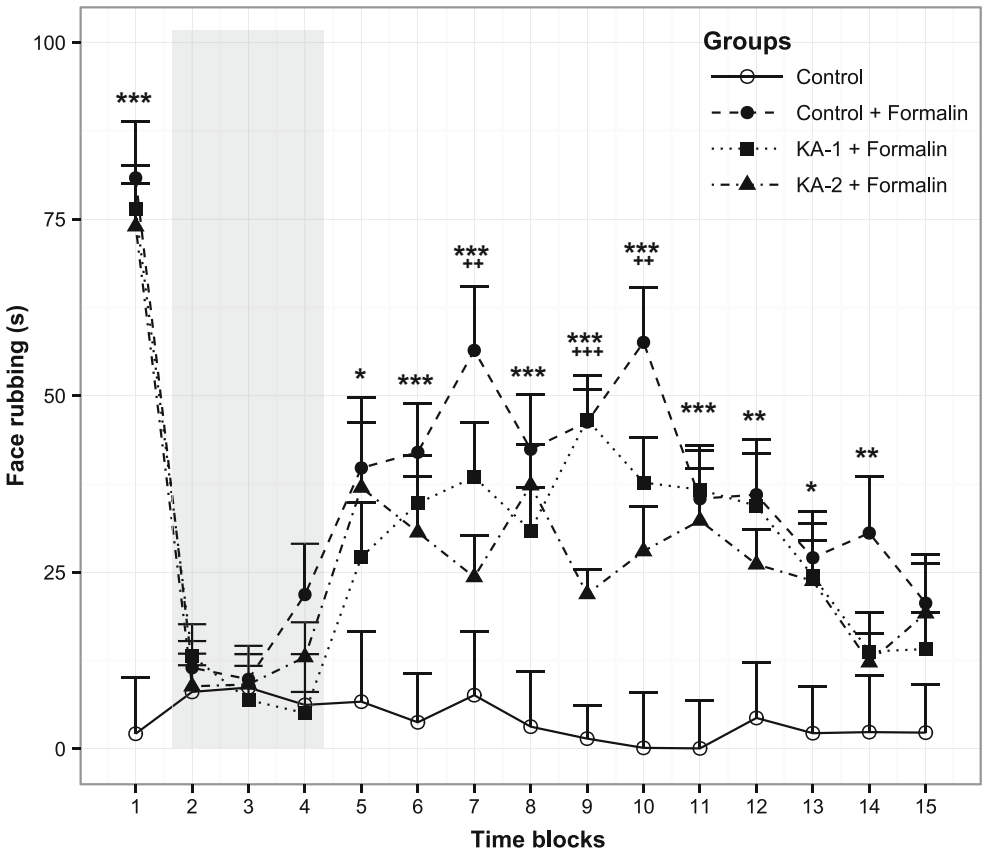

Fig. 2 The effects of formalin and KYNA amide treatments on Sprague–Dawley male rats in the orofacial formalin tests. Following formalin injection, the recording period was 45-min and this time period was divided into 15 × 3-min time blocks. Phase 1 (time block 1) of the test is before the grayed out area, while Phase 2 (time blocks 5–15) is after the grayed out area. The two phases of formalin action in the control + formalin group are well demonstrated when compared to the control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The KA-2 pretreatment significantly reduced the formalin-induced nociceptive behavior relative to the control + formalin group ($^{++}p < 0.01$; $^{+++}p < 0.001$). Sprague–Dawley male rats: $n = 13$–15 in each group; data are shown as mean ± SEM; *KA-1 N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride; *KA-2 N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

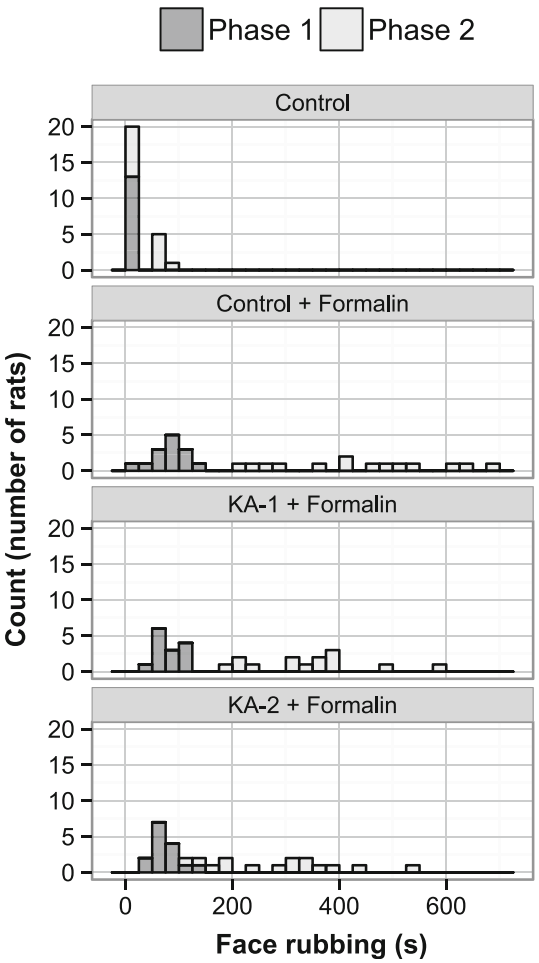

Fig. 3 Distribution of rubbing activity by groups in Phase 1 and 2 of the orofacial formalin test. The recording period was 45-min, Phase 1 is the first 3-min, while Phase 2 is the overall rubbing activity from min 12 to the end of the examination. Sprague–Dawley male rats: $n = 13$–15 in each group; *KA-1* *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride; *KA-2* *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

3.75 nM, respectively, while in the CNS samples they were 0.4 and 1 nM, respectively. The LOD and LLOQ for the samples in MS detection were 0.001 and 0.015 μM, respectively.

Precision

Replicate HPLC analysis showed that the relative standard deviation was ≤2.2 % for the peak area response and ≤0.1 % for the retention time for KYNA and the KYNA amides.

Recovery

The relative recoveries were estimated by measuring spiked samples of KYNA and the KYNA amides at two concentrations with three replicates of each. No significant differences were observed for the lower and higher concentrations. The recoveries for the serum samples ranged from 103 to 108 %, 81 to 94 % and 79 to 80 % for KYNA, KA-1 and KA-2, respectively. The recoveries for the CNS samples ranged from 82 to 92 % and 78 to 84 % for KYNA and KA-2, respectively.

Data evaluation

To compare the means of jaw rubbing counts (orofacial formalin tests) in the different treatment groups (control, control–formalin, KA-1, KA-1–formalin, KA-2, KA-2–formalin) on rats during 15 3-min time periods ($n = 13$–15 animals in each group), two-way repeated measures ANOVA were run. Treatment with six groups was used as between-subject factor and time with 15 time points as within-subject factor for the analysis.

When Levene's test for homogeneity of variance was significant in the Phase data (Phase 1 is the first 3 min, while Phase 2 is the overall rubbing from min 12 to the end of the examination, min 45, i.e., which is from the fifth 3-min time period to the 15th), the Welch test was run to compare mean rubbing counts. Pairwise comparisons were estimated by the Sidak test. The effect size (EF, f) was calculated according to Cohen J. (Cohen 1988) with regard to the ANOVA tests comparing the effects of compounds on behavioral data in Phase 1 and 2.

In the case of the immunohistochemistry data, the numbers of c-Fos-containing nuclei were assessed. Three-way repeated measures ANOVA was used to analyze group means across 15 adjacent measuring sites (consequent slices from the medullary segment of the CNS containing the TNC) among sides (contralateral and ipsilateral) and between pretreatment groups (control, KA-1 and KA-2) all with formalin injections. Measuring sites and sides were used as within-subject factor, while pretreatment was the between-subject factor in the general linear model.

In the ANOVA models when Mauchly's test of sphericity was significant, the Greenhouse–Geisser correction was performed. In the event of the significant interaction of factors, effects could not be reported independently. Moreover, owing to the significant interaction, group differences could be examined separately over the within-subject factor based on estimated marginal means for multiple comparisons with the Sidak adjustment.

Statistical analyses were carried out with IBM SPSS Statistics, version 21 (IBM Corporation, Armonk, NY, USA) software. All tests were two-sided, and $p < 0.05$ was considered to be statistically significant. The pharmacokinetic data were evaluated with PKSolver, a freely available menu-driven add-in program for Microsoft Excel (Zhang et al. 2010).

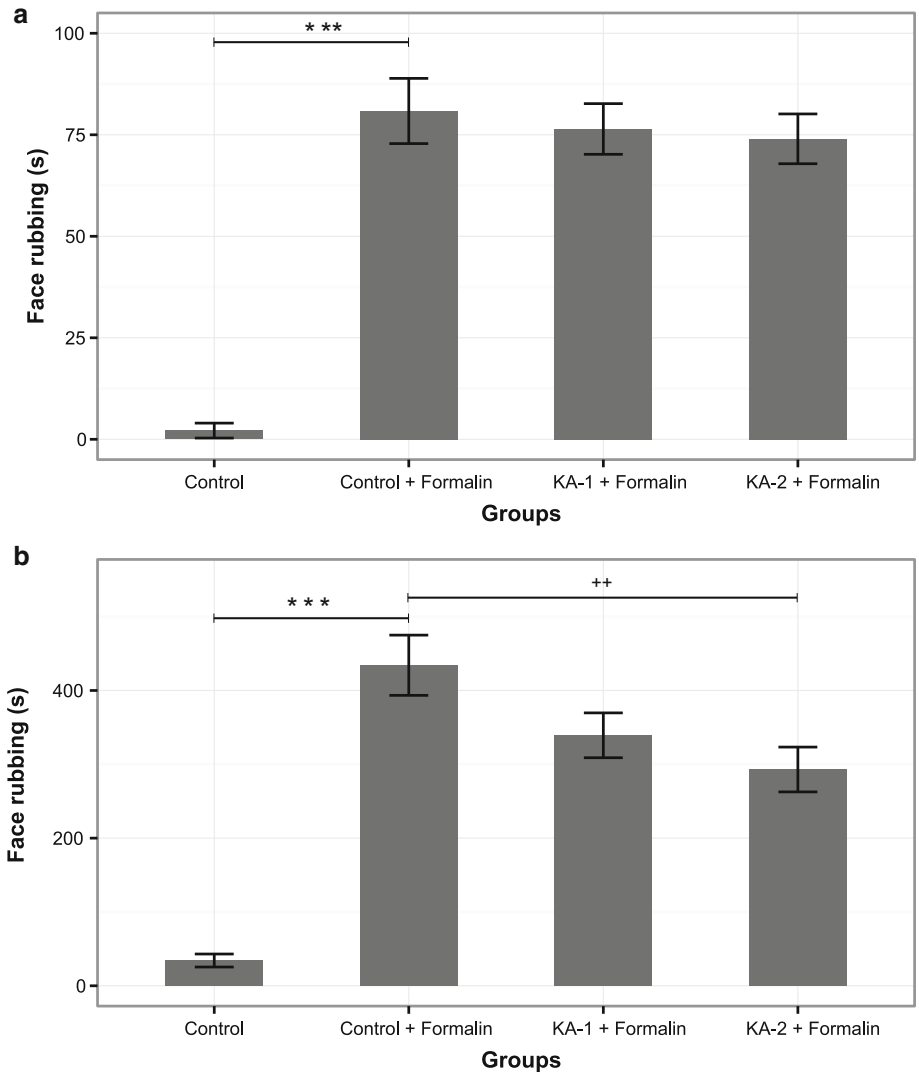

Fig. 4 Diagrams showing the rubbing activity in the first (**a**) and the second (**b**) phase in the orofacial formalin test. In the control + formalin group, the subcutaneous formalin injection induced a significant increase in rubbing activity in both Phase 1 and 2 (***$p < 0.001$) as compared to the control group. In Phase 2, pretreatment with KA-2 had a significant effect on mitigating the formalin-induced increase in the time spent with rubbing ($^{++}p < 0.01$) as compared with the control + formalin group. Sprague–Dawley male rats: $n = 13$–15 in each group; data are presented as mean ± SEM; *KA-1* *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride; *KA-2* *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

Results

Behavioral assessment

The results of orofacial formalin testing are demonstrated in Figs. 2, 3 and 4. To simplify the presentation of group comparisons for better transparency, the groups of KA-1 and KA-2 were omitted from Figs. 2, 3 and 4. Pairwise comparisons revealed that face rubbing activity in the control, KA-1 and in the KA-2-treated groups was significantly lower in both Phase 1 and Phase 2 compared with the control–formalin (ES of Phase 1, −1.88 and Phase 2, −2.17), KA-1–formalin (ES of Phase 1, −1.75 and Phase 2, −1.56) and KA-2–formalin (ES of Phase 1, −1.64 and Phase 2, −1.26) groups. With regard to the comparison of the face rubbing activity in the KA-1–formalin (ES of Phase 1, 0.11 and Phase 2, 0.51) and KA-2–formalin (ES of Phase 1: 0.16 and Phase 2: 0.76) groups with that in the control–formalin group, although KA-1 decreased face rubbing activity as well (demonstrated by the corresponding left-skewed histogram in Fig. 3), only KA-2 treatment resulted in a significant reduction in the middle of Phase 2 (Figs. 2, 4).

Immunohistochemistry

The results of immunohistochemical analysis are demonstrated in Figs. 5 and 6. The comparison of the ipsilateral and the contralateral sides of the slices from the medullary segment of the rat CNS containing the TNC demonstrated that the mean number of c-Fos IR neurons was significantly higher on the ipsilateral side than on the contralateral side in the control–formalin, KA-1–formalin and KA-2–formalin groups (the two latter groups are not demonstrated to simplify the presentation of group comparisons for better transparency in Fig. 6). With regard to the comparison of the ipsilateral sides of the KA-1–formalin and KA-2–formalin groups with the control–formalin group from the

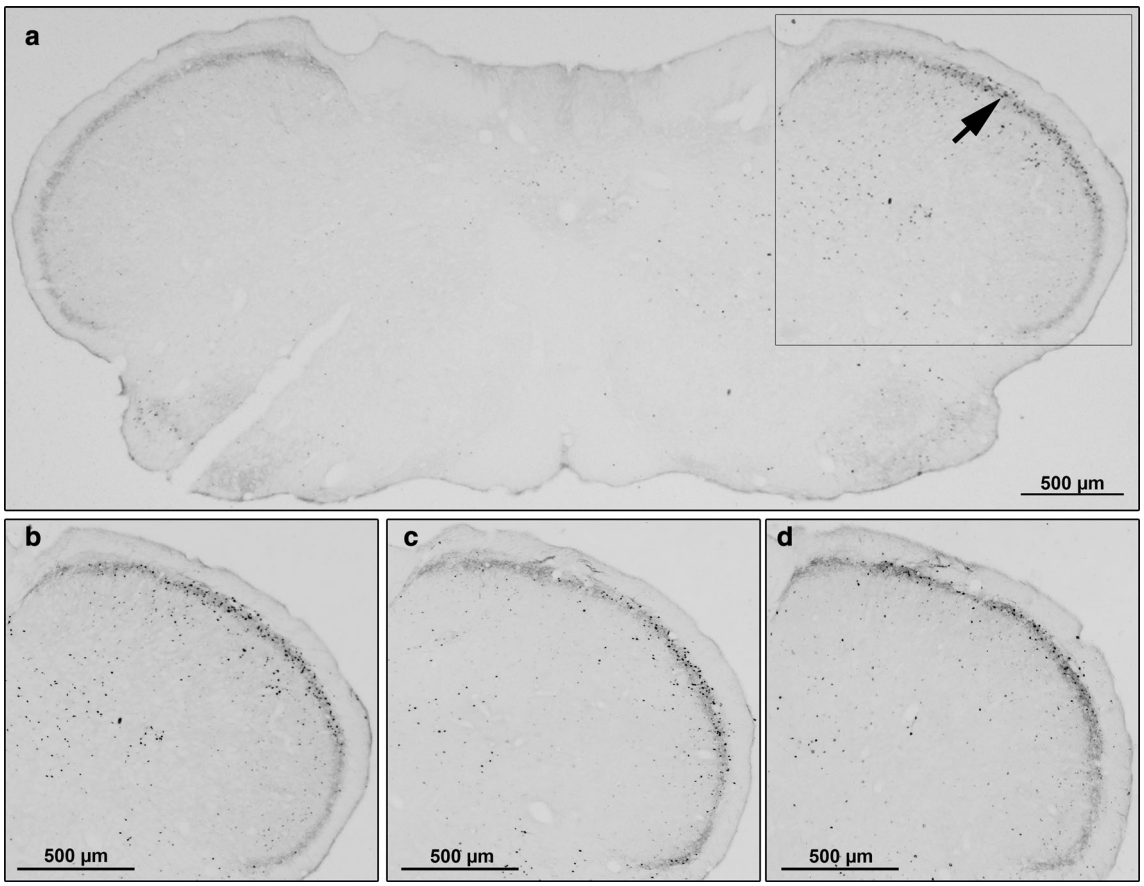

Fig. 5 Representative histological images following c-Fos immunostaining in Sprague–Dawley male rats. Subcutaneous formalin injection induced an increase in the number of c-Fos IR neurons on the ipsilateral side demonstrated on the transverse section of medulla containing the TNC from the control + formalin subgroup (**a**, **b**; contralateral side is indicated with an incision on the ventral side of the section). The pretreatments (KA-1: **c**; KA-2: **d**), reduced the amount of IR cells in the superficial layers of TNC as compared with control + formalin group (**b**). *Black arrow* c-Fos IR neurons in the superficial laminae; *black frame* magnified area, which can be seen in images **b**–**d**; *TNC* trigeminal nucleus pars caudalis, *IR* immunoreactive, *KA-1 N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, *KA-2 N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

aspect of the mean number of c-Fos IR neurons, the treatments, preferentially with KA-2, resulted in significant reductions in IR cell count at certain levels of the assessed region (the c-Fos changes followed the somatotopic representation of the trigeminal nociceptors in the injected whisker pad area; Strassman and Vos 1993). Besides the observed significant differences, the effects of the treatment are well demonstrated by the curves in Fig. 6.

HPLC measurements and pharmacokinetics

The concentrations of KYNA and KYNA amides measured in rat serum and CNS samples by HPLC are demonstrated in Table 1. The time-course profile of the KYNA amides in the rat serum revealed that after a steep increase in the concentration, a subsequent steep decrease occurred in the first hour, followed by a prolonged further gradual decrease (Fig. 7). Although the serum concentration of KA-2 did not show such a high level as that of KA-1, a slightly slower decrease in concentration was observed. These observations are consistent with the calculated serum pharmacokinetic parameters (C_{max}, T_{max}, area under the curve (AUC_{0-t}), the half-life ($t_{1/2}$), apparent total clearance (CL/F_{obs}) and apparent volume of distribution (Vz/F_{obs})) of KA-1 and KA-2, demonstrated in Table 2. However, the increase in serum KYNA concentration following the i.p. injection of KA-2 was considerably higher (an approximately 200-fold maximal increase) as compared with that of KA-1 (an approximately 70-fold maximal increase), also well reflected by the above-mentioned pharmacokinetic parameters. To avoid the influence of the basal serum KYNA level on the calculated pharmacokinetic parameters, these basal concentrations were subtracted from the corresponding subsequent concentrations in the calculation of the pharmacokinetic parameters. The pharmacokinetic data therefore reflect only the KYNA amide-induced changes in KYNA concentrations. In the fifth hour, KA-1 and KA-2 were still present at 3.1 (0–61.5) µM and 0.5 (0.5–0.5) µM in the serum,

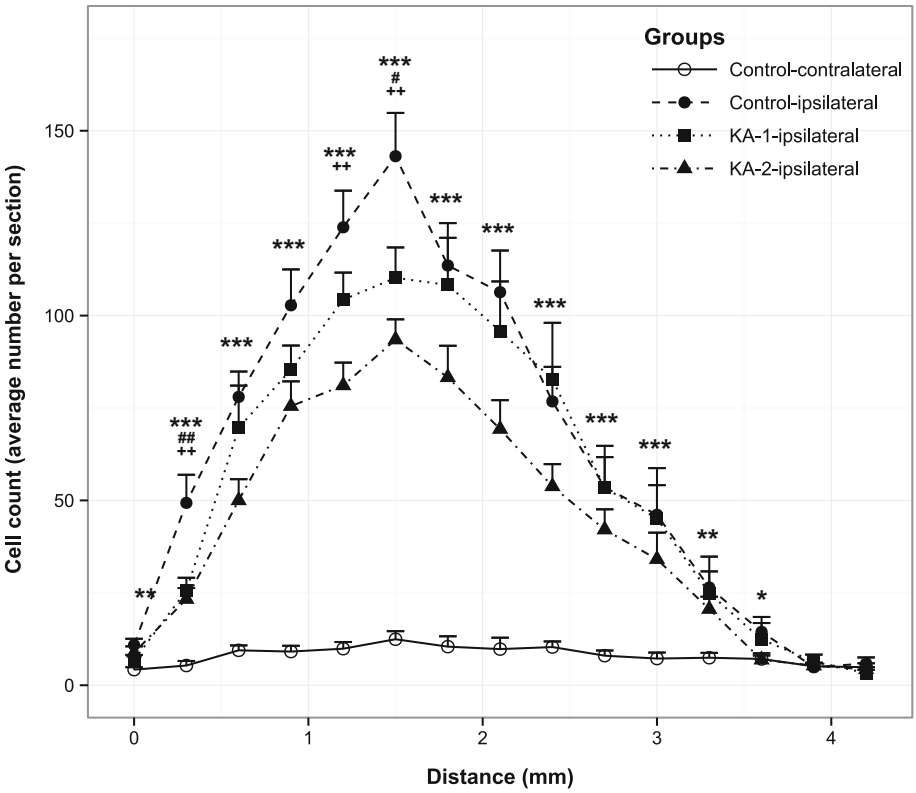

Fig. 6 The average number of c-Fos immunoreactive cells per section of TNC in Sprague–Dawley male rats after formalin injection with KYNA amide pretreatments. The distance was calculated caudally from the starting point of TNC. Subcutaneous formalin injection resulted in a higher number of c-Fos-IR neurones on the ipsilateral side compared with the contralateral side in the control + formalin group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). The pretreatments (KA-1: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; KA-2: $^{++}p < 0.01$), reduced the amount of IR cells in the superficial layers of TNC as compared with the ipsilateral side in the control + formalin group. Sprague–Dawley male rats: $n = 9$–10 in each group; data are shown as mean ± SEM; *IR* immunoreactive, *TNC* trigeminal nucleus pars caudalis, *KA-1* *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, *KA-2* *N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

respectively, while the KYNA levels had approximately returned to the baseline level, preferentially in the case of KA-1 treatment. In the rat CNS samples, the KA-1 concentration was under LOD. However, KA-2 was present in detectable amounts in the CNS, reaching its maximum concentration (6.44 (5.62–7.85) pmol/g ww) after an hour which subsequently gradually decreased, but in the fifth hour it was still present at 1.98 (1.50–2.90) pmol/g ww. The CNS pharmacokinetics of KYNA following KA-1 and KA-2 administration showed quite similar profiles, characterized by an approximately maximal tenfold increase in basal concentration within the first hour. Vz/F_{obs} was relatively high in KA-1 and especially in KA-2. The apparent clearance was also high in the case of KA-2 relative to KA-1.

Discussion

Headache, one of the most common disorders of the nervous system, is a major health problem worldwide. The global prevalence for the adult population of active headache disorder is 46 % for headache in general, 11 % for migraine, 42 % for tension-type headache and 3 % for chronic daily headache (Stovner et al. 2007). The treatment of primary headache disorders is challenging requiring both acute and preventive therapeutic strategies (Weatherall 2015). The efficacy of these treatments is not always satisfactory and the contraindications and side effects often limit the options of the physician (Diener et al. 2015; Obermann et al. 2015). There is therefore a constant need to study and develop new molecules. In addition to the currently available drugs, e.g., NSAIDs, triptans, anticonvulsants, verapamil, propranolol, etc. (National Institute for Health and Care Excellence 2012), the pipeline of pharmaceutical research in this field involves the development of novel agents acting on the glutamatergic system due to its essential role in the nociceptive process (Dickenson et al. 1997; Diener et al. 2015). Animal and human studies have revealed that glutamate receptors are present in various parts of the trigeminal system (Quartu et al. 2002; Sahara et al. 1997; Tallaksen-Greene et al. 1992), and stimulation of the trigeminal nerve results in elevated

Table 1 The concentration of KYNA and KYNA amides in Sprague–Dawley male rat serum and CNS samples

Serum					CNS			
Time (min)	KA-1 treatment		KA-2 treatment		KA-1 treatment		KA-2 treatment	
	KA-1 (μM)	KYNA (nM)	KA-2 (μM)	KYNA (nM)	KA-1 (pmol/g ww)	KYNA (pmol/g ww)	KA-2 (pmol/g ww)	KYNA (pmol/g ww)
0 (control)	0	75.7 (72.8–90.4)	0	75.7 (72.8–90.4)	0	5.33 (1.00–13.17)	0	5.33 (1.00–13.17)
15	87.4 (0–191.2)	5517.9 (3287.2–6290.1)	2.6 (2.2–20.9)	287.9 (239.1–6753.5)	<LOD	58.98 (53.37–277.22)	<LOD	17.41 (15.02–19.31)
30	35.6 (0–140.4)	1159.3 (523.8–2397.9)	20.9 (17.4–22.6)	16,709.5 (13,796.6–17,519)	<LOD	44.69 (29.43–104.13)	4.45 (3.88–4.67)	34.77 (25.01–50.53)
60	39.6 (0–114.4)	464.1 (304.9–1342.3)	13.7 (13.7–14)	3375.8 (3332.7–4604.1)	<LOD	58.61 (25.97–103.53)	6.44 (5.62–7.85)	65.70 (41.54–110.68)
120	19.5 (0–87.2)	561 (353.2–570.4)	3.2 (3–4.7)	334.6 (232.4–422.2)	<LOD	12.00 (11.07–15.68)	3.28 (2.75–3.31)	15.30 (10.81–18.47)
300	3.1 (0–61.5)	103.6 (99.1–128.2)	0.5 (0.5–0.5)	49.3 (110.3–150.1)	<LOD	13.73 (13.16–19.79)	1.98 (1.50–2.90)	22.43 (19.63–24.62)

The concentrations were measured with HPLC after pretreatment with KA-1 and KA-2. Sprague–Dawley male rats: $n = 5$ in each group; data are shown as median (interquartile range)

KYNA kynurenic acid, *KA-1 N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, *KA-2 N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, *LOD* limit of detection

glutamate levels in the TNC (Oshinsky and Luo 2006). The peripheral application of glutamate to deep craniofacial tissue proved to be able to activate and sensitize nociceptive afferents and neurons in the upper cervical cord (Lam et al. 2009a, b). These findings suggest that excitatory amino acid receptors (and NMDA in particular) play an important role in pain processing and the sensitization process which is also present in migraineurs (Vikelis and Mitsikostas 2007). Ketamine, an NMDA antagonist, is so far the only promising treatment option for patients with severe or long-lasting migraine aura (Afridi et al. 2013). Another novel substance, tezampanel, which acts on the AMPA and kainate subtypes of ionotropic glutamate receptors (Alt et al. 2006), has also shown promising results in acute migraine therapy (Sang et al. 2004).

As regards the preclinical models of headache disorders, NTG is the most frequently used substance to trigger a delayed migraine-like attack (Sicuteri et al. 1987). Animal experiments have revealed that NTG can activate the trigeminal system and stimulate the second-order trigeminal neurons, which lead to increased c-Fos and neuronal nitric oxide synthase (nNOS) expression in the affected area (Párdutz et al. 2000; Tassorelli et al. 1997). NTG is also able to sensitize the trigeminal system in humans (Di Clemente et al. 2009). Together, these results confirm that NTG administration can model the central trigeminal nociceptor sensitization demonstrated in migraine patients (Burstein et al. 2000). With regard to the above-mentioned therapeutic strategy with the aim of neurotransmission modulation via the glutamatergic system, earlier studies revealed that KA-1 is able to attenuate the effects of NTG on the number of nNOS, calcium/calmodulin-dependent protein kinase II type alpha (CamKIIα) and calcitonin gene related peptide (CGRP) IR cells in the TNC, markers related to the activation and sensitization of the nociceptors (Vámos et al. 2010, 2009). KA-2 was later also tested in the NTG model and proved to be able to increase the KYNA concentration both in the C1–C2 region and in the serum. Pretreatment with KA-2 (0.5 and 1 mmol/kg) significantly reduced the effects of NTG on the CGRP-, c-Fos-, nNOS- and CaMKIIα-related changes in the C1–C2 region (Fejes-Szabó et al. 2014). To date, there has been only one study of the pharmacokinetic properties of KYNA amides, which found that only a small proportion of KA-1 decays into KYNA in the serum of C57B/6 mice (Zádori et al. 2011a).

In this study, we used another well-known model of trigeminal nociception, the orofacial formalin test. Formalin solution administered s.c. into the upper lip of rats causes tissue injury, inflammation and nociception (Clavelou et al. 1995). Immunohistochemical studies have revealed that formalin induces c-Fos and nNOS expression in the TNC, similarly as in the NTG model, which suggests

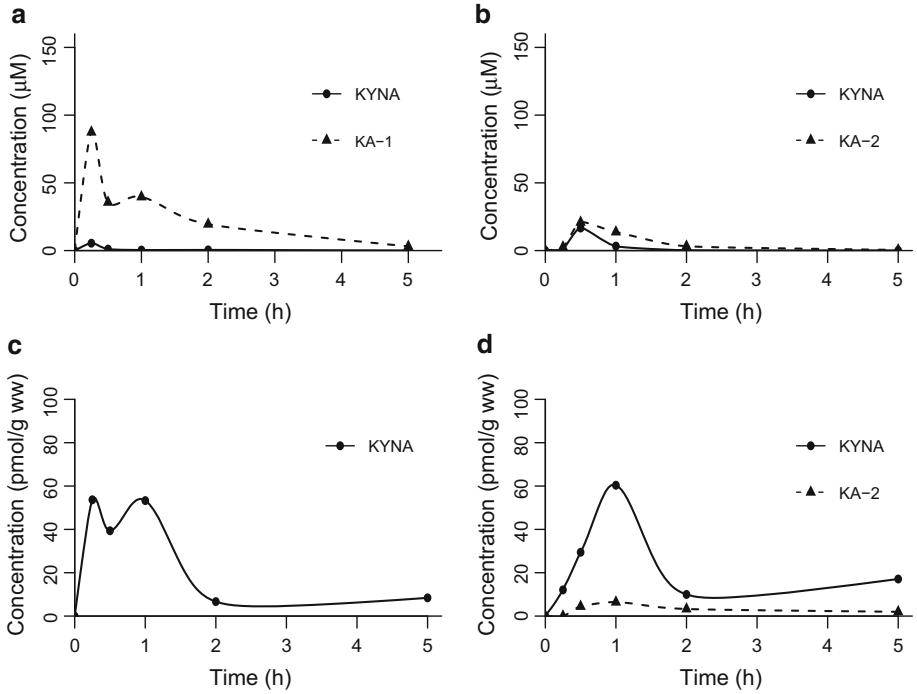

Fig. 7 The concentrations of KYNA and KYNA amides in serum and CNS samples of Sprague–Dawley male rats. **a**, **b** Concentrations of KYNA and KYNA amides in rat serum with the course of time after injection. **c**, **d** Concentrations of KYNA and KYNA amides in the CNS samples of the same animals. KA-1 concentrations were under the limit of detection in the CNS samples. Sprague–Dawley male rats: $n = 5$ in each group; data are shown as medians; *KYNA* kynurenic acid, *KA-1 N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, *KA-2 N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

Table 2 Pharmacokinetic parameters of KYNA and KYNA amides in rat serum and CNS samples after intraperitoneal injection of KA-1 and KA-2

Serum					CNS				
Pharmacokinetic parameters	KA-1 treatment		KA-2 treatment		Pharmacokinetic parameters	KA-1 treatment		KA-2 treatment	
	KA-1	KYNA	KA-2	KYNA		KA-1	KYNA	KA-2	KYNA
$t_{1/2}$ (h)	1.09	0.91	0.84	0.64	$t_{1/2}$ (h)	NA	1.67	2.61	3.45
t_{max} (h)	0.25	0.25	0.50	0.50	t_{max} (h)	NA	0.25	1	1
C_{max} (μM)	87.44	5.44	20.87	16.63	C_{max} (pmol/g)	NA	53.66	6.44	60.37
AUC_{0-t} (μM h)	99.22	2.61	23.35	7.89	AUC_{0-t} (pmol/g h)	NA	86.46	16.24	97.77
Vz/F_{obs} ((μmol/kg)/(μmol/l))	15.17	–	50.02	–	Vz/F_{obs} ((pmol/kg)/(pmol/l))	NA	–	158,879.15	–
Cl/F_{obs} ((μmol/kg)/(μmol/l)/h)	9.61	–	41.81	–	Cl/F_{obs} ((pmol/kg)/(pmol/l)/h)	NA	–	42,220.03	–

The KYNA amides were applied in a dose of 1 mmol/kg. Sprague–Dawley male rats: $n = 5$ in each group

KYNA kynurenic acid, *KA-1 N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride, *KA-2 N*-(2-*N*-pyrrolidinylethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride

the activation and sensitization of the area (Hunt et al. 1987; Párdutz et al. 2000). The behavioral effect of formalin is biphasic, with a short period of rubbing activity immediately after injection and then a tonic prolonged (20–22 min) second phase after a quiescent period (Raboisson and Dallel 2004). Using the formalin model, our aim was to compare the two compounds (KA-1 and KA-2) in an assessment method which involves both peripheral and central components of pain processing, with special interest in the pharmacokinetic explanation of the observed differences.

Our results indicated that KA-2 has more significant beneficial effects on the formalin-induced behavioral and immunohistochemical alterations. We carried out a comparative pharmacokinetic study to clarify this difference between the two analogs. With regard to the serum concentrations of the analogs following their i.p. administration, the levels of KA-2 were considerably lower than those of KA-1 from the aspects of peak concentration and AUC_{0-5h}. In contrast, KA-1 could not be detected in the examined CNS region, and the concentration of KA-2 was likewise relatively low. On the other hand, the serum pharmacokinetic data revealed that KA-2 decays into KYNA in larger amount as compared with KA-1, but nevertheless, in the examined CNS region, there is no major difference between KYNA levels following the treatments with KA-1 or KA-2. Summarizing these findings, the peak elevation of KYNA in the CNS

(approximately tenfold of the basal concentration) is considerably lower (by one order of magnitude) than that of peak elevation of KYNA in the serum (approximately 70–200-fold of the basal concentration) following the administration of KA-1 and KA-2, and with respect to CNS samples, there are no differences between KYNA levels. In view of these findings, the difference in the observed effects in behavioral and immunohistochemical studies could be explained by the differences in serum KYNA levels. From the aspect of a structure–activity relationship, the difference in peripheral conversion may stem from the structures of the two analogs, e.g., in the case of KA-2 the strained pyrrolidine moiety may influence the faster hydrolysis of the amide bond relative to the N,N-dimethyl function (KA-1). These findings suggest that the difference in the beneficial effects of the two analogs may be explained by the peripheral effect of elevated KYNA concentrations on formalin-induced pathological alterations. The molecular background would be the inhibition of NMDA receptor-mediated neurotransmission at the strychnine-insensitive glycine-binding site (Szalárdy et al. 2012) which is present on the peripheral process of the trigeminal nociceptors (Quartu et al. 2002; Watanabe et al. 1994). The observed peak serum KYNA concentration following KA-2 treatment during the experiment (16.71 μM) would be relevant with regard to the inhibition of glutamatergic neurotransmission via the above-mentioned possibility (Szalárdy et al. 2012).

In conclusion, our results draw attention to the role of influencing the glutamatergic system in the alleviation of peripheral sensitization, which can be utilized during future drug development. The possibility of targeting the peripheral component of pain processing would provide an option of pharmaceutical drug design without the obligation of good penetration through the BBB, but other pharmacokinetic parameters, such as solubility and clearance, must be kept in mind. The present results and previous preclinical findings indicate that the KYNA amides, via their probable direct effects (KA-1; Zádori et al. 2011a) or serving as prodrugs (KA-2 in the current pharmacokinetic study), would be promising drug candidates in neurological disorders, including those involving pain and headache, with a high socioeconomic burden.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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IV.

The Effects of Hypokalaemia on the Hormone Exocytosis in Adenohypophysis and Prolactinoma Cell Culture Model Systems

Authors

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Key words
- adenohypophysis cell cultures
- extracellular ion milieu
- hypokalaemia
- prolactinoma

Abstract

The extracellular ion milieu determines the exocytosis mechanism that is coupled to spontaneous electrical activity. The K^+ ion plays crucial role in this mechanism: as the potassium current is associated with membrane hyperpolarization and hormone release through protein cascade activation. The primary aim of this study was to investigate the response mechanisms of normal adenohypophysis and adenohypophyseal prolactinoma cell populations at different extracellular K^+ levels with an otherwise isoionic milieu of all other essential ions. We focused on prolactin (PRL) and adrenocorticotrophic hormone (ACTH) release.

In our experimental study, female Wistar rats (n = 20) were treated with estrone-acetate (150 µg/kg b.w./week) for 6 months to induce prolactinomas in the adenohypophysis. Primary, monolayer cell cultures were prepared by enzymatic and mechanical digestion. PRL and ACTH hormone presence was measured by radioimmunoassay or immuno-chemiluminescence assay. Immunocytochemistry was used to assess the apoptotic cells.

Differences between the effects of hypokalaemia on normal adenohypophysis cultures and prolactinoma cell populations were investigated. Significant alteration ($p < 0.001$, $n = 10$) in hormone exocytosis was detected in K^+ treated adenohypophyseal and prolactinoma cell cultures compared to untreated groups. Immunocytochemistry showed that Bcl-2 expression was reduced under hypokalaemic conditions.

The decrease in hormone exocytosis was tightly correlated to the extracellular K^+ in both cell types, leading to the conclusion that external K^+ may be the major factor for the inhibition of hormone release. The significant increase in hormone content in supernatant media suggests that hypokalaemia may play important role in apoptosis.

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Introduction

There is a persistent and dynamic contact among the living structure and its environment, thus in this context, the cell and its extracellular milieu comprise an operational unit.

2 structurally and functionally distinct entities of hypophysis are the neurohypophysis and the adenohypophysis: both of which are under strict hypothalamic control. The present study is focused on the multiple cell types of the heterogeneous anterior pituitary gland that produce peptide hormones essential for reproduction, lactation, growth, development, response to stress, and metabolic homeostasis [1–4]. Prolactin (PRL) and adrenocorticotropic hormone (ACTH) are studied in this paper.

We intended to investigate response mechanisms in the function of normal, monolayer, primary adenohypophysis cell cultures (Adh) at low extracellular [K^+]. The novelty of our experimental method is that other essential ions were under homeostatic, isoionic conditions. It would be interesting to determine whether environmental stress, namely extracellular hypo [K^+] may modify the cells' rapid accommodation and basic regulatory functions. *In this paper 2 cell cultures were studied, an Adh cell culture and another one formed from a monolayer derived from estrone-acetate induced prolactinomas and their adjoining adenohypophsial cells (PRLoma).*

The effect of extracellular hypoionic conditions on cellular functions is intriguing and likely important factor in a number of pathologies. Several studies revealed that altered extracellular [K^+] plays a crucial role in endocrine-related diseases; for example in chronic kidney disease [5] and in cardio-renal decompensation syndrome

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[6]. It is also known that hypo [K^+] is involved in cell proliferation [7] (e.g. in small-cell lung cancer [8]) by the activation of voltage-gated K^+-channel (K_v) pathways leading to plasmalemma hyperpolarization.

It is documented that hypokalaemia may induce cell aging and cell death through mitogen-activated protein kinases (MAPK), particularly the p38 and c-Jun N-terminal kinases (JNK) [9]. In connection with kinase activation, the generation of reactive oxygen species (ROS) is known to trigger apoptosis [10,11] leading to apoptosis signal regulating kinase 1 (ASK1) activation [12]. Furthermore, the caspase cascade is activated by death signals such as the members of the Bcl-2 family, leading to degradation of cellular structures [13].

Although most pituitary neoplasms are benign, they are associated with high morbidity and mortality. Prolactinomas are the most common benign pituitary adenomas in the general population [14]. Hyperprolactinaemia can be caused by physiological processes [3,15], pharmacological interventions [16], and pathological effects [17,18]. One possible explanation for PRL oversecretion is fluctuation in membrane hyperpolarization that decreases the driving force for Ca^{2+} ions [19]. Steroid hormones, mainly estrogen, are known to potentiate the expression and synthesis of PRL in a manner, which is dependent on elevated intracellular Ca^{2+} concentration and Ca^{2+} influx [20,21]. A rapid increase in the rate of PRL synthesis was detected both *in vivo* and *in vitro* with estradiol treatment [22,23].

Methods

Experimental protocol

Female Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120–250 g, aged 4–6 weeks at the beginning of the research) were used for hypophysis cell culture model systems. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary. During the research period, rats were kept under controlled relative air humidity of 55–65% and 22±2°C ambient temperature. Experimental animals lived under automated diurnal conditions (12 h dark and 12 h light system) in groups of 10 animals for 6 months. Standard pellet food and tap water were available ad libitum. Female Wistar rats (n=20) were treated subcutaneously with estrone-acetate (CAS registry number: 901-93-9, Sigma, Germany; 150 µg/kg b.w./week) for 6 months to induce adenohypophyseal prolactinomas.

After pentobarbital anaesthesia (4.5 mg/kg b.w. Nembutal, Abbott, USA) the animals were killed and decapitated. Tissues were separated under a preparative microscope. Primary, monolayer cell cultures were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2%/Sigma, Germany/ for 30 min; collagenase/Sigma, Germany/: 30 µg/ml for 40 min; dispase/Sigma, Germany/: 50 µg/ml for 40 min in phosphate-buffered saline/PBS-A/; temperature: 37 °C). Mechanical dispersion was achieved with nylon blutex sieves (∅: 83 and 48 µm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2\times10^5/cm^3$. The dissociated cells were placed onto 24 well-plastic plates (5% collagen coated/ Nunc., Germany/; Dulbecco's Modified Essential Medium/ DMEM/+20% Fetal Calf Serum/FCS/+antibiotics/Penicillin+Streptomycin: 1.0 µg/cm^3). The cells were cultured at 37 °C in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking for PRL and ACTH protein release. After functional standardization, the basal ACTH and PRL levels were determined in both normal Adh and PRLoma (Tyrode's medium/ Sigma, Germany/). In the medium, only the [K^+] was modified; all other essential anions and cations were under homeostatic (e.g. isoionic) conditions. The hormone release of primary cell cultures was detected under hypokalaemic conditions of varying degrees ([K^+]: 0; 0.5; 1.0; 1.5; 2.0 mM; n=10 in each group). Samples were taken at 10, 20, 30, 60 and 90 min after treatments to measure hormone kinetics.

The PRL and the ACTH content were detected in the supernatant media. From the supernatant media, 500 µl samples were removed by Gilson pipette at appropriate times and stored at −80 °C until peptide radioimmunoassay (RIA) [24,25] and immuno-chemiluminescence assay (LIA) were performed.

A rat PRL RIA KIT (Institute of Isotopes Ltd., Budapest, Hungary) was used to determine the supernatant PRL content; all components were stored at 2–8 °C, where they were stable. Non-specific binding, defined as the proportion of tracer bound in the absence of antibody, was determined to be <5%. The sensitivity of the RIA procedure was 0.07 ng/tube. The intra-assay precision obtained was 0.92±0.03 ng, PRL data are given in ng PRL/mg protein.

The ACTH levels of supernatant media were measured by LIA with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL) and DPC kit (L2KAC-02; Euro/DPC Ltd, Glyn Rhonwy, UK). ACTH data are given in pg ACTH/mg protein.

A modified Lowry Method [26] and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

Immunocytochemistry

At 90 min of the experiment, the 0 mM [K^+] manipulated monolayers and the controls were fixed by 4% paraformaldehyde and stored until staining. Immunostaining with anti Bcl-2 (Santa Cruz Biotechnology Inc., 1:25 dilution, N-19, sc-492) for 60 min was performed (samples were washed in Tris buffered saline/TBS, 0.05 M, pH 7.4 and 0.85% NaCl/ for 5 min before treatment) after incubating of monolayers with peroxidase blocking reagent for 5 min. After additional washes TBS (5 min) bound antibodies were visualized using 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma, Germany) for 2 min. Samples were then washed in TBS and image was captured using a phase contrast invertoscope (Zeiss) equipped with an Olympus camera (Olympus C-7070).

Statistical analysis

To compare various effects of treatment in Adh and PRLoma over time, two-way repeated measures ANOVA was used for each independent set of data: ACTH secretion of Adh or PRLoma, or PRL release of Adh or PRLoma. Treatment was considered as a between-subject factor and time (5 time points: 10, 20, 30, 60, 90 min) as a within-subject factor for the analysis. We compared the ACTH and PRL secretion of untreated Adh with that of the PRLoma group under hypokalaemia over time using repeated measures ANOVA. Not only group differences, but also individual within-subject variation in time can be modeled.

Significant interaction was found between the 2 investigated factors ($p<0.001$), thus both effects could not be reported independently. When Mauchly's test of sphericity was significant, the Greenhouse-Geisser correction was performed. Pairwise

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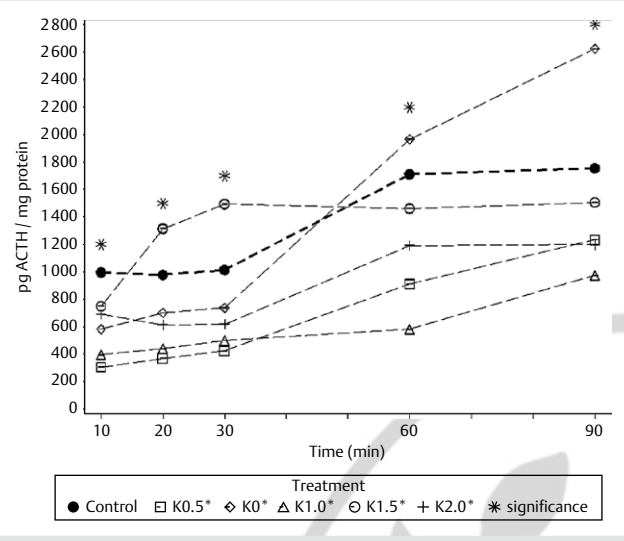

Fig. 1 The effects of different potassium concentrations on the release of ACTH in Adh. Asterisks indicate the significance between the hormone release of various treated groups vs. the ACTH release of normal Adh as the control group.

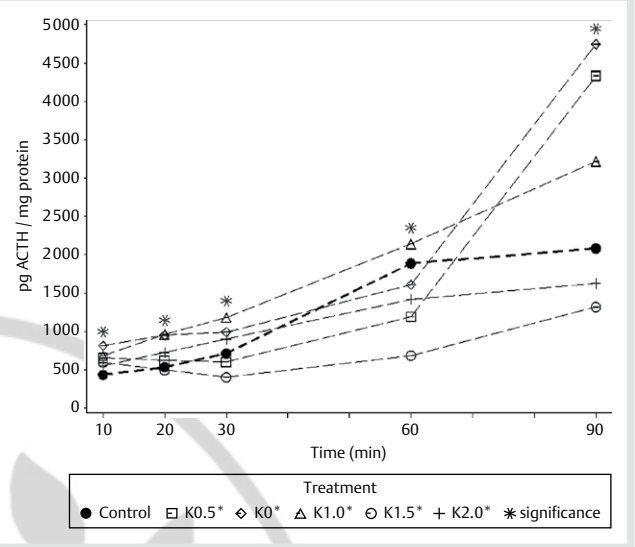

Fig. 2 The effects of different potassium concentrations on the release of ACTH in PRLoma. Asterisks indicate the significance between treated groups vs. the PRL release of untreated PRLoma as a control group.

comparisons of group means were performed, based on estimated marginal means with Sidak adjustment for multiple comparisons. Data are represented as means and S.E.M.

Statistical analyses were carried out using SPSS, version 17 (SPSS Inc., Chicago) software. All tests were two-tailed, and $p < 0.05$ was considered to be statistically significant. All graphs were made with SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

Results

Cell culture standardization resulted that the ACTH positive cells were accounted for approximately 15.81% in Adh and 18.43% in the *in vivo* estron-acetate pretreated, than cultured adenohypophysis tissues (the monolayer contained prolactinoma cells and the adjoining adenohypophysial tissue). The percentage of PRL positive cells was 24.0% in normal Adh and 52.2% in PRLoma. Our *in vitro* experiments revealed statistical differences between treated and untreated groups. **Fig. 1, 2** show the ACTH release of both the control systems and the treated groups. The basal ACTH level of normal Adh and PRLoma is labelled as $CAdh_{ACTH}$ and $CPRLoma_{ACTH}$ respectively.

The basal PRL release of the control Adh ($CAdh_{PRL}$) and PRLoma ($CPRLoma_{PRL}$) systems and the treated primary cell cultures are represented in **Fig. 3, 4**.

In **Fig. 5, 6**, the PRL and ACTH release of CAdh and CPRLoma was compared with that of the cell cultures treated with 0 mM [K^+]. All p-values for within group and between group effects were determined to be statistically significant ($p < 0.001$).

The ACTH release of induced prolactinoma and adjoining adenohypophysial cell cultures and normal adenohypophysis cultures by the effects of different potassium concentration

At 10, 20 and 30 min of the experiment (**Fig. 1**) the ACTH secretion of the groups treated with 0 mM [K^+] decreased appreciably (583.5 ± 1.86, 701.4 ± 1.65, 738.1 ± 1.26 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) compared with the $CAdh_{ACTH}$ (996.9 ± 2.55, 978.4 ± 1.23, 1014.5 ± 1.89 pg hormone/mg protein; means ± S.E.M.). In the supernatant media, the hormone content was increased significantly (1966.4 ± 1.36, 2625.7 ± 0.97 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) at 60 and 90 min under hypokalaemic conditions compared with $CAdh_{ACTH}$ (1711.3 ± 2.07, 1754.5 ± 1.34 pg hormone/mg protein; means ± S.E.M.).

As shown in **Fig. 1**, the ACTH release of Adh treated with 0.5 mM [K^+] diminished significantly ($p < 0.001$) depending upon the duration of exposure (306.0 ± 1.96, 369.8 ± 1.26, 424.6 ± 1.45, 911.2 ± 1.17, 1235.4 ± 1.25 pg hormone/mg protein; means ± S.E.M.).

The hormone levels of cell cultures treated with 1.0 mM [K^+] (**Fig. 1**) were reduced significantly (396.7 ± 1.82, 439.3 ± 1.58, 499.6 ± 1.10, 582.3 ± 1.75, 973.5 ± 1.48 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) depending upon the duration of exposure, correlating with the $CAdh_{ACTH}$.

It was observed that the ACTH release of cell cultures treated with 1.5 mM [K^+] was decreased significantly (1461.8 ± 3.61, 1506.8 ± 1.48 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) at 60 and 90 min correlating with the $CAdh_{ACTH}$. The secretion of ACTH was decreased significantly as a consequence of 2.0 mM [K^+] treatment depending upon the duration of exposure (691.2 ± 1.58, 614.4 ± 1.66, 618.7 ± 1.57, 1192.9 ± 1.75, 1199.3 ± 1.46 pg hormone/mg protein; means ± S.E.M., $p < 0.001$).

In **Fig. 2**, the ACTH release of PRLoma was increased significantly (813.4 ± 1.19, 951.9 ± 3.03, 992.6 ± 1.92, 1610.5 ± 1.91, 4746.2 ± 3.61 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) in the 0 mM [K^+] group compared with that of $CPRLoma_{ACTH}$ (435.1 ± 1.39, 536.3 ± 3.10, 713.6 ± 1.82, 1887.9 ± 2.52, 2083.0 ± 3.42 pg hormone/mg protein; means ± S.E.M.).

The hormone release of ACTH treated with 0.5 mM [K^+] increased (4333.7 ± 5.37 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) at 90 min of the experiment compared with the $CPRLoma_{ACTH}$ group.

As depicted in **Fig. 2**, the hormone levels in the PRLoma group treated with 1.0 mM [K^+] were elevated significantly (685.4 ± 1.53, 962.0 ± 2.05; 1181.3 ± 1.83, 2142.0 ± 1.72, 3217.7 ± 2.54 pg hormone/mg protein; means ± S.E.M., $p < 0.001$) compared to $CPRLoma_{ACTH}$.

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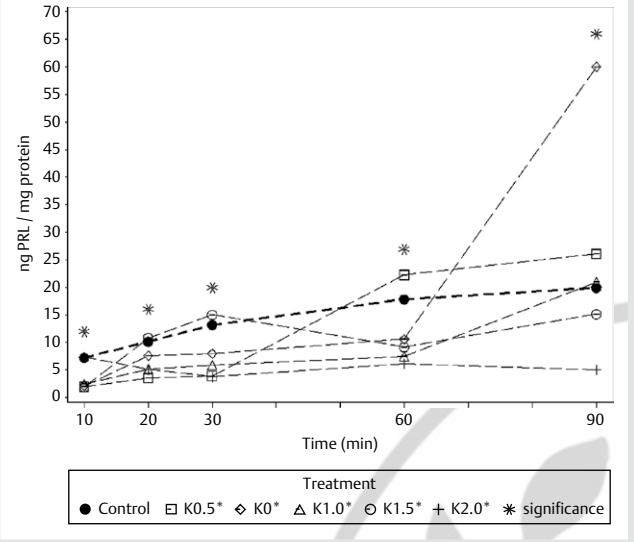

Fig. 3 The effects of different potassium concentrations on the release of PRL in Adh. Asterisks indicate the significance between treated groups vs. the PRL release of untreated Adh as control group.

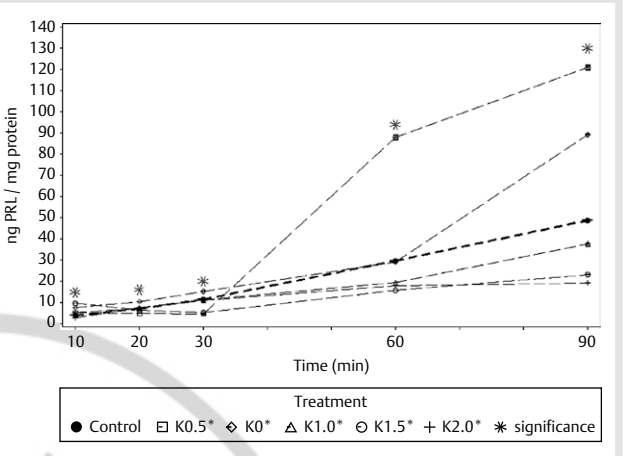

Fig. 4 The effects of different potassium concentrations on the release of PRL in PRLoma cell populations. Asterisks indicate the significance between treated groups vs. the PRL release of PRLoma cultures as control group.

In the 1.5 mM [K^+] manipulated groups the ACTH secretion was decreased (495.6±2.71, 403.1±2.18, 685.0±2.68, 1319.7±2.25 pg hormone/mg protein; means±S.E.M., $p<0.001$) depending upon the duration of exposure. The hormone levels of treated PRLoma under 2.0 mM [K^+] were decreased significantly (1418.9±2.13, 1626.1±2.24 pg hormone/mg protein; means±S.E.M., $p<0.001$) at 60 and 90 min of the experiment.

The effects of different potassium concentrations on the release of PRL in pretreated and normal adenohypophysis cultures

The PRL levels of the 0 mM [K^+] group decreased (**Fig. 3**; 2.11±0.01, 7.65±0.01, 7.95±0.01, 10.61±0.02 ng hormone/mg protein; means±S.E.M., $p<0.001$) at 10, 20, 30 and 60 min of experiment compared with $CAdh_{PRL}$ (7.24±0.01, 10.15±0.01, 13.18±0.02, 17.84±0.02 ng hormone/mg protein; means±S.E.M.). In contrast to this, the hormone secretion of treated cell cultures increased (60.04±0.02 ng hormone/mg protein; means±S.E.M., $p<0.001$) at 90 min, compared to the untreated groups (19.93±0.01 ng hormone/mg protein; means±S.E.M.).

Notable enhancement was detected in the PRL secretion of 0.5 mM [K^+] manipulated groups (22.25±0.05, 26.09±0.02 ng hormone/mg protein; means±S.E.M., $p<0.001$) at 60 and 90 min, in contrast to the $CAdh_{PRL}$.

As shown in **Fig. 3**, PRL release at 90 min was increased significantly (21.0±0.02 ng hormone/mg protein; means±S.E.M., $p<0.001$) by the effects of 1.0 mM [K^+] compared with the $CAdh_{PRL}$.

In **Fig. 3** significant decrease (1.97±0.01, 9.14±0.01, 15.14±0.01 ng hormone/mg protein; means±S.E.M., $p<0.001$) was noticed in the PRL release at 10, 60 and 90 min of the treatment by the application of 1.5 mM [K^+].

As shown in **Fig. 3**, the PRL secretion was reduced significantly (7.41±0.01, 5.11±0.01, 3.77±0.01, 6.13±0.01, 5.07±0.01 ng hormone/mg protein; means±S.E.M., $p<0.001$) depending upon the duration of exposure to 2.0 mM [K^+].

In **Fig. 4**, an increase in PRL release (7.64±0.01, 10.48±0.02, 15.32±0.05, 89.23±0.17 ng hormone/mg protein; means±S.E.M., $p<0.001$) was observed in the 0 mM [K^+] groups compared to $CPRLoma_{PRL}$ (3.86±0.01, 7.11±0.02, 11.52±0.01, 48.78±0.02, ng hormone/mg protein; means±S.E.M.) at 10, 20, 30 and 90 min of the experiment.

Fig. 4 shows that the levels of PRL were reduced significantly (4.82±0.02, 4.73±0.02 ng hormone/mg protein; means±S.E.M., $p<0.001$) at 20 and 30 min compared with the $CPRLoma_{PRL}$. Then the PRL level was increased significantly (88.07±0.25, 121.0±0.25 ng hormone/mg protein; means±S.E.M., $p<0.001$) by the effects of 0.5 mM [K^+] at 60 and 90 min of treatment.

The PRL secretion of the 1.0 mM [K^+] groups was reduced significantly (19.34±0.02, 37.67±0.21 ng hormone/mg protein; means±S.E.M., $p<0.001$) at 60 and 90 min, in contrast to $CPRLoma_{PRL}$.

Fig. 4 shows the PRL release of PRLoma was increased (9.63±0.02 ng hormone/mg protein; means±S.E.M., $p<0.001$) by 1.5 mM [K^+] treatment at 10 min; however, the PRL secretion in the cardinal points of the research protocol decreased significantly (6.28±0.04, 5.32±0.03, 15.77±0.02, 23.24±0.07 ng hormone/mg protein; means±S.E.M., $p<0.001$).

The hormone secretion was reduced significantly (10.94±0.03, 17.93±0.03, 19.29±0.03 ng hormone/mg protein; means±S.E.M., $p<0.001$) by the effects of 2.0 mM [K^+] depending upon the duration of exposure.

The effects of hypokalaemia on the ACTH and PRL release in adenohypophysis containing prolactinoma vs. normal adenohypophysis

We next examined the correlation between the normal and the altered endocrine regulation modified by hypokalaemia. As shown in **Fig. 5**, under hypokalaemia the ACTH release of PRLoma was increased significantly at 90 min of the experiment, in contrast to $CAdh_{ACTH}$. A similar interaction is depicted in **Fig. 6**: the PRL release increased significantly in PRLoma compared with $CAdh_{PRL}$.

Immunostaining

Immunocytochemical staining with anti-Bcl-2 was shown in **Fig. 7**. It was observed that the levels of Bcl-2 were higher in control groups than in 0 mM [K^+] manipulated groups.

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Discussion and Conclusion

In this paper, *in vitro* model systems (primary monolayer cell cultures) were used and their cellular functions were standardized to investigate cellular phenomena. We decided to focus on the alteration of ACTH and PRL release in Adh and PRLoma under hypokalaemic conditions. Differences between the above mentioned primary cell cultures were investigated. The role of the extracellular ionic milieu in cell function can be defined by hormone exocytosis, sensitivity of intracellular receptors, or the discrete alteration of intracellular messenger molecules.

Inhibitory signals depend upon extracellular K^+ conductance. A reduction of the external [K^+] depolarizes the membrane [27], while an increase in external [K^+] hyperpolarizes the plasmalemma. In the anterior lobe of the pituitary gland, hormone exocytosis is associated with electrical and protein cascade signaling pathways. Recently published literature has revealed that in lactotrophs and corticotrophs the spontaneous electrical activity couples to hormone secretion [28].

The electrical properties of cell membrane activate cell surface receptors, which mediate variable cellular processes, including G-protein utilization and cyclic mononucleotide accumulation. Activation of the Gq/G_{11} protein induces membrane-bound phospholipase-C that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate (IP_3) and diacylglicerol (DAG) [29]. In lactotrophs and corticotrophs, IP_3 is essential to mediate the mobilization of non-mitochondrial Ca^{2+}. DAG activates Ca^{2+} dependent protein kinase C and protein kinase B, which phosphorylates voltage-sensitive

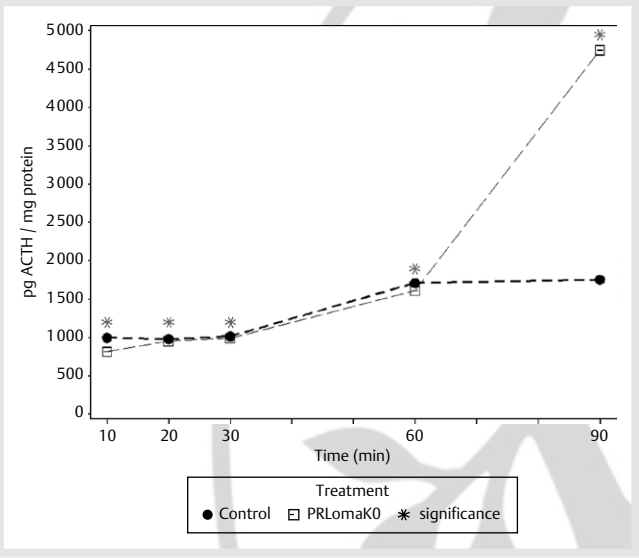

Fig. 5 The effects of hypokalaemia on the ACTH release of PRLoma vs. normal Adh hormone release. Asterisks indicate the significance among the hormone release of hypokalaemic PRLoma group (PRLomaK0) vs. normal Adh as control group, n = 10 in each group.

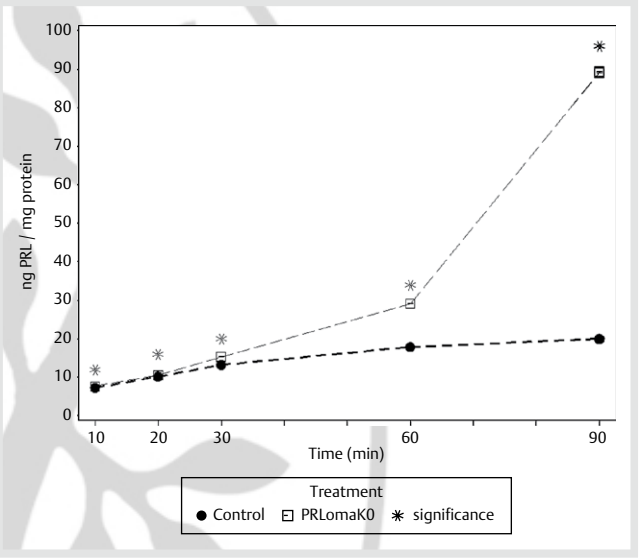

Fig. 6 The effects of hypokalaemia on the PRL release of PRLoma vs. normal Adh hormone release. Asterisks indicate the significance among the hormone release of hypokalaemic PRLoma group (PRLomaK0) vs. untreated Adh as control group, n = 10 in each group.

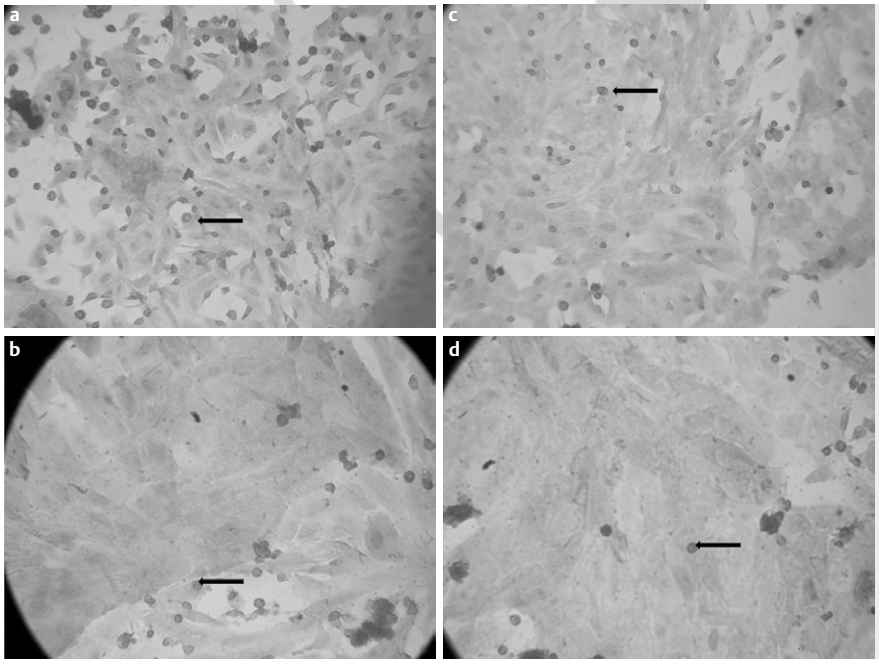

Fig. 7 Immunocytochemical analysis of Bcl-2 protein in rat adenohypophysis monolayer cultures. The cells were immunostained with antisera specific for Bcl-2. Antibodies were detected by DAB method; **a, b** control monolayer cultures; **c, d** monolayer cultures under hypokalaemic conditions. Arrows indicate Bcl-2.

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Ca^{2+}-channels resulting in an increased Ca^{2+} influx [30]: enhancing hormone exocytosis through SNARE complex activation [31].

Since the extracellular environment is constantly fluctuating, the cell must adapt to it [32,33]. A reduced rate of hormone exocytosis was observed as a result of higher, but still hypoionic extracellular ion milieu. Interestingly, some reports suggest an evoked hormone release in hypophysis by the effects of higher extracellular [K^+] [34–36]. Under hypokalaemic conditions potassium channels, to maintain the equilibrium of K^+, may open and cause efflux of K^+ leading to cell membrane hyperpolarization [27,37,38]. According to our hypothesis hyperpolarization may block AC activity through $G_{i\text{-}3\alpha}$ activation that leads to a reduction of IP_3 metabolism and a decreased intracellular Ca^{2+} concentration. Diminution of Ca^{2+} influx inhibits the SNARE mediated fusion of ACTH and PRL containing vesicles to the plasma membrane. The discrete elevation of ACTH and PRL production by the effects of increased extracellular [K^+] suggested that hyperpolarization showed both time and cell type dependence.

The results showed that there was relation between the hormone release of PRLoma by K^+ depletion and the hormone exocytosis of untreated normal Adh. Under hypokalaemic conditions an increase in hormone content was observed in the supernatant media of PRLoma in the latest part of the experiment (at 90 min; occasionally at 60 min). The cell aging machinery may play role in this phenomenon. Apoptosis is known to be crucial in controlling cell number and proliferation [39]. Different extracellular stimuli activate tyrosine kinases through phosphorylation such as MAPK, which induces cell death. Recent studies suggest that specifically p38 and the stress kinase JNK [9] and ASK1 play key role in the apoptosis machinery mediated by hypoionic conditions. Under hypokalaemia ASK1 is activated, which then triggers JNK and p38 activation. These crucial events induce the pro-apoptotic protein Bad activation and Bcl-2 diminution. Activated Bad then triggers Bax-Bak oligomerization leading to cell aging, and cell death [40], which can be observed in the kinetics of ACTH and PRL release. The hormone content of the supernatant media increased significantly depending upon the duration of exposure. This phenomenon may be explained by membrane disintegration through caspase cascade signalling system [41], thus several types of caspases mainly the caspase-12 pathway recruits to dismantle the cellular structures [13] including the hormone containing vesicles.

Our results indicate that a strict association exists among certain biophysical properties, especially the extracellular K^+ milieu, hormone vesicle exocytosis and apoptosis. Understanding of hormone secretion and extracellular ion milieu has improved dramatically over the past few years: yet, there is much that remains to be explored. Considering this, it is important to improve our knowledge of the relationship between the hormone release of untreated cultures and PRLoma under K^+ depletion.

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The Hormone Exocytosis in Prolactinoma and Normal Adenohypophysis Cell Cultures by the Effects of Hypocalcaemia

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Abstract

The biological systems are opened, complex objects, which can regularly exchange feedbacks with their environment. The calcium ion is a universal messenger, which can regulate several cellular functions e.g. exocytosis machinery. The primary aim of this study was to investigate the response mechanisms of normal adenohypophysis and adenohypophyseal prolactinoma cell populations at different extracellular Ca^{2+} levels with an otherwise isoionic milieu of all other essential ions. We focused on prolactin (PRL) and adrenocorticotrophic hormone (ACTH) release.

In our experimental study, female Wistar rats (n=10) were treated with estrone-acetate (150 µg/kg b.w/week) for 6 months to induce prolactinomas in the adenohypophysis. Primary, monolayer cell cultures were prepared by enzymatic and mechanical digestion. PRL and ACTH hormone presence was measured by radioimmunoassay or immuno-chemiluminescence assay. Repeated measurements of ACTH and PRL hormone release in different treatment groups on cell cultures during 80 minutes were compared using marginal models.

Differences between the effects of hypocalcaemia on normal adenohypophysis cultures and prolactinoma cell populations were investigated. Significant alteration ($p<0.001$, n=12) in hormone exocytosis was detected in Ca^{2+} treated adenohypophyseal and prolactinoma cell cultures, compared to untreated groups.

Diminution of Ca^{2+} may inhibit the SNARE mediated fusion of hormone containing vesicles to plasma membrane. In conclusion, the main finding of this study is that a strict correlation exists among certain biophysical properties, especially extracellular Ca^{2+} milieu and hormone vesicle exocytosis.

Keywords: Adenohypophysis cell cultures; Prolactinoma; Extracellular ion milieu; Hypocalcaemia; Prolactin; Adrenocorticotrophic hormone

Introduction

The biological systems are opened, thus they regularly exchange feedbacks with their environment. In the adaptation processes the hypophyseal hormones, primarily adrenocorticotrophic (ACTH) [1] and prolactin (PRL) [2] play major role. Firstly the hypothalamo-pituitary-adrenal (HPA) axis activity is essential in the adaptability of living organisms and their capacity to construct new operating conditions via ACTH release of corticotroph cells [3]. The HPA axis regulates the circadian rhythm, activates in response to stress and activates the release of adrenal corticosteroids. Secondly PRL is a common mediator of the immune-neuroendocrine system and affects the different reproductive states. PRL secretion is also modified by environmental inputs such as light, sound and stress [2].

To examine these hormone effects in the level of organism it is important to investigate the mechanisms of endocrine cells. A complex dynamical system can be described with its internal structure and the relationships among the internal structure and its external environment, therefore in the level of cells the extracellular homeostasis is determined by the ion regulation, e.g. Ca^{2+} regulation. In cellular level the calcium ion (Ca^{2+}) is a universal messenger, which can act an extracellular and intracellular messenger to regulate a diverse array of cellular functions [4,5]. It has been known, that changes in [Ca^{2+}] interfere with gating properties of plasma membrane channels, which in turn, affect spontaneous and receptor-controlled changes in the intracellular [Ca^{2+}] [6,7].

Upon entering the cytoplasm, most Ca^{2+} is trapped by Ca^{2+} binding proteins [4,8]. Intracellular Ca^{2+} sensors are e.g. calmodulin, Ras, Raf, MEK, MAPK [8].

Ca^{2+}-channels in many different cell types activate on membrane depolarization and mediate Ca^{2+} influx in response to action potentials [9]. Ca^{2+} entering the cells through voltage-gated Ca^{2+}-channels serves (CaV) as the second messenger of electrical signaling, initiating many different cellular events [10,11].

The β subunit of the Ca^{2+}-channels bounds to calmodulin via their carboxy-terminal domain. Calmodulin is essential for exocytosis machinery. C1 domain of calmodulin bounds to diacylglycerol (DAG), meanwhile C2 domain interacts with 2 or 3 Ca^{2+}, than activates phospholipases and protein-kinases [5,12].

The exocytosis machinery is triggered by membrane depolarization followed by Ca^{2+} entry (Figure 1). In resting cells the intracellular [Ca^{2+}] is low. By the effects of extracellular stimuli, alterations of the electrical properties of cell membrane induce the voltage-gated Ca^{2+} channels and mediate G-protein utilization. Gα subunit of Gq/G11 protein activates the phospholipase-C that cleaves phosphatydil-inositol 4,5-bisphosphate into inositol [1,4,5] trisphophate (IP3) and DAG [7,13]. These intracellular messengers induce higher intracellular [Ca^{2+}] that trigger the fusion of hormone containing vesicles to plasma membrane.

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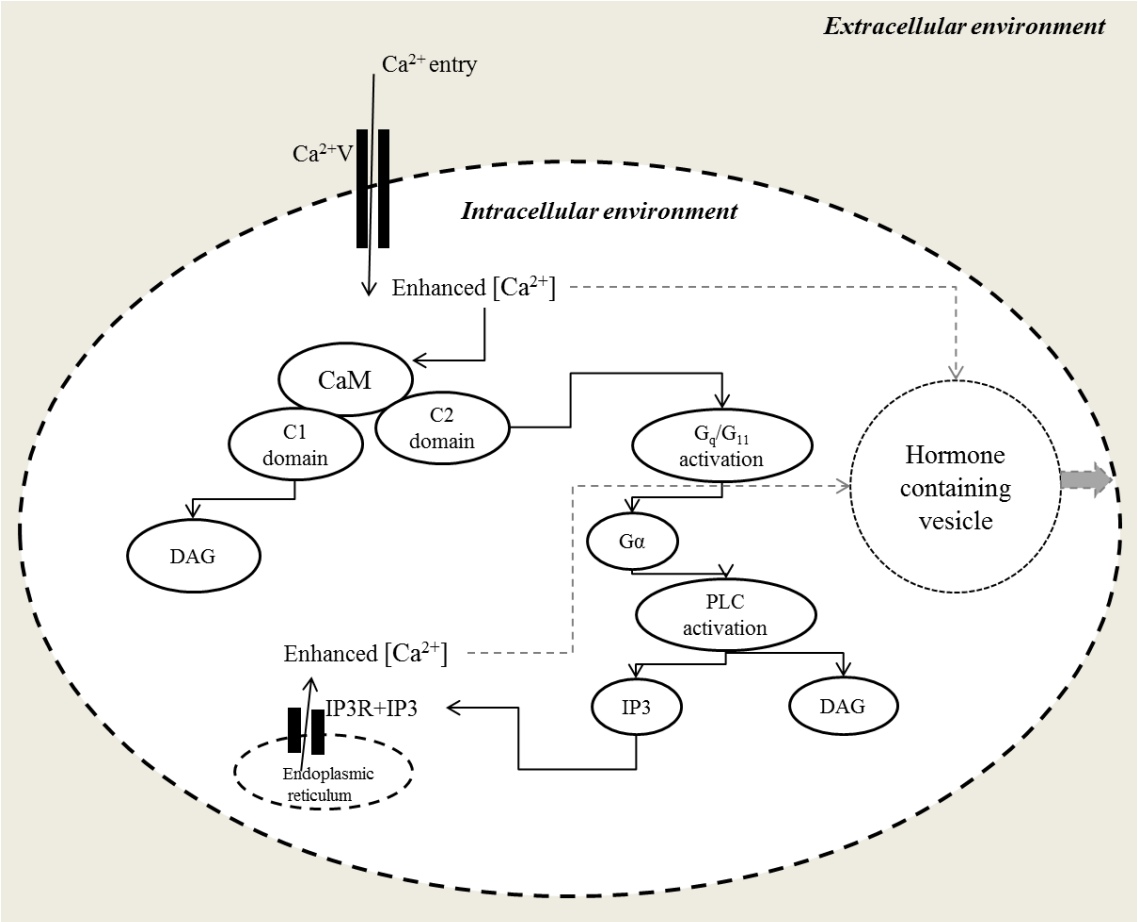

Figure 1: The possible mechanism of hormone exocytosis.
Ca^{2+} enters the cells via voltage-gated Ca^{2+}-channels (Ca^{2+}V). Ca^{2+} bounds to calmodulin (CaM) than the C1-domain of the active CaM activates diacyl-glycerol (DAG); meanwhile the C2 domain mediates G_q/G_{11} proteins. The Gα-subunit of G proteins enhance the activation of phospholipase-C (PLC), that cleaves phosphyatydil-inositol 4,5 bisphosphate into inositol (1,4,5)-trisphosphate and DAG. IP3 bounds to IP3 receptors, which release Ca^{2+} from the endoplasmic reticulum. The higher intracellular [Ca^{2+}] supports the fusion of hormone containing vesicles to plasma membrane

Ca^{2+}-channels interaction with proteins such as syntaxin, SNAP25 has begun to increase. Synaptotagmin plays major role in membrane fusion and transmitter release as well as in hormone exocytosis [14,15]. Then the excitosome can fuse to the plasma membrane [16].

To investigate the hormone secretion mechanisms of endocrine cells is essential to understand disorders, which are manifested in overexpression of these hormones. In these days it has been recognized that pituitary adenomas are frequently in general human population. PRL secreting prolactinomas are the most common pituitary adenomas and account for up to 45% of these tumors. In young adults, prolactinomas occur more frequently in women than men, while this sex imbalance is not apparent in the middle aged population [17,18].

This study examines the extracellular environment of cells and their adaptation mechanisms as a complex system. We wanted to investigate the cell membrane function and/or mechanisms under hypocalcaemic effects, because the effect of extracellular hypoionic conditions on cellular (exocytosis) functions is intriguing and likely important factor in a number of endocrine disorders, and since most of the published work, investigates the release of neurotransmitter vesicles in neurons and this present work is potentially interesting due to its focus on hormone release.

Materials and Methods

Experimental model

Experimental protocol: Female Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for hypophysis cell culture model systems. The animal care and research protocols were in full accordance with the guidelines of University of Szeged, Hungary. During the research period, rats were kept under controlled relative air humidity of 55-65% and 22 ± 2°C ambient temperature. Experimental animals lived under automated diurnal conditions (12 h dark and 12 h light system) in groups of 10 animals for 6 months. Standard pellet food and tap water were available *ad libitum*. Female Wistar rats (n=10) were treated subcutaneously with estrone-acetate (CAS registry number: 901-93-9, Sigma, Germany; 150 μg/kg b.w./week) for 6 months to induce adenohypophyseal prolactinomas.

After pentobarbital anaesthesia (4.5 mg/kg b.w. Nembutal, Abbott, USA) the animals were killed and decapitated. Tissues were separated under a preparative microscope. Primary, monolayer cell cultures were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2%/Sigma, Germany/for 30 min; collagenase/Sigma, Germany/:30 μg/ml for 40 min; dispase/

Sigma, Germany/:50 µg/ml for 40 min in phosphate-buffered saline/PBS-A/; temperature: 37°C). Mechanical dispersion was achieved with nylon blutex sieves (Ø: 83 and 48 µm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be $2 \times 10^5/cm^3$. The dissociated cells were placed onto 24 well-plastic plates (5% collagen coated /Nunc., Geramany/; Dulbecco's Modified Essential Medium/DMEM/+20% Fetal Calf Serum/FCS/+antibiotics/Penicillin+Streptomycin: 1.0 µg/ml/). The cells were cultured at 37°C in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking for PRL and ACTH protein release. After functional standardization, the basal ACTH and PRL levels were determined in both normal Adh and PRLoma (Tyrode's medium/Sigma, Germany/) [19]. In the medium, only the [Ca^{2+}] was modified; all other essential anions and cations were under homeostatic (e.g. isoionic) conditions. The hormone release of primary cell cultures was detected under hypocalcaemic conditions of varying degrees ([Ca^{2+}]: 0; 0.5; 1.0; 1.5 mM; n=12 in each group). Samples were taken at 10, 20, 30, 60 and 90 minutes after treatments to measure hormone kinetics.

The PRL and the ACTH content were detected in the supernatant media. From the supernatant media, 500 µL samples were removed by Gilson pipette at appropriate times and stored at -80°C until peptide radioimmunoassay (RIA) [20] and immuno-chemiluminescence assay (LIA) were performed.

A rat PRL RIA KIT (Institute of Isotopes Ltd., Budapest, Hungary) was used to determine the supernatant PRL content; all components were stored at 2-8°C, where they were stable. Non-specific binding, defined as the proportion of tracer bound in the absence of antibody, was determined to be <5%. The sensitivity of the RIA procedure was 0.07 ng/tube. The intra-assay precision obtained was 0.92 ± 0.03 ng. PRL data are given in ng PRL/mg protein.

The ACTH levels of supernatant media were measured by LIA with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL) and DPC kit (L2KAC-02; Euro/DPC Ltd, Glyn Rhonwy, UK). ACTH data are given in pg ACTH/mg protein.

A modified Lowry Method [21] and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

Statistical analysis: Repeated measurements of ACTH and PRL hormone release in different treatment groups on cell cultures during 80 min (time points at 10, 20, 30, 60 and 90 min; n=12 in each group) were compared using marginal models (a.k.a. population average models) [22,23]. PRLoma with 1 group and treatment with 5 groups were used as between-subject fixed factors and time with 5 time points as within-subject fixed factor for the analysis. Restricted maximum likelihood estimation and Kenward-Roger method for adjusting the degrees of freedom were applied. In case of ACTH, unstructured covariance matrix, for PRL data, the heterogeneous first order autoregressive covariance matrix resulted the best fit among different structures (variance components, compound symmetry, first order autoregressive, toeplitz, unstructured and their heterogeneous versions), based on Akaike's information criterion (AIC) statistic [24]. Pairwise comparisons were estimated by least squares means using Sidak p-value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions. In case of extreme values, winsorization technique was applied by shifting the strongly outlying data toward the center to protect parameter estimation against the emergence of unexpectedly large errors [25,26].

Statistical analyses were performed in SAS (Version 9.3 SAS Institute Inc., Cary, NC, USA), where p-values of <0.05 were considered to indicate statistical significance (SAS, 2011).

Results

Cell culture standardization resulted that the ACTH positive cells were accounted for approximately 15.81% in Adh and 18.43% in the *in vivo* estron-acetate pretreated, than cultured adenohypophysis tissues (the monolayer contained prolactinoma cells and the adjoining adenohypophysial tissue). The percentage of PRL positive cells was 24.0% in normal Adh and 52.2% in PRLoma. Significant interaction was found between time, PRLoma and treatment groups ($p<0.0001$) for ACTH and PRL as well. Pairwise comparisons revealed that all treatment groups differ significantly ($p<0.0001$) from the control at every time points.

The effects of hypocalcaemia on the release of ACTH and PRL in normal adenohypophysis

Figure 2 shows the ACTH release of both the normal AdH control systems and the treated groups.

The basal PRL release of the control AdH systems and the treated primary cell cultures are represented in Figure 3.

Our experiments revealed statistical differences between treated and untreated groups. In Figure 2 the ACTH content of absolute control groups in supernatant media were compared with that of the groups treated with 0 and 0.5 mM [Ca^{2+}]. During the experiment the ACTH secretion of treated groups decreased appreciably (in groups treated with 0 mM [Ca^{2+}]: 293.92 ± 1.97, 291.4 ± 2.45, 314.08 ± 1.72, 408.75 ± 1.21, 440.08 ± 1.07 and in groups treated with 0.5 mM [Ca^{2+}]: 370.75 ± 1.37, 323.08 ± 1.18, 304.92 ± 1.45, 330.42 ± 1.04, 383.58 ± 1.0; pg hormone/mg protein; means ± S.E.M, $p<0.0001$) compared with controls (531.75 ± 3.57, 570.83 ± 5.02, 757.25 ± 2.78, 1476.50 ± 4.30, 1853.42 ± 82.72; pg hormone/mg protein; means ± S.E.M). It was observed that at 60 and 90 min of the experiment the hormone content was higher in 0 mM groups than in 0.5 mM groups.

The hormone levels of cell cultures treated with 1.0 mM [Ca^{2+}] (Figure 3) were reduced significantly (392.25 ± 0.76, 370.0 ± 1.55, 387.92 ± 6.32, 1010.0 ± 2.18, 1194.25 ± 1.53 pg hormone/mg protein; means ± S.E.M, $p<0.0001$) depending upon the duration of exposure, correlating with the control groups. The hormone levels of supernatant media in 1.5 mM [Ca^{2+}] treated groups were decreased significantly (444.50 ± 1.25, 427.67 ± 0.93, 439.25 ± 1.07, 1014.42 ± 1.49, 1194.25 ± 1.53 pg hormone/mg protein; means ± S.E.M, $p<0.0001$).

As shown in Figure 3 the PRL secretion was reduced significantly by the effects of 0 and 0.5 mM [Ca^{2+}] depending upon the duration of exposure (in groups of treated with 0 mM [Ca^{2+}]: 4.52 ± 0.02, 4.11 ± 0.02, 3.82 ± 0.02, 3.89 ± 0.02, 3.88 ± 0.01 and in groups treated with 0.5 mM [Ca^{2+}]: 5.25 ± 0.01, 3.77 ± 0.01, 2.96 ± 0.02, 3.03 ± 0.01, 6.78 ± 0.02 ng hormone/mg protein; means ± S.E.M, $p<0.0001$) compared with control groups (7.19 ± 0.01, 10.16 ± 0.01, 13.21 ± 0.01, 17.88 ± 0.02, 19.88 ± 0.02 ng hormone/mg protein; means ± S.E.M).

Figure 3 shows that the levels of PRL were reduced significantly by the effects of 1.0 mM [Ca^{2+}] (6.13 ± 0.02, 5.86 ± 0.02, 5.29 ± 0.02, 8.78 ± 0.01, 12.79 ± 0.01 ng hormone/mg protein; means ± S.E.M, $p<0.0001$) compared with the controls. The PRL secretion in the cardinal points of

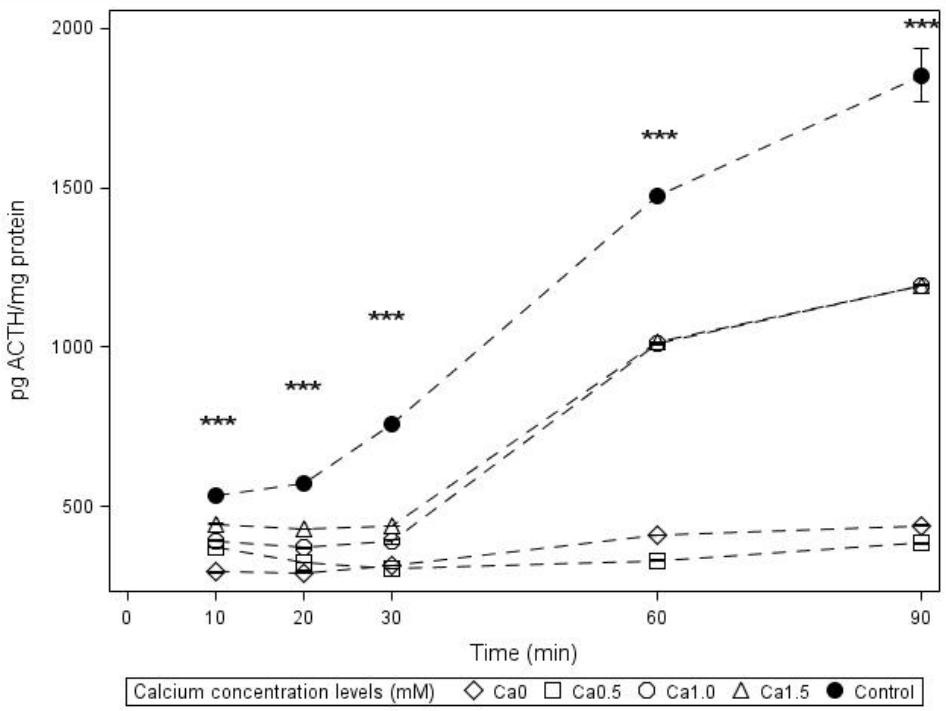

Figure 2: The effects of hypocalcaemia on the ACTH release in adenohypohysis cell cultures.
Cell cultures were treated as follows [Ca^{2+}]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated groups versus the ACTH release of normal Adh as the control group. The level of significance was chosen as p<0.0001

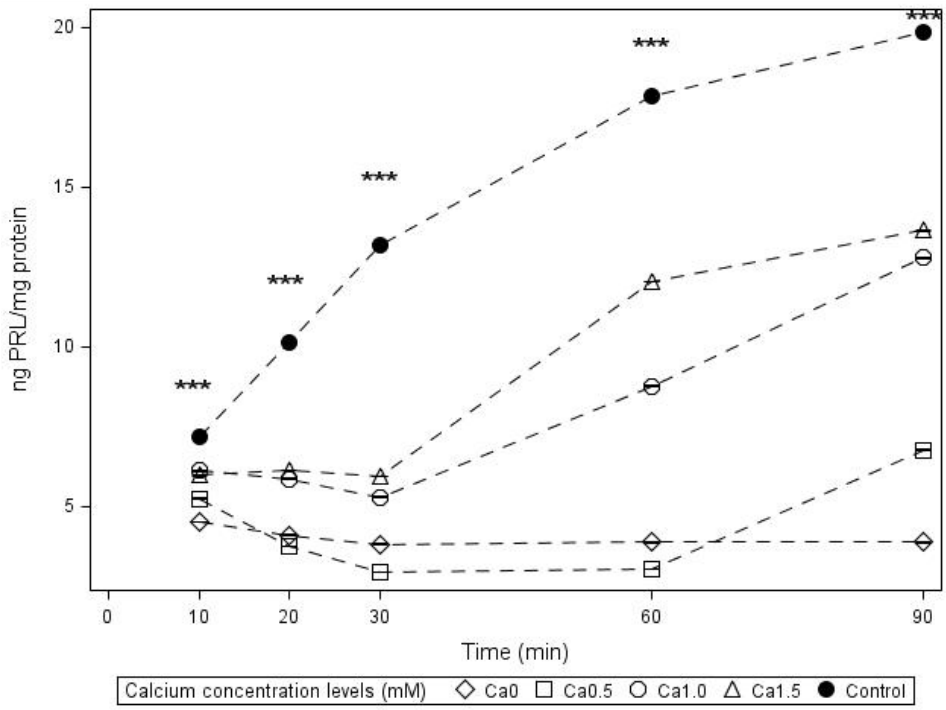

Figure 3: The effects of hypocalcaemia on the prolactin release in adenohypophysis monolayer cell cultures.
Cell cultures were treated as follows [Ca^{2+}]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated groups versus the PRL release of normal Adh as the control group. The level of significance was chosen as p<0.0001

the research protocol decreased significantly (5.98 ± 0.01, 6.13 ± 0.01, 5.96 ± 0.02, 12.04 ± 0.02, 13.64 ± 0.02 ng hormone/mg protein; means ± S.E.M, p<0.0001) by the effects of 1.5 mM [Ca^{2+}].

The effects of hypocalcaemia on the release of ACTH and PRL in the prolactinoma cell cultures

Figure 4 shows the ACTH release of both the PRLoma cell culture control systems and the Ca^{2+} treated PRLoma groups.

The basal PRL release of the control PRLoma systems and the treated primary PRLoma cell cultures are represented in Figure 5.

As shown in Figure 4, at 10 min of the experiment the ACTH of PRLoma cell cultures treated with 0 mM [Ca^{2+}] was enhanced (598.75 ± 2.63 ng hormone/mg protein; means ± S.E.M, p<0.0001) and also enhanced in 1.5 mM [Ca^{2+}] treated groups (469.83 ± 1.16 ng hormone/mg protein; means ± S.E.M, p<0.0001) compared with the control groups (441.0 ± 1.5 ng hormone/mg protein; means ± S.E.M). In the supernatant media the hormone levels were decreased significantly in both treated groups compared with the controls depending upon the duration of exposure. It was observed that the hormone release of PRLoma cell cultures treated with 0 mM [Ca^{2+}] was increased significantly at 90 min of the experiment (2113.17 ± 7,8 ng hormone/mg protein; means ± S.E.M, p<0.0001) correlating with the control group (1946.5 ± 1946.5 ng hormone/mg protein; means ± S.E.M).

In Figure 5, it can be observed that the PRL release of PRLoma cell cultures, under different hypocalcaemic conditions, was decreased significantly depending upon the duration of exposure compared with the control group. Interestingly notable enhancement was detected in the PRL secretion in groups treated with 1.5 mM [Ca^{2+}] (5.6 ± 0.02 ng hormone/mg protein; means ± S.E.M, p<0.0001) in contrast to control (3.82 ± 0.01 ng hormone/mg protein; means ± S.E.M). At 90 min of the experiment the PRL content was increased significantly as a consequence of 0 mM [Ca^{2+}] (49.05 ± 0.03 ng hormone/mg protein; means ± S.E.M, p<0.0001), correlating with the control. (48.63 ± 0.04 ng hormone/mg protein; means ± S.E.M, p<0.0001).

Discussion

To investigate cellular phenomena, *in vitro* model systems, namely primary monolayer cell cultures from normal, healthy AdH and PRLoma adenohypophysis were used and their cellular functions were standardized. In this paper the alteration of the kinetics of ACTH and PRL release in AdH and PRLoma cell cultures were studied under hypocalcaemic conditions. ACTH is essential for the HPA axis regulation [27,28]. At the surface of adrenal cortex cells the ACTH receptors are highly expressed, thus ACTH-signalling pathway is crucial for the growth and proliferation of adrenocortical cells [29]. The association of hormone molecules to the receptors can initiate G-protein than adenylyl cyclase stimulation [30,31]. This process induces generic alterations in the HPA axis, which is important to stress response mechanisms [30]. PRL is synthesized and secreted by the anterior lobe of the pituitary gland. PRL has several biological functions, it plays role in the reproduction, osmoregulation, growth, synergism with steroids, immunoregulation and protection [32,33]. PRL has also been suggested to be involved in parental behaviour [33]. The adaptability of living organisms is maintained by the HPA axis and PRL release systems [34,35]. Plasma PRL levels were also studied due to the large amount of evidence showing that this hormone and ACTH are activated by stress to the organism [1,36,37]. Therefore these hormones

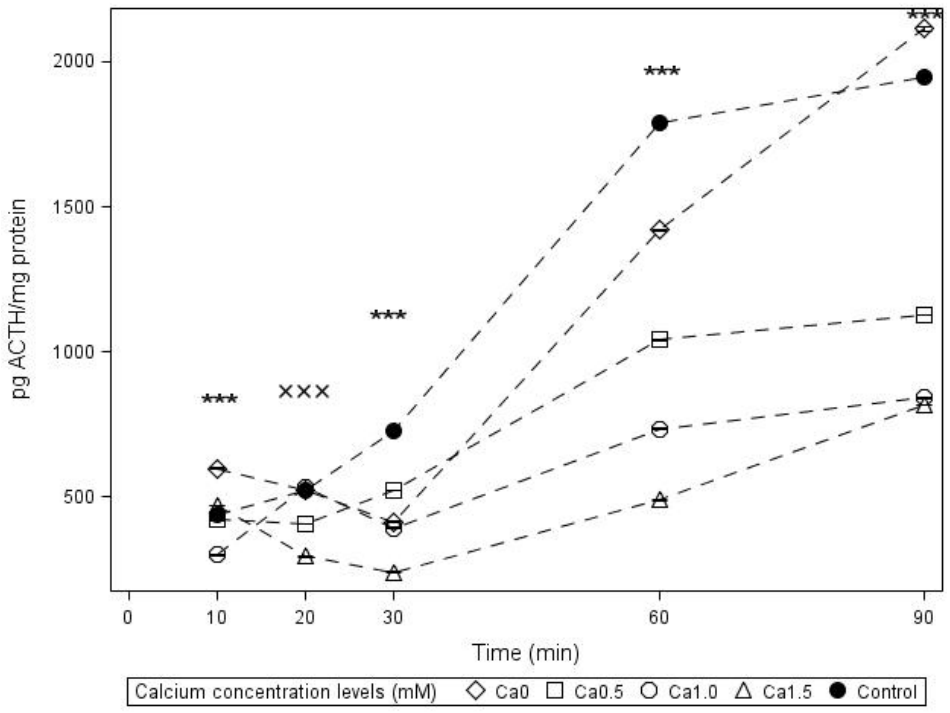

Figure 4: The effects of different calcium ion concentrations on the release of ACTH in prolactinoma cell cultures.
Cell cultures were treated as follows [Ca^{2+}]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated PRLoma groups versus the ACTH release of PRLoma control group. The level of significance was chosen as p<0.0001
XXX indicates that the control is not significantly different from the data of hormone release by the effects of 0 mM [Ca^{2+}]

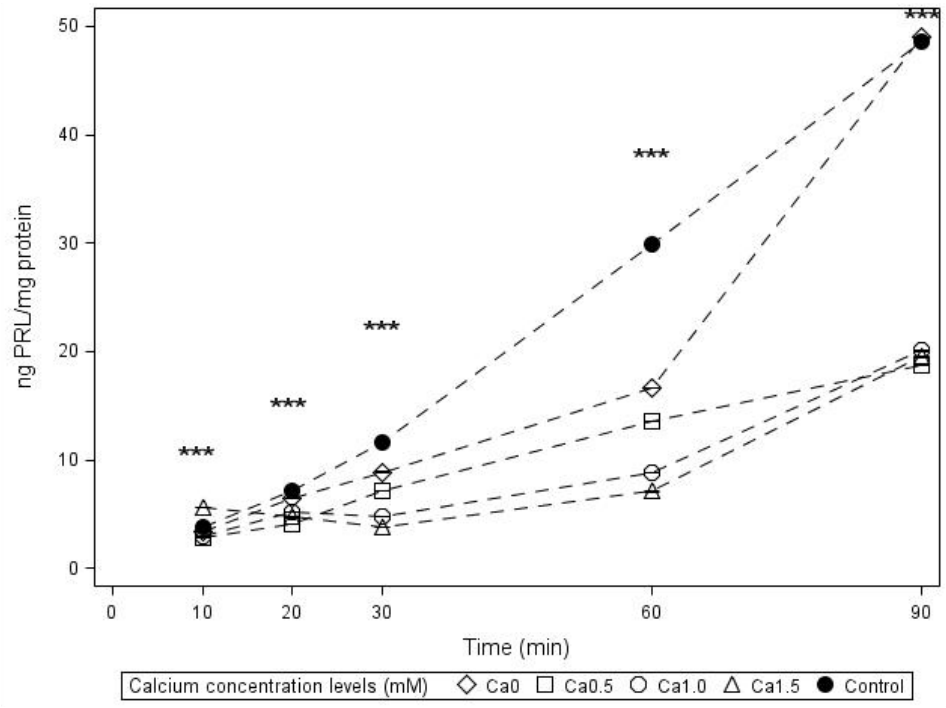

Figure 5: The effects of different calcium ion concentrations on the PRL hormone release in PRLoma cell cultures.
Cell cultures were treated as follows [Ca^{2+}]:0, 0.5, 1.0, 1.5 mM. After treatment samples from the supernatant media were taken in every 10, 20, 30, 60, 90 min
*** indicate the significance between the hormone release of various treated PRLoma groups versus the PRL release of PRLoma control group. The level of significance was chosen as $p<0.0001$

are essential for the vertical homeostatic regulation, which is related to extracellular Ca^{2+} concentrations. PRL secreting tumors are the most frequently occurring pituitary tumors. Abnormal, tumorous cells are more active than normal cells; however our results showed that their behaviour was similar under hypocalcaemia.

Significant differences between the control and the treated primary cell cultures were investigated in both groups. The role of changes to the extracellular milieu in cell function can be defined by hormone exocytosis, sensitivity of intracellular receptors, or the discrete alteration of intracellular messenger molecules.

Since the extracellular environment is constantly fluctuating, cells must adapt to it [1]. A reduced rate of hormone exocytosis was observed as a result of hypocalcaemia. The extracellular hypocalcaemia induced generic alterations in the end-differentiated cell functions. The homeostatic [Ca^{2+}] is essential for the normal exocytotic processes. For the altered extracellular conditions the AdH and PRLoma cells responded with the reduction of hormone release. The results showed that the intracellular Ca^{2+} allocation derived from Ca^{2+} pools (endoplasmic reticulum and mitochondria) resulted hormone release by the exposition of 0 mM [Ca^{2+}]. At the endpoint of the experiment the PRL and ACTH release were higher in PRLoma cultures by the effects of 0 mM [Ca^{2+}]. Our earlier studies suggest that apoptosis mechanisms may play role in this phenomenon [17].

The results showed reduced exocytotic activity by the effects of higher, but still hypocalcaemic extracellular ion milieu. The Ca^{2+} influx via CaV is seemed not sufficient to the Ca^{2+} message. The decreased intracellular Ca^{2+} concentration may block the PLC activation. This mechanism leads to reduced IP3 and DAG conversion and then blocks the release of Ca^{2+} from intracellular pools. Diminution of Ca^{2+} may inhibit the SNARE mediated fusion of hormone containing vesicles to plasma membrane.

Conclusion

In conclusion, the main finding of this study is that a strict correlation exists among certain biophysical properties, especially extracellular Ca^{2+} milieu and hormone vesicle exocytosis.

To better understand the relationship between thy hypocalcaemia and hormone release of PRLoma and normal AdH is essential, because PRL and ACTH hormones play crucial role in the adaptation processes of living organisms. Our results provide strong evidence that sustained lack of Ca^{2+} inhibits the hormone release in both cell types.

Declaration of Interest

The authors report no declarations of interest.

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