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Impact of repeated asenapine treatment on FosB/ΔFosB expression in neurons of the rat central nucleus of the amygdala: colocalization with corticoliberine (CRH) and effect of an unpredictable mild stress preconditioning

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Objectives. FosB/ΔFosB expression in the central amygdalar nucleus (CeA) in response to repeated asenapine (ASE) treatment (an atypical antipsychotic used for the treatment of schizophrenia) was studied in normal rats and rats preconditioned with chronic unpredictable variable mild stress (CMS). The goal of this study was to reveal whether repeated ASE treatment for 14 days may: 1) induce FosB/ΔFosB expression in the amygdala, 2) activate CRH-synthesizing neurons in the CeA, and 3) interfere with 21 days lasting concomitant CMS preconditioning.

Methods. Four groups of animals were studied: controls and ASE-, CMS-, and CMS+ASE-treated ones. CMS consisted of the restrain, social isolation, crowding, swimming, and cold and lasted 21 days. The ASE and CMS+ASE groups were from the 7th day of the experiment treated with ASE (0.3 mg/kg, subcutaneously - s.c.) twice a day, i.e. together for 14 days. Controls and CMS groups were treated with saline (300 μl/rat, s.c.) twice a day for 14 days. All the animals were sacrificed on the 22nd day, i.e. 16-18 hours after the last treatments. Single FosB/ΔFosB, FosB/ΔFosB colocalizations with CRH, and CRH immunolabeled perikarya were investigated in the CeA using a combined light and fluorescent immunohistochemistry.

Results. The distribution aspect of the black FosB/ΔFosB profiles was homogeneous over the whole CeA and no significant differences in the number of FosB/ΔFosB profiles between the individual groups of the rats really occurred. The level of colocalization pattern of FosB/ΔFosB in CRH perikarya was also very similar between the individual groups and in each case it reached approximately 10% of double-labeling. No differences were also seen in the number of CRH immunolabeled perikarya. The density of CRH nerve projections within the CeA was very alike in the individual groups of animals investigated.

Conclusions. The study provides a new anatomical/functional finding about the lack of the stimulatory effect of the repeated ASE treatment on the expression of FosB/ΔFosB, FosB/ΔFosB/CRH colocalizations, and CRH immunolabeled perikarya number in the CeA. In addition, CMS preconditioning itself neither stimulated nor inhibited FosB/ΔFosB expression, nor altered the impact of ASE on the activity of CRH neurons in the CeA.

Key words: asenapine, FosB/ΔFosB, CRH, immunohistochemistry, central amygdala, unpredictable mild stress, rat

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The amygdaloid complex, i.e. corpus amygdaloideum, is formed by a group of amygdaloid nuclei located deep and medially within the temporal lobe. They are displaying extensive intranuclear and extranuclear (with cortical and subcortical regions) connections (Sah et al. 2003). From the functional point of view, the amygdala is involved in many of emotions such as fear, anger, anxiety, sadness, and pleasure and motivations, particularly those that are related to survival. The amygdala is also responsible for determining what memories are stored and where the memories are stored in the brain and for the controlling of aggression, stress, and addictive disorders (Gray and Bingaman 1996; Scott et al. 1997; Curtis et al. 2002; Cook 2004; Klinge et al. 2010).

It has been demonstrated that antipsychotic drugs may affect the firing of amygdalar neurons, especially those in the CeA. Rebec et al. (1981) have demonstrated by electrophysiological analysis that clozapine (antipsychotic drug with low extrapyramidal side-effect liability) may significantly increase the cell firing in the CeA, while haloperidol (classical antipsychotic drug) is without effect. Also clozapine in comparison with haloperidol has been shown to block the motor activity elevation induced by the intra-amygdaloid administration of amphetamine (a psychostimulant drug) (Wang and Rebec 1998). These data indicate that classical and atypical antipsychotic drugs may elicit even differential impact on the activity of neurons in the amygdaloid structures.

ASE is a novel atypical antipsychotic drug being developed for the acute treatment of schizophrenia and bipolar disorder (Alphs et al. 2007; Traynor 2010). ASE improves positive and negative symptoms in patients with schizophrenia (Potkin et al. 2007) and exhibits high affinity for many serotonin-, dopamine-, norepinephrine- and histamine-receptor subtypes (Shahid et al. 2009). Likewise other atypical antipsychotics, ASE has been shown to increase the release of dopamine, norepinephrine, and acetylcholine in the prefrontal cortex (Huang et al. 2008; Franberg et al. 2012). Previously, we have shown that acute ASE treatment induces a significant activation of neurons in the rat the striatum, septum, nucleus accumbens, and prefrontal cortex (Majercikova et al. 2014), i.e. in the brain areas which are the most frequently investigated for evaluation of antipsychotics effect related to mental disorders (Robertson and Fibiger 1996; Suzuki et al. 1998). However, little is known about the effect of ASE repeated administration on the activity of other brain structures.

CRH is a 41-amino acid peptide (Vale et al. 1981), predominantly known as a substance to be produced by parvicellular neurons of the hypothalamic paraventricular nucleus and playing important role in controlling the secretion of adrenocorticotropin hormone from the anterior pituitary and modulating the activity of the hypothalamic-pituitary-adrenal (HPA) axis following exposure to a stressor (Aguilera et al. 1996; Rabadan-Diehl et al. 1996). The extrahypothalamic CRH, particularly of the limbic system, also appears to play a role in the stress response (Cook 2004). The immunohistochemically identified CRH-containing neurons, located in the amygdala, are mainly concentrated in the lateral subdivision of the CeA (Swanson et al. 1983; Bale and Vale 2004; Asan et al. 2005). They represent a part of an endogenous CRH circuitry within the brain that mediates neuroendocrine, autonomic, and behavioral changes in response to stress. The functional involvement of the amygdalar CRH neurons has been shown to be associated with mediation of stress effects on memory consolidation (Roozendaal et al. 2002) and action in the stress responsiveness (Gray and Bingaman 1996; Curtis et al. 2002; Cook 2004). CRH-containing neurons of the amygdala have been shown to be directly modulated by alterations in circulating glucocorticoids through glucocorticoid receptors, which are expressed in amygdaloid CRH-containing neurons and release of endogenous CRH has been measured in the amygdala during stress (Gray and Bingaman 1996).

Stress is a natural element of human life, which plays a role in the development and pathophysiology of different psychological disorders, including depression, anxiety, and psychosis (Southwick at al. 2005). A complex of mild stressors used in animal models can mimic the human natural environment (Nyuyki et al. 2012). It is known, that stress-induced Fos expression can be modulated by different drug classes like antidepressants, anxiolytics, and glucocorticoids (Imaki et al. 1995). However, it is not clear whether effect of antipsychotics may be modulated by stress.

FosB and ΔFosB are members of the Fos family of transcription factors that are one of the best characterized transcription factors induced by chronic stimulation. There are many studies utilizing FosB/ΔFosB detection for revealing the effect of chronic or repeated administration of drugs (Chocyk et al. 2006; Marrtila et al. 2006; Nunez et al. 2010; Kaplan et al. 2011). Generally, FosB/ΔFosB is considered for a well acceptable morphological tool in the displaying the long-lasting effect of many biologically active drugs.

The present study was aimed to reveal whether repeated ASE treatment for 14 days may: 1) activate neurons in the CeA and 2) interfere with 21 days lasting concomitant CMS preconditioning. Single FosB/ΔFosB expression and FosB/ΔFosB colocalization with CRH neurons were investigated in the CeA using a combined light and fluorescent immunohistochemistry (Majercikova et al. 2012).

Materials and Methods

Experimental animals. Adult male Wistar rats (n=28) weighing 200-210 g were used. They were housed two per cage in a room with controlled temperature (22±1°C), light (12-hour light/dark cycle with lights on at 06:00 h), and humidity (55%). Animals were provided with a regular rat chow (dry pellets) and tap water ad libitum. Principles of the laboratory animal care and the experimental procedures used were approved by Animal Care Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Slovak Republic. The investigation conditions were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Stress model. The rats were divided into 4 groups: controls (n=6), ASE (n=6), CMS (n=8), and CMS+ASE (n=8). The CMS consisted of the following sequence of stressors: restrain (RE, plastic restrainers), social isolation (SI), crowding (CR), cold (CO, 4°C cold room), and swimming (SW, 30 cm high x 20 cm width glass cylinders). The sequence of the stressors and the time of exposure were following: RE (30 min, 09:00 a.m.) – SI (overnight) – CR (overnight) – RE (30 min, 12:00 a.m.) – CO (30 min, 09:00 a.m.) – SI (overnight) – CO (30 min, 12:00 a.m.) – RE (45 min, 12:00 a.m.) – CO (45 min, 09:00 a.m.) – SI (overnight) - RE (45 min, 12:00 a.m.) – SW (15 min, 09:00 a.m.) – SW (5 min, 09:00 a.m.) – SI (overnight) – RE (60 min, 12:00 a.m.) – CO (45 min, 09:00 a.m.) – SI (overnight) – RE (60 min, 12:00 a.m.) – CR (overnight) – CO (60 min, 12:00 a.m.) – SI (overnight). The animals received one stress challenge per day. In order to minimize the stressors predictability, the particular stressor was applied each day at different time.

CMS lasted 21 days and the rats received saline (300 µl/rat, s.c.) from the 7th day, twice a day. The ASE and CMS+ASE groups were, from the 7th day of the CMS, treated with ASE (0.3 mg/kg b.w., Sigma St. Louis MO, A7861) dissolved in 1 ml of saline twice a day, i.e. together for 14 days. Controls and CMS groups were treated with saline for 14 days. All the animals were sacrificed on the 22nd day (between 8:00 to 10:00 a.m.), i.e. 16-18 hours after the last treatments. The dose of the ASE (0.3 mg/kg b.w.) used was selected based on the data of a pilot study and its effectivity demonstrated in the forebrain structures (Majercikova et al. 2014).

Immunohistochemistry. The animals were anesthetized with sodium pentobarbital (50 mg/kg of body weight, i.p.) and perfused via the left heart ventricle with 50 ml of cold isotonic saline containing 450 µl of heparin (5000 IU/l, Zentiva, Slovakia) followed by 250 ml of fixative containing 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate buffer (PB, pH 7.4) (Slavus, Slovakia). The brains were removed and postfixed in a fresh fixative overnight. Then, the brains were washed in 0.1 M PB at 4°C overnight, and infiltrated with 30% sucrose (Slavus, Slovakia) cryoprotectant for 2 days. Four sets of 30 µm thick coronal sections were collected. The sections were processed as free floating. They were repeatedly washed in cold 0.1 M PB and preincubated with 0.3% H_2O_2 in 0.1 M PB for 15 min at room temperature (RT). Then, the sections were rinsed 3 x 10 min in 0.1 M PB and incubated with a rabbit polyclonal FosB/ΔFosB protein antiserum (1:1000, ab11959, Abcam, Cambridge, UK) diluted in 0.1 M PB containing 4% normal goat serum (Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Sigma-Aldrich), and 0.1% sodium azide (Sigma-Aldrich) for 48 h at 4°C. After several rinsing in PB, the sections were incubated with biotinylated goat anti-rabbit IgG (1:500, Vector-Stain Elite ABC Kit, Vector Lab., Burlingame, CA, USA) in PB for 90 min at RT. Next PB rinsing was followed by incubation with the avidin-biotin peroxidase complex (1: 250) for 90 min at RT. After several washings in 0.05 M sodium acetate buffer (SAB, pH 6.0), FosB/ΔFosB antigenic sites were visualized by nickel-enhanced 3,3´-diaminobenzidine tetrahydrochloride (0.0625% DAB, 2.5% nickel chloride, Sigma-Aldrich, No 7718-54-9), in SAB containing 0.0006% H_2O_2. Developing time was 6-8 min. The heavy metal-intensification of DAB yielded to black staining in the FosB/ΔFosB labeled nuclei. FosB/ΔFosB containing sections were further incubated with rabbit polyclonal anti-CRH antibody (gift from Dr. Greti Aguilera, NICHD, NIH, USA) 48 h at RT, followed by incubation with goat anti-rabbit IgG labeled with Alexa Fluor555 (1:500, Molecular Probes, A-21437) overnight. Finally, the sections were mounted in semi-darkness onto slides, left to dry in RT, coverslipped with Pertex (Stockholm, Sweden), and stored in dark boxes.

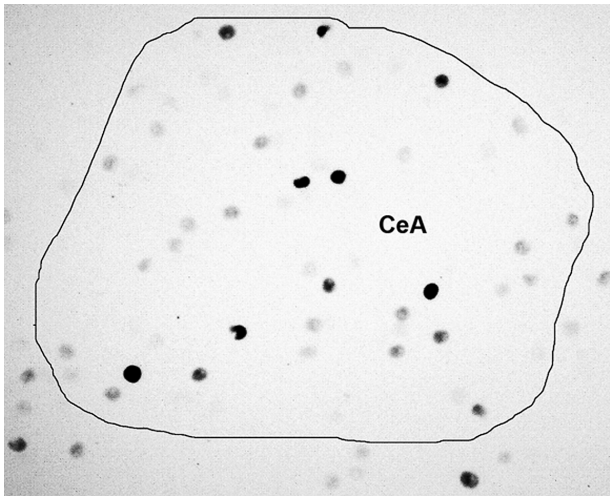

Fig. 1. DAB-nickel stained histological section, indicating the homogeneous distribution pattern of the FosB/ΔFosB profiles within the CeA (encircled area) characteristic for all groups of rats investigated. Abbreviation: CeA = central amygdaloid nucleus.

Fos counting. The FosB/ΔFosB counting was performed unilaterally (in Adobe Photoshop 7.0 program) from photomicrographs captured from 4 representative sections in Axio-Imager A1 light microscope (Carl Zeiss, Jena) coupled to a video camera and monitor. Identification of single FosB/ΔFosB, colocalized FosB/ΔFosB/CRH, and immunoreactive CRH cells was perfomed from photomicrographs captured on the above mentioned Zeiss microscope using a simultaneous light and fluorescent illuminations, as described previously (Majercikova et al. 2012). Actually, the parallel illumination of both types of lights was adjusted to the level which allowed clearly distinguish the nickel immunolabeled FosB/ΔFosB nuclei within the fluoresceine-labeled perikarya.

Statistical analysis. Statistical analysis was carried out by one way analysis of variance (ANOVA) and Bonferroni multiple comparison post hoc test. All data were expressed as mean ± SEM. The value of $p < 0.05$ was considered as statistically significant.

Results

On the DAB-nickel stained histological sections, the expression of the long-lived FosB/ΔFosB protein was perceived over the whole CeA in all four groups of animals investigated (Fig. 1). The distribution aspect of the black FosB/ΔFosB profiles was quite even over the whole CeA, without revealing any pattern of a specific space accumulation or concentration of FosB/ΔFosB profiles (Fig. 1). The average number of FosB/ΔFosB-containing cells in the individual groups of rats occurred between 17 and 24 when considering from the unilateral counting point of view. There were no significant differences in the FosB/ΔFosB expression levels between the individual groups of the rats studied (Fig. 2A).

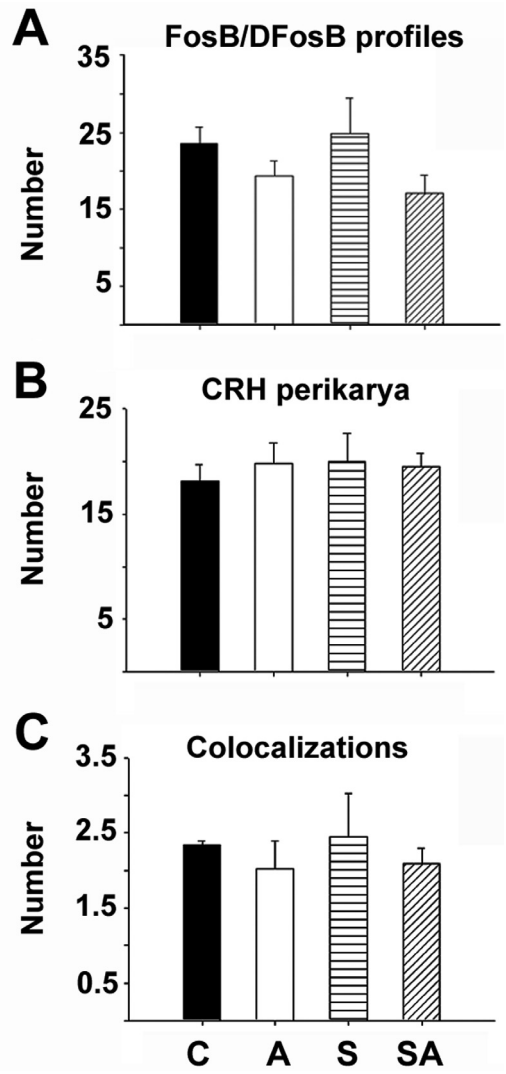

Fig. 2. As it is demonstrated in graphs, neither asenapine, CMS, nor CMS plus asenapine treatments affected the single FosB/ΔFosB expression (A-graph), the number of CRH-immunopositive perikarya (B-graph), and FosB/ΔFosB-CRH colocalization rate (C-graph) in the CeA in the individual groups of the rats studied. Abbreviations: C = controls, A = asenapine, S = CMS, SA = CMS + asenapine, FosB/DFosB = FosB/ΔFosB.

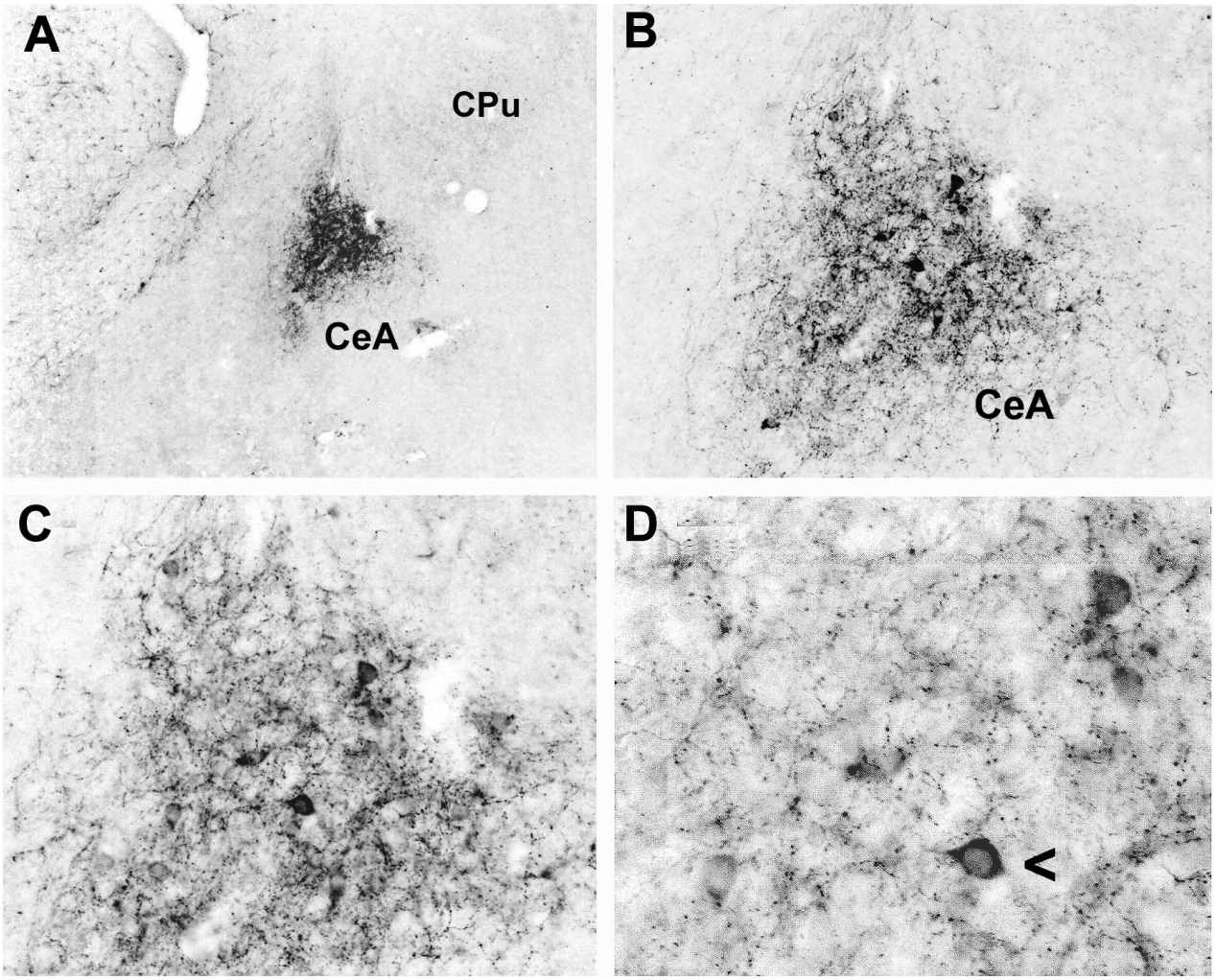

Fig. 3. Histological sections counterstaining with CRH antibody revealing the shape and the location of the CeA (a, b) and the presence of a number of CRH-labeled somata (b, c). CRH perikarya were formed by large round or oval perikarya, bearing a well distinguishable pale spherical nucleus (d). Abbreviations: CeA = central amygdaloid nucleus, CPu = nucleus caudatus-putamen, < = CRH-immunolabeled perikaryon.

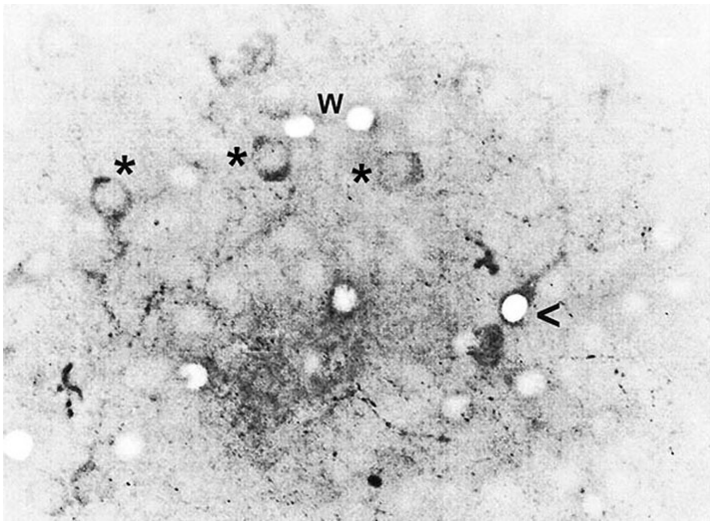

Fig. 4. Representative sample of the section demonstrating the colocalization pattern of FosB/ΔFosB in CRH perikarya, characteristic for all groups of rats investigated. Abbreviations: W = FosB/ΔFosB profiles (white circles), * = CRH perikarya without FosB/ΔFosB, < = FosB/ΔFosB-CRH colocalization.

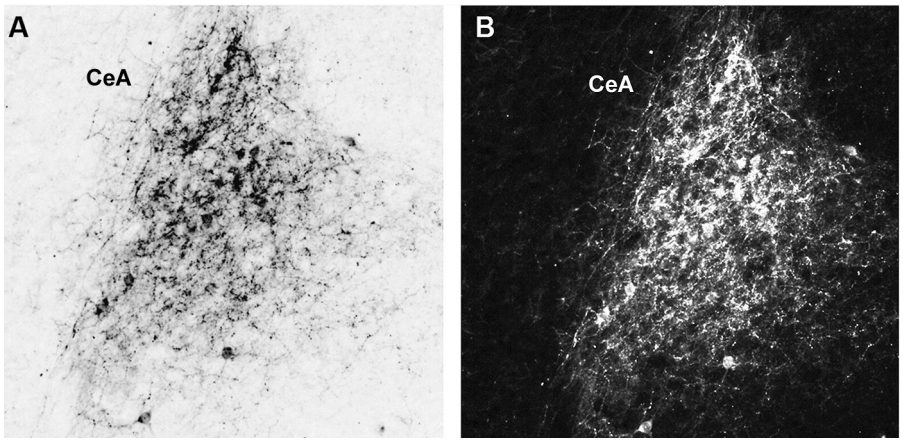

Fig. 5. Representative sample of the CRH pattern of innervation within the CeA characteristic for all groups of rats investigated. As it shown on the inversed (A) and fluorescent (B) photomicrographs, several CRH perikarya are observable within the network of the CRH fibers and terminals. Abbreviation: CeA = central amygdaloid nucleus.

On the FosB/ΔFosB stained histological sections, counterstaining with CRH antibody revealed the shape and the location of the CeA (Fig. 3A,B) and the presence of a number of CRH-labeled somata (Fig. 3B,C) These CRH neurons were almost uniform, formed by large round or oval perikarya, bearing a well distinguishable pale spherical nucleus (Fig. 3D). There were really no differences observed in the number of immunolabeled CRH somata between the individual groups of the rats studied (Fig. 2B). Colocalization pattern of FosB/ΔFosB in CRH perikarya (Fig. 4) was also very similar between the individual groups of the rats studied and in all groups of animals. Sections revealed approximately 10% of FosB/ΔFosB-CRH double-labeling in each group of rats (Fig. 2C). Generally, neither ASE, CMS, nor CMS+ASE affected the single FosB/ΔFosB expression, FosB/ΔFosB-CRH colocalization rate, or the number of CRH-immunopositive perikarya in the individual groups of the rats studied (Fig. 2A,B,C).

In all four groups of animals, a quite distinctive pattern of CRH innervation within the CeA was observed (Fig. 5). However, there were no differences seen in the density of CRH nerve projections or terminals, estimated under the light microscopy, between the individual groups of animals, demonstrating that neither ASE, CMS, nor CMS+ASE affected significantly the density of the CRH innervation field in the CeA area.

Discussion

The present study provides a new anatomical/functional finding about the lack of the stimulatory effect of the repeated ASE treatment on the expression of FosB/ΔFosB profiles, FosB/ΔFosB/CRH colocalizations or CRH immunoreactive perikarya number in the CeA. It is also demonstrated that CMS preconditioning neither provoked FosB/ΔFosB expression nor altered the impact of ASE treatment on the activity of CRH neurons in the CeA.

There is a number of studies dealing with impact of antipsychotic drugs on the Fos expression (a common marker used to map neuronal activity in the brain) in the forebrain structures (Deutch et al. 1992; MacGibbon et al. 1994; Wan et al. 1995; Deutch and Duman 1996; Young et al. 1999; Oka et al. 2004) and less in the other brain areas (Kiss et al. 2010). Previously, we have shown that acute ASE treatment, acting via a broad spectrum of receptors, including adrenoceptors and serotonin- dopamine- histamine-, and muscarinic-receptors (Shahid et al. 2009), has a profound effect on the activity of Fos expression in the forebrain structures including striatum, septum, accumbens nucleus, and prefrontal cortex (Majercikova et al. 2014). In addition, ASE administration stimulated number of neurons in the basal nucleus of Meynert (submitted for publication). However, there exists considerably less amount of data regarding the long-lasting effect of antipsychotics on the activity of brain neurons.

In the present study, FosB/ΔFosB transcription factor was used for the detection of the persistent cells activation in the CeA cells after repeated ASE treatment for 14 days without or with CMS preconditioning. FosB/ΔFosB together with c-Fos, Fra-1 and Fra-2, belongs to the Fos family of transcription factors. ΔFosB is a truncated splice variant of fullength FosB (Piechaczyk and Blanchard

1994; Herdegen and Leah 1998). Many antibodies, however, bear the ΔFosB together with FosB as FosB/ΔFosB. In contrast to other members of the Fos family, ΔFosB is only lightly or even no induced in the brain after a single drug administration. However, with respect to its quite long half-life, ΔFosB may persist in cells for weeks, even months, after the drug withdrawal. Recently, it has been reported that the transcription factor, ΔFosB, is robustly and persistently induced in forebrain structures by several chronic stimuli, such as drugs of abuse, antipsychotic drugs, natural rewards, and stress (Lobo et al. 2013). Furthermore, chronic alterations in dopaminergic neurotransmission have been shown to produce a persistent elevation of ΔFosB-like protein in both the rodent and primate striatum (Doucet et al. 1996). Atkins et al. (1999) have demonstrated region-specific induction of ΔFosB in forebrain by repeated administration of typical versus atypical antipsychotic drugs. All these studies indicate that forebrain structures are not sensitive only to the acute antipsychotics treatment but also to the chronic or repeated administration of antipsychotics.

On the other hand, the response of many of other brain structures to the repeated antipsychotics treatments has been not studied so extensively. The CeA serves as the major output nucleus of all the amygdaloid nuclei. CeA is involved in the regulation of autonomic components of emotions (e.g. changes in heart rate, blood pressure, and respiration) primarily through output pathways to the lateral hypothalamus and brain stem. CeA is also responsible for conscious perception of emotion primarily through the ventral amygdalofugal output pathway to the anterior cingulate cortex. The CeA is essential for acquiring and expressing conditional fear after overtraining (Zimmerman et al. 2007). Both typical and atypical antipsychotic drugs have been shown to may activate cells in the CeA (Morelli and Pinna 1999; Pinna and Morelli 1999; Ma et al. 2003; Park et al. 2011; Zhao and Li 2012). In the present study, neither ASE nor CMS+ASE induced stimulation of the FosB/ΔFosB expression in the CeA, indicating that ASE does not belong to those atypical antipsychotic drugs which may activate cells in the CeA.

One of the chemical components of the CeA neurons is CRH (Treweek et al. 2009). The CeA is a part of an endogenous brain CRH circuitry and CRH-expressing neurons of the amygdala communicate with widespread regions of the neural axis (Gray and Bingaman 1996). Direct injections of CRH into the amygdala produce anxiety-like behaviors. Release of endogenous CRH can be measured in the amygdala during stress (Gray and Bingaman 1996). Psychological stressors induce an increase of both CRH mRNA levels and CRH content in the CeA (Makino et al. 1999). However, CMS, as a psychological stressor applied in the present study, did not increase the number of CRH-immunoreactive perikarya itself or in combination with ASE, in the CeA. Although CMS activated FosB/ΔFosB expression in the parvocellular subdivision of the PVN, i.e. in PVN area containing mainly CRH-producing cells (yet unpublished results), no response was seen in the CeA, whereas between the PVN and CeA an anatomical/functional link exists and many sorts of stressor induce distinct stimulation of the PVN and CeA CRH neurons. Indeed, in many cases parallel stimulation of FosB/ΔFosB in the PVN and CeA, including chronic morphine administration has been reported (Nunez et al. 2010; Garcia-Perez et al. 2012).

CRH innervation in the CeA arises from several sources, i.e. from the local CRH neurons, which possess features of local-circuit neurons, part of the CRH axon terminals identified in electron micrographs arises from neurons in the lateral hypothalamic area and the dorsal raphe nucleus (Uryu et al. 1992) and the others from the hypothalamic paraventricular nucleus (PVN). In the present study, light microscopic evaluation of the CRH innervation density did not truly visually revealed differences between the individual groups of rats studied, thus excluding the effect of CMS, ASE, and CMS plus ASE on the density of the CeA CRH innervations field, at all.

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