Brief Communications

Novel Higher-Order Epigenetic Regulation of the *Bdnf* Gene upon Seizures

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Studies in cultured cells have demonstrated the existence of higher-order epigenetic mechanisms, determining the relationship between expression of the gene and its position within the cell nucleus. It is unknown, whether such mechanisms operate in postmitotic, highly differentiated cell types, such as neurons *in vivo*. Accordingly, we examined whether the intranuclear positions of *Bdnf* and *Trkb* genes, encoding the major neurotrophin and its receptor respectively, change as a result of neuronal activity, and what functional consequences such movements may have. In a rat model of massive neuronal activation upon kainate-induced seizures we found that elevated neuronal expression of *Bdnf* is associated with its detachment from the nuclear lamina, and translocation toward the nucleus center. In contrast, the position of stably expressed *Trkb* remains unchanged after seizures. Our study demonstrates that activation-dependent architectural remodeling of the neuronal cell nucleus *in vivo* contributes to activity-dependent changes in gene expression in the brain.

Introduction

Brain-derived neurotrophic factor (Bdnf) and its tropomyosin-related kinase receptor type B (Trkb) constitute the key trophic system in the brain, involved in neuronal differentiation, survival, and synaptic plasticity (Huang and Reichardt, 2001; Bramham and Messaoudi, 2005), as well as in brain diseases (Heinrich et al., 2011; Nagahara and Tuszynski, 2011). The mechanisms of *Bdnf* and *Trkb* expression are quite deeply understood at the level of gene regulatory sequences and transcription factors, and with regard to classic epigenetic mechanisms such as DNA methylation and posttranslational modifications of histones (Aid et al., 2007; Lei and Parada, 2007). Recent studies in non-neuronal cell types point to the existence of important, yet poorly understood, higher-order epigenetic mechanisms determining the relationship between the position of the gene within the cell nucleus, and the level of its expression (Lanctôt et al., 2007; Misteli, 2007; Geyer et al., 2011), including the gene association/dissociation from the nuclear envelope (Zuleger et al., 2011). Such mechanisms have not been studied with respect to the regulation of gene expression in neurons. Accordingly, we asked whether the intranuclear positions of *Bdnf* and *Trkb* change as a result of increased neuronal activity (seizures), and what functional consequences such movements may have.

Materials and Methods

Animals. The experiments were performed in 144 male Wistar rats, weight 170–250 g, obtained from Mossakowski Medical Research Centre, Polish Academy of Sciences. Animals were kept under a 12 h light/dark cycle, with unlimited food and water supplies. All procedures were performed with the consent of the Ethical Committee at the Nencki Institute.

Induction of seizures. Seizures were evoked by three 5 mg/kg doses of kainate (Sigma-Aldrich) (0.5% solution in saline, pH 7), administered intraperitoneally in 1 h intervals, and scored as described by Hellier et al. (1998). The animals were taken for further studies regardless of whether they fulfilled the criterion of the full status epilepticus or not (Hellier et al., 1998). The seizures were terminated by injection of diazepam after 2 h of status epilepticus or 3 h after the last dose of kainate. The neuronal damage was revealed by staining with Fluoro-Jade B (Millipore) according to the method of Schmued et al. (1997). There was no neuronal damage in the dentate gyrus at any time point.

FISH, immuno-FISH, and immunocytochemistry. These procedures were performed in 30-μm-thick brain cryosections of the 4% paraformaldehyde-perfused, 52 kainate-treated and 26 control animals, according to the protocol of Cremer et al. (2008). As templates for *Bdnf* and *Trkb* probes, CH230-449H21 and CH230-285J18 BACs (respectively) were obtained from Children's Hospital Oakland Research Institute, and verified on rat metaphase spreads. The probes were labeled using the standard nick-translation procedure. Biotinylated probes were

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detected by means of Alexa Fluor 488-conjugated avidin (Invitrogen), followed by FITC-conjugated rabbit anti-avidin antibody (Sigma-Aldrich). Digoxigenin-labeled probes were detected using Rhodamine-conjugated sheep anti-digoxigenin antibody (Abcam) followed by Rhodamine-conjugated donkey anti-sheep antibody (Roche). In the case of Immuno-FISH, the FISH was followed by a standard immunofluorescent staining protocol (Wilczynski et al., 2008). Rabbit polyclonal anti-phosphorylated RNA polymerase II C-terminal domain S2 or S5 (Abcam), followed by DyLight 488-conjugated donkey anti-rabbit (Jackson ImmunoResearch) were used. Nuclei were counterstained using TOPRO-3 or Hoechst 33342 (Invitrogen).

Image acquisition. Fluorescent specimens were examined under a TCS SP5 confocal microscope (Leica), by sequential scanning of images, with a pixel size of 80 nm and an axial distance of 200 nm, using a PlanApo oil-immersion 63× (1.4 numerical aperture) objective. The stacks were 3D deconvolved using Huygens Professional software (Scientific Volume Imaging).

Quantitative image analysis. The 3D morphological analysis of neuronal nuclei was performed using custom-written software, Segmentation magick. The program performs segmentation of nuclei in confocal *Z*-stack. Using a continuous boundary tracing algorithm, it quantifies spatial positions and intensities of fluorescent signals in different channels as well as calculates the distances between the alleles and nuclear periphery, nuclear volume, and form factor. The accuracy of the program was tested using artificial image stacks containing nucleus-like objects. The quantitative assessment of colocalization (see Fig. 2) was performed using the ImageJ plugin Colocalization color map.

Real-time reverse transcriptase-PCR for Bdnf mRNA. Total cellular RNA was isolated from the hippocampi of 6 controls and 24 kainate-treated rats, reverse-transcribed, and subjected to reverse transcriptase (RT)-PCR according to the method of Rylski et al. (2008). Forward and reverse primers, respectively, were: 5′-CCATAAGGACGCGG ACTTGTAC and 5′-AGACATGTTTGCGGCA TCCAGG. Cycling conditions for *Bdnf* gene amplification were: 40 cycles at 95°C for 15 s, annealing at 60°C for 1 min.

Chromatin immunoprecipitation assay. The procedure was performed according to the method of Rylski et al. (2008) in samples from 18 control and 18 kainate-treated rats, using 4 μg of goat anti-lamin A/C or B (Santa Cruz Biotechnology), or normal isotype control antibody (Abcam). Each chromatin immunoprecipitation assay (ChIP) experiment was repeated 3 times. The primer pairs and annealing temperatures were as follows: (1) for a 145 bp fragment of exon 2 of the *Bdnf*: F1 (GCATAGGAAGGTGCTTTCACTG) and R1 (GACTTCTCCTAACC CCAAGAGG), 60°C; (2) for a 122 kb fragment of exon 9 of *Bdnf*: F3 (GCAG TCAAGTGCCTTTGGAG) and R3 (GTGACCCACTCGCTAATACTGT), 63°C; (3) for a *Trkb* fragment: F2 (CTTATGCTTGCTGGTCTTGG), and R2 (TCTGGGTCAATGCTGTTAGG), 60°C.

Each PCR was done in 4 replicates.

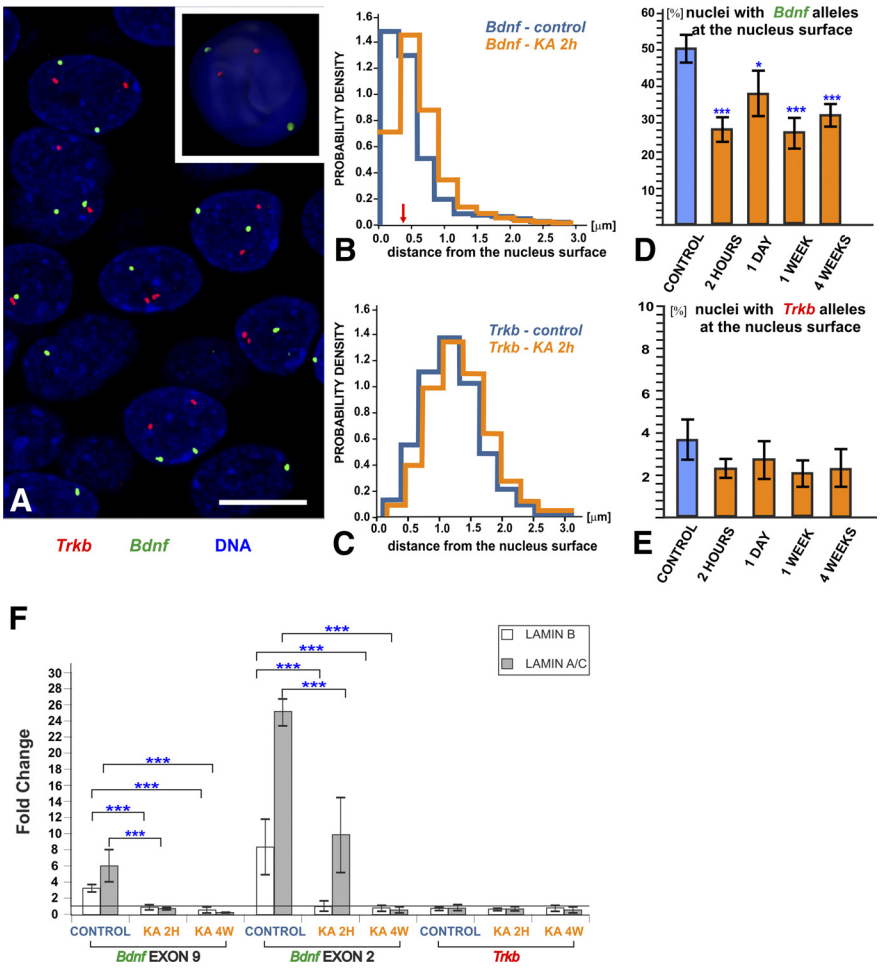

Figure 1. Spatial distributions of *Bdnf* and *Trkb* in the nuclei of rat hippocampal dentate gyrus neurons; effects of seizures. ***A***, FISH labeling (maximum projection of five optical sections) of the control rat dentate gyrus for *Bdnf* (green) and *Trkb* (red); DNA was counterstained using TOPRO 3 (blue). Inset, A neuronal nucleus from control tissue with typical arrangement of Bdnf and Trkb alleles; it represents one of the 4571 nuclei used for and statistical analysis, obtained from 63 animals. Scale bar, 10 μm. ***B***, ***C***, Quantitative analysis of the intranuclear positions of Bdnf (***B***) and Trkb (***C***) alleles in the nuclei of the dentate gyrus granule neurons from control animals (blue lines), and animals killed 2 h after the onset of the status epilepticus (orange lines). The minimal distance between the respective alleles and the nucleus surface is presented in the normalized histogram. Red arrow in ***B*** indicates the distance of 350 nm from the nuclear envelope; the alleles that are positioned within this distance are considered to be present at the nuclear envelope. ***D***, ***E***, Quantitative analysis of the intranuclear positions of *Bdnf* (***D***) and *Trkb* (***E***) in the nuclei of the dentate gyrus neurons from control animals (blue bars), and animals killed 2 h, 1 d, 7 d, and 28 d after the onset of the status epilepticus (orange bars). The mean percentages of the nuclei with the minimum distance between respective alleles and the nucleus surface <350 nm are shown; Kruskal–Wallis test: $p < 0.001$, *U* test: $^{*}p < 0.05$, $^{***}p < 0.001$. ***F***, Detachment of Bdnf from nuclear lamina verified by chromatin immunoprecipitation assay. The results of ChIP analysis with anti-lamin antibodies demonstrate significant enrichment of *Bdnf* exons 2 and 9 in the immunoprecipitates from hippocampi of control rats compared with kainate-treated rats. Neither in control nor after seizures the enrichment of the *Trkb* sequence was observed. Kruskal–Wallis test: $p < 0.001$, *U* test: $^{***}p < 0.001$.

Results

The quantitative 3D analysis of a double-color FISH for *Bdnf* and *Trkb* performed in thick slices through the control hippocampal dentate gyrus (Fig. 1*A*) revealed a highly nonrandom spatial distribution of *Bdnf* alleles in the nuclei of the granule neurons of the control rats. The alleles were most frequently positioned at the nuclear margin, with 50.5 ± 9.7% of nuclei having at least one allele located 350 nm or less from the nuclear border (Fig. 1*B*, blue). This distance is an approximate microscope resolution limit in three dimensions, hence it has been chosen as an indicator of the allele proximity to the nuclear margin. In contrast, the *Trkb* alleles were most frequently positioned in the nuclear interior (Fig. 1*C*, blue).

To examine whether the spatial arrangement of studied genes may be modified by neuronal excitation-transcription response leading to synaptic plasticity, we analyzed their intranuclear po-

sitions in animals subjected to status epilepticus evoked by kainate. We focused initially on the time point of 2 h after the beginning of seizures, when a strong transcriptional response involving immediate-early genes (e.g., *c-Fos* and *Bdnf*) occurs (Zagulska-Szymczak et al., 2001). We observed a distinct repositioning of the Bdnf alleles from the nucleus surface toward the nucleus center (Fig. 1*C*, orange). The percentage of the nuclei having the alleles closer than 350 nm to the nuclear margin decreased almost twice (Fig. 1*D*). No repositioning was found in the case of *Trkb* alleles (Fig. 1*C*, orange, *E*). To determine the duration of the observed phenomenon, we analyzed the nuclei of granule neurons at 1, 7, and 28 d after status epilepticus. The repositioning of the *Bdnf* alleles persisted throughout the whole period (Fig. 1*D*). No significant changes in nuclear shape and volume were detected, that could contribute to the observed positional changes of Bdnf alleles.

To verify whether the *Bdnf* alleles located at the nuclear border are physically bound to the nuclear lamina, and whether their repositioning is associated with the loss of the binding, we performed chromatin immunoprecipitation assay using anti-lamin A/C and anti-lamin B antibodies, followed by the analysis of *Bdnf* exon 2 and 9 content in the immunoprecipitate (Fig. 1*F*). These exons were chosen because (1) they encompass almost the entire gene; (2) the sequences around their 5′ termini are enriched in CpG pairs (Lubin et al., 2008), known to be implicated in chromatin binding to the lamina (Guelen et al., 2008); and (3) they are known to be transcriptionally responsive to status epilepticus evoked by kainate (Aid et al., 2007). In control animals, the chromatin immunoprecipitated with anti-lamin antibodies was enriched severalfold in sequences of *Bdnf* exons 2 and 9, compared with the chromatin immunoprecipitated with a non-immune antibody. In contrast, lamin-associated chromatin from animals having status epilepticus (2 h) was not enriched for any of the *Bdnf* sequences tested. When compared directly to each other, the amount of *Bdnf* sequence immunoprecipitated from control animals was significantly higher than that from kainate-treated animals. The quantitative differences in ChIP results between the exons, e.g., higher association of exon 2 with lamin A/C vs lamin B, evident in control and the 2 h time point, compared with the equal association of exon 9 with both lamins, may suggest a higher affinity of exon 2 chromatin to lamin A/C binding proteins, such as LAP2α (Dechat et al., 2000), or may reflect the positioning of the gene relative to lamin A/C- and lamin B-enriched microdomains (Shimi et al., 2008). Importantly, there was no enrichment of the *Trkb* coding sequence in either control conditions or after seizures, consistent with the lack of association of this gene with the nuclear lamina found using FISH analysis. Therefore, we conclude that the activity-dependent *Bdnf* gene repositioning is associated with loss of its binding to the nuclear lamina. Essentially identical ChIP results were obtained after 28 d post-kainate, confirming that the repositioning of the *Bdnf* gene is a long-term phenomenon.

Another important question was whether any mechanistic connection between the observed translocation of *Bdnf* alleles and the changes in expression of the gene can be proposed. Accordingly, first we analyzed the expression of *Bdnf* and *Trkb* at the level of mRNA by RT-PCR. Two hours after the beginning of status epilepticus, the *Bdnf* mRNA in the hippocampus increased 12-fold (Fig. 2*A*), whereas the mRNA for *Trkb* was unchanged, in agreement with Wetmore et al. (1994). *Bdnf* mRNA was increased severalfold after 1 and 7 d, whereas only a modest (1.75-fold) increase was observed after 28 d (Fig. 2*A*).

To assess *Bdnf* functional status at the level of a single nucleus, we performed immuno-FISH analysis, with simultaneous detection of alleles and activated RNA Polymerase II phosphoepitopes p-Ser2 or p-Ser5 (Buratowski, 2009) (Fig. 2*B–E*). The quantitative analysis revealed that both in controls and 2 h after beginning of seizures, the alleles that were internally located were associated with significantly higher p-Ser2 (Fig. 2*F*) and p-Ser5 (Fig. 2*G*) immunoreactivities than the nuclear surface-bound *Bdnf* alleles. In addition, both phosphoepitopes were upregulated 2 h after seizures, compared with control, at both internal and peripheral positions. In contrast, 28 d post-status epilepticus the internally positioned alleles had higher p-Ser5, but not p-Ser2, immunoreactivity. In addition, the intensities of the signals from both phosphoepitopes returned to control levels. However, when we reexposed the animals at day 28 post-status epilepticus, once again, to the same dose of kainate, the internally positioned alleles displayed an even higher degree of p-Ser5 immunoreactivity after 2 h than in animals treated for the first time (Fig. 2*G*). Notably, none of the animals reexposed to kainate reached the full-blown status epilepticus, likely due to increased inhibition of the dentate gyrus occurring several weeks after status epilepticus (Sloviter et al., 2006).

Thus, we conclude that massive detachment of *Bdnf* alleles from the nuclear lamina, occurring 2 h after the beginning of seizures, can be functionally related to the striking upregulation of *Bdnf* transcription upon neuronal activation. The repositioned alleles become transcriptionally silenced with time, yet they appear to remain in the state of sensitization to the subsequent bursts of neuronal activity.

Discussion

Higher-order chromatin organization (Lanctôt et al., 2007; Misteli, 2007) has been proposed to represent another level of epigenetic phenomena that adds to, and is probably mechanistically interconnected with, DNA and histone covalent modifications, ATP-dependent nucleosome-remodeling, and/or noncoding RNAs, as well as with transcription factor binding (Cohen and Greenberg, 2008; Dulac, 2010; Fischer et al., 2010; Meaney and Ferguson-Smith, 2010; Qureshi and Mehler, 2010; Roth et al., 2010; Barco and Marie, 2011). With regard to the Bdnf gene, an activity-dependent transcription from its several alternative promoters has been associated with DNA demethylation and histone acetylation, and concomitant dissociation of transcriptional repressors such as HDAC1, MBD1, MeCP2, and REST/NRSF (Aid et al., 2007; Tian et al., 2009; Boulle et al., 2012). Here, we report an additional, novel epigenetic phenomenon important for neuronal activity-dependent regulation of Bdnf gene expression, associated with spatial positioning of its gene within the neuronal nucleus.

It is well established that nuclear lamina acts as a repressive environment for transcription (Zuleger et al., 2011). The detachment of chromatin segments associated with their transcriptional derepression is well known to operate in non-neuronal cell types, frequently in association with cellular differentiation (Lanctôt et al., 2007; Misteli, 2007). With respect to neural lineage, Williams et al. (2006) and Peric-Hupkes et al. (2010) demonstrated prominent rearrangements of chromatin interactions with the nuclear lamina during differentiation of embryonic stem cells into neuronal progenitors. Our findings demonstrate that transcription-associated gene repositioning can occur in mature neurons *in vivo*, as a result of enhanced activity.

The molecular mechanisms underlying the events occurring at the lamina are poorly understood, yet it appears that classic epigenetic mechanisms could be involved. For example, it was suggested that lamina recruits histone deacetylases, thereby creating a zone negatively affecting transcription (Somech et al.,

2005). The other mechanism could be DNA methylation followed by binding of MeCP2, which, in addition to its role as a repressor, is able to interact with lamin-B receptor (Guarda et al., 2009). Notably, *Bdnf* expression is strongly dependent on both aforementioned epigenetic phenomena (Aid et al., 2007; Tian et al., 2009; Boulle et al., 2012).

The association of the Bdnf gene activation with its detachment from the lamina is in agreement with the recent results by Saha et al. (2011). The authors found that *Bdnf* belongs to so-called delayed immediate-early genes, whose induction, in contrast to that of *c-Fos* or *Arc*, normally does not rely on the instantaneous activation of poised RNA Polymerase II; consequently, *Bdnf* requires >5 min to begin transcription upon neuronal activation. Our study suggests that the delay results, at least in part, from the molecular events associated with the allele detachment.

Although *Bdnf* detachment and repositioning persists for several weeks, the amount of activated RNA Polymerase II found at the repositioned alleles eventually returns to control levels, and the activity of the locus (measured by mRNA quantity) remains only slightly elevated. This may indicate that a homeostatic mechanism is being activated by a cell to diminish Bdnf expression. However, in contrast to the lamina-attached alleles, the repositioned alleles may be associated with the poised RNA Polymerase II being ready for immediate elongation once the neuronal activation occurs, e.g., remain in the state of sensitization. This scenario is fully consistent with very robust upregulation of the activated RNA Polymerase II (especially elongation-associated p-Ser5; Buratowski, 2009) at the repositioned alleles after reexposure to kainate.

Finally, potential mechanisms of Bdnf allele movements have to be addressed. A directed looping of chromatin fibers upon transcriptional activation has been suggested (Chambeyron and Bickmore, 2004). However, available evidence from live cell studies rather supports that *Bdnf* repositioning can be explained by constrained Brownian motions of chromatin domains, which occur within a range of 1 μm (Strickfaden et al., 2010). This agrees with our observation that the shift of Bdnf loci is largely restricted within a range <1 μm. Such Brownian movements of chromatin domains harboring the Bdnf gene may then suffice to enable their sticking to a limited number of (specialized) transcription factories, which are distributed throughout the nuclear space and involved in facilitating cooperation of specific sets of genes (Schoenfelder et al., 2010). Sticking to a transcription factory may then be maintained regardless of a decrease of the transcription level over time, as observed in our study. It remains to be experimentally determined whether repositioning of the Bdnf gene is a direct cause or consequence of the transcriptional activation.

Our work sets a new direction of research on activity-dependent gene expression, expanding the current paradigm of neuronal molecular epigenetics. Further studies are needed to evaluate the extent of activity-dependent gene repositioning in neuronal nuclei. It is also necessary to assess whether this epigenetic mechanism is associated with physiological forms of neuronal stimulation and to what extent it can participate in processes of normal synaptic plasticity.

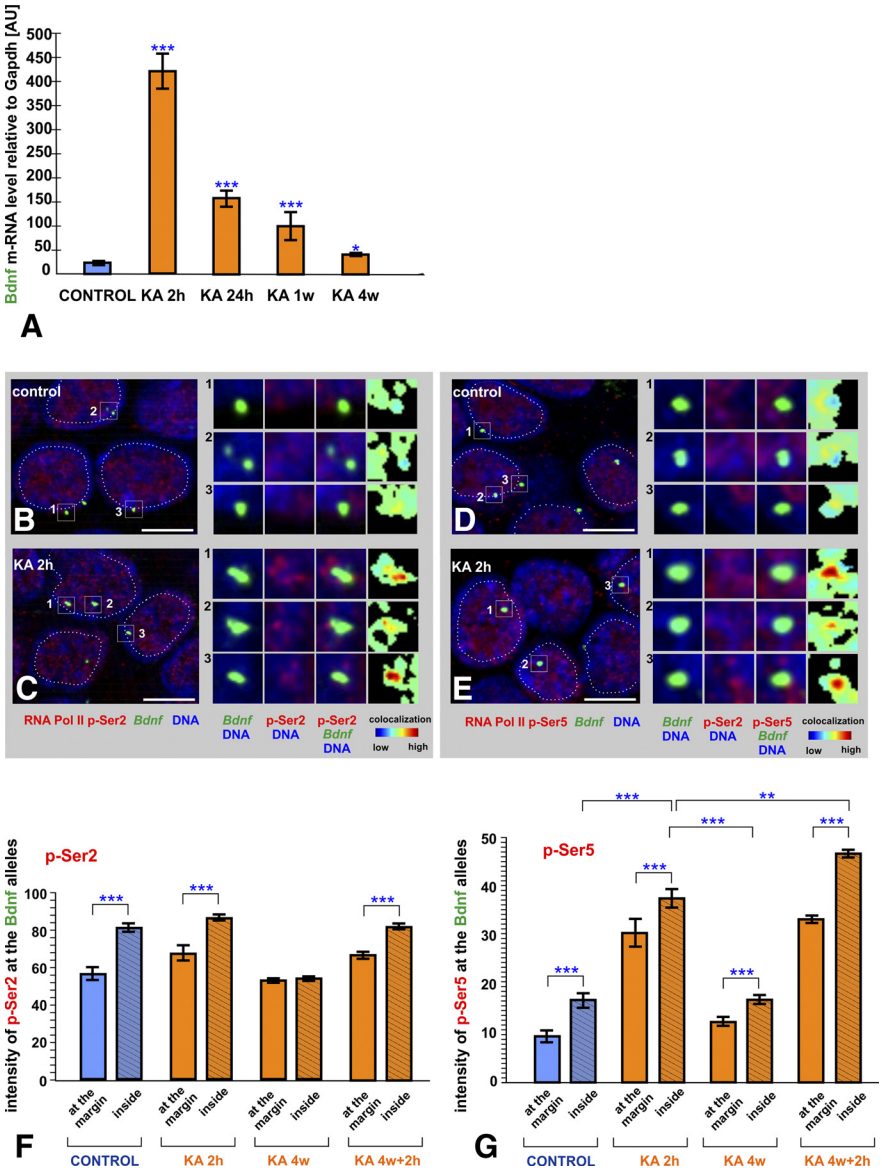

Figure 2. The relationship between the intranuclear position of the Bdnf alleles and their activity, investigated by colocalization with immunoreactivity of activated RNA Polymerase II, and by RT-PCR. ***A***, RT-PCR quantification of relative induction of Bdnf mRNA level relative to GAPDH mRNA in control hippocampi and upon seizures. ***B–E***, Immuno-FISH for *Bdnf* (green) and activated RNA Polymerase II (red), p-Ser2 (***B***, ***C***) or p-Ser5 (***D***, ***E***). ***B***, ***D***, Control animals; ***C***, ***E***, animals killed 2 h after the onset of the status epilepticus. Two-channel- and three-channel overlays, and colocalization maps are shown. The approximate position of the border between the nuclear periphery and interior is indicated by dotted line. Scale bar, 5 μm. ***F***, ***G***, Quantitative analysis of the intensity of RNA Polymerase II p-Ser 2 (***F***) and p-Ser5 (***G***) signals colocalizing with the Bdnf alleles, in relation to the allele position. Clear bars, The alleles positioned <350 nm from the nucleus surface; dashed bars, >350 nm; blue bars, the control; orange bars, 2 h, 28 d, and 28 d + 2 h (the animals reexposed to kainate) time points. $^{**}p < 0.01$, $^{***}p < 0.001$, *t* test. For clarity, the depicted differences are restricted to internal alleles.

Notes

Supplemental material for this article is available at http://walczak.nencki.gov.pl. Content of supplemental materials: (1) supplemental.pdf file containing supplemental figures demonstrating the validation of the FISH probes (Fig. S1), the relationship of *Bdnf* and *TrkB* genes to the nucleolus (Fig. S2); (2) a schematic presentation of 3D segmentation procedure (Fig. S3), and (3) the program Segmentation magick together with instructions and segmentation examples (see readme.pdf file for details), available at http://walczak.nencki.gov.pl. This material has not been peer reviewed.

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