c-Fos and FosB/ Δ FosB colocalizations in selected forebrain structures after olanzapine, amisulpride, aripiprazole, and quetiapine single administration in rats preconditioned by two different mild stressors sequences

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Objective. Olanzapine (OLA), amisulpride (AMI), aripiprazole (ARI), and quetiapine (QUE) belong to antipsychotics, which administration represents still most reliable way for the treatment of schizophrenic and bipolar disorders. The intention of the present study was to explore whether the acute administration of a particular antipsychotic, indicated by the presence of c-Fos, will: a) stimulate neurons already activated by a long lasting homogeneous or heterogeneous stress preconditioning, indicated by the FosB/ Δ FosB (Δ FosB) expression, or b) have a stimulatory effect only on a not activated, so called silent neurons. The pattern of Δ FosB and c-Fos spatial relationship was investigated in three forebrain structures, including the septal ventrolateral nucleus (seVL), the striatal dorsolateral area (stDL), and the shell of the nucleus accumbens (shell).

Methods. The rats were divided into 10 groups and exposed to two types of stressors. Half of them was exposed to a sequence of homogeneous stressor – handling (HDL) and the other half to a heterogeneous stressor (CMS) daily for 20 days. CMS consisted of five types of stressors: crowding, air-puff, wet bedding, predator stress, and forced swimming applied in an unexpected order. On the 21st day of the experiment, the rats were free of the stress exposure and on the 22nd day, both groups of animals receive a single intraperitoneal injection of vehicle (4% DMSO in saline, 0.1 ml/100 g) or OLA (5 mg/kg), AMI (20 mg/kg), ARI (10 mg/kg), and QUE (15 mg/kg). 90 min after the drugs administration the animals were transcardially perfused, brains removed, cut into 30 μ m thick coronal sections, and double stained: first with Δ FosB antibody linked with Alexa488 fluorescent dye and second with c-Fos antibody linked to Alexa596 one. Quantitative evaluation of Δ FosB and c-Fos colocalizations was performed on fluorescence photomicrographs transformed into a final picture containing only yellow, green, and red colored circles.

Results. The data of this investigation demonstrate that Δ FosB and c-Fos colocalizations occurred in each of the three areas investigated, i.e. seVL, stDL, and shell ones, in both HDL as well as CMS preconditioned rats. The levels of Δ FosB and c-Fos colocalizations varied in the individual forebrain areas studied. From the total 22 areas measured, level of c-Fos colocalization prevailed over Δ FosB in 18 ones. However, neither c-Fos nor Δ FosB reached 100% level of colocalization in any of the forebrain areas investigated.

Conclusion. The present findings indicate that Δ FosB and c-Fos colocalizations occurred in each of the three areas investigated, i.e. seVL, stDL, and shell, in both HDL and CMS preconditioned rats, whereas the parallel occurrence of free c-Fos as well as c-Fos colocalized with Δ FosB might speak out for a possible involvement of the c-Fos activated by antipsychotics applied in dual, i.e. short- and long-lasting, functions.

Key words: c-Fos, Δ FosB, olanzapine, amisulpride, aripiprazole, quetiapine, mild stressors, rat

Antipsychotics (APs) administration is still most reliable way for the treatment of schizophrenic and bipolar disorders symptoms (Taylor et al. 2018). By blocking neurotransmitter receptors, which include dopamine, serotonin, and acetylcholine receptors subclasses, whereby, all of them occur in the circuitry of the basal ganglia and prefrontal cortex, APs alter the second-messenger systems in neurons (Graybiel et al. 1990; Cleghorn et al. 1991; Ellenbroek 1993). Olanzapine (OLA), amisulpride (AMI), and quetiapine (QUE) belong to the second, while aripiprazole (ARI) is affiliated to the third generation of atypical APs (Mailman and Murthy 2010). OLA and QUE are classified as MARTA (Multi-Acting Receptor Targeted Antipsychotics). QUE is characteristic by fast dissociation from D₂ receptors (Kapur and Seeman 2000; Seeman 2014). AMI has high-affinity for dopaminergic D₂/D₃ and lower affinity for 5-HT₇ receptors (Schoemaker et al. 1997; Abbas et al. 2009), and ARI is characterized by a partial agonistic activity on the D₂ receptor subpopulations and antagonism on 5-HT_{1A} receptors (Swainston-Harrison and Perry 2004). It has been shown that acute treatment with many neuroleptics of typical or atypical types enhances the expression of c-Fos-like transcription factor in the rat striatum, septum, accumbens nucleus, and prefrontal cortex (Majercikova et al. 2014; Osacka et al. 2018; Kiss et al. 2019), whereas, both types of neuroleptics do so in different anatomical patterns (Hiroi and White 1991; Robertson and Fibiger 1992; Herdegen and Leah 1998; Slattey et al. 2005; Yanagida et al. 2016).

All the above-mentioned antipsychotics have been shown to induce c-Fos protein expression in different brain areas (Wisden et al. 1990). The effect of OLA on c-Fos expression has been investigated in an atropine-induced disruption of the degraded contingency effect (Carnicella et al. 2010) and on the rat maternal behavior (Zhao and Li 2012). The effect of AMI on c-Fos expression has been demonstrated in the interaction between trace amine-associated receptor 1 and dopamine D₂ receptor (Espinoza et al. 2011) and limbic selectivity in comparison with haloperidol, risperidone, and clozapine (Natesan et al. 2008). The effect of QUE on c-Fos expression has been investigated in the ventrolateral striatum after pimozide induced tremulous jaw movements (Betz et al. 2009). The effect of ARI on c-Fos expression has been reported in striatum of caffeine-induced hyperlocomotion (Batista et al. 2016) and prefrontal cortex of prenatal valproic acid-induced behaviors abnormalities (Hara et al. 2017).

Actually, the c-Fos and related gene products belong to the transcription factors candidates that could create linkage between the acute cell surface receptor occupancy by APs and the gradual, long-lasting neural changes induced by their chronic treatment (Hiroi and Graybiel 1996). The long-lasting effect induced by APs repeated treatment can be monitored by the deltaFosB (ΔFosB), expression, a truncated splice variant of the FosB/ΔFosB gene (Kovacs 1998; Majercikova and Kiss 2015; Majercikova et al. 2016; Kiss and Majercikova 2017; Osacka et al. 2019). The functional significance of ΔFosB has been implicated in different experiments. The formation of ΔFosB in multiple brain regions and the molecular pathway leading to the formation of AP-1 complexes is well understood (Milde-Langosch et al. 2004). The ΔFosB is an essential transcription factor implicated in the molecular and behavioral pathways of addiction following repeated drug exposure (Zachariou et al. 2006). Overexpression and excessively high level of ΔFosB expression following repeated drug exposure triggers the development of addiction-related neuroplasticity throughout the reward system and produces a behavioral phenotype. The ΔFosB also mediates epigenetic desensitization of the c-Fos gene after chronic amphetamine exposure (Renthal et al. 2008).

Stress has been shown to be a risk factor for the development in various mental disorders, likewise schizophrenia (Jones and Fernyhough 2007) and an interconnection between the long-lasting stress challenges and emotional, social, and physiological breakdowns have been shown in rodents (Amat et al. 2005; Tsankova et al. 2006). It is strongly assumed that the combination of different mild stressors in animal models may more or less mimic the natural environment affecting the everyday life of the human being (Nyuyki et al. 2012). An interference of stressors with the effect of drugs or psychopharmacologic agents with harmful effect on the human health has also been presumed (Post 1992; Tennant 2002).

The goal of the present study was to reveal whether the acute effect of olanzapine, amisulpride, aripiprazole, and quetiapine, indicated by the presence of c-Fos, will: a) stimulate neurons already activated by a 20 day HDL or CMS preconditioning, indicated by the ΔFosB expression, or b) have a stimulatory effect only on a not activated, so called silent neurons. The pattern ΔFosB and c-Fos spatial relationship was investigated in three forebrain structures, including the septal ventrolateral nucleus (seVL), the striatal dorsolateral area (stDL), and the shell of the nucleus accumbens (shell) employing dual fluorescence labeling technique.

Materials and methods

Animals. Adult male Sprague Dawley rats ($n=55$), purchased from Velaz (Prague, Czech Republic) weighing 280–300 g, were housed three or four per cage in a room with controlled temperature ($22\pm 1^\circ\text{C}$), light (12-h 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 the State Veterinary and Food Administration of the Slovak Republic Committee (Approval protocol number 1461/17-221). The investigation conditions were in accordance with the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Stress preconditioning and antipsychotics treatments. The rats were divided into 10 groups and exposed to two kind of stressors. Half of them was exposed daily to a homogeneous stressor – handling (HDL): 1) vehicle (VEH, $n=5$), 2) AMI ($n=6$), 3) OLA ($n=5$), 4) QUE ($n=6$), 5) ARI ($n=5$), and half of them was exposed daily to a heterogeneous stressors (CMS): 6) CMS+VEH ($n=5$), 7), CMS+AMI ($n=6$), 8) CMS+OLA ($n=6$), 9) CMS+QUE ($n=6$), and 10) CMS+ARI ($n=5$). CMS was represented by five types of stressors: crowding (CR, instead of 3–4, 7–8 rats were placed into the cage for 24 h), air-puff (AP, air noise divided into 45 episodes, each of them lasting 1 min, randomized by a computer for 24 h), wet bedding (WB, 500 ml of water poured into the cage for 24 h), predator stress (PS, $5 \times 5 \times 10$ cm large wood piece wrapped by a rag saturated by a cat odor put into the cage for 24 h), and forced swimming (FS, immersion of the rats in a 45 cm tall and 25 cm wide glass cylinder filled with $24\pm 1^\circ\text{C}$ water for 10 min). The animals were exposed to both the HDL and CMS stressors for 20 days. In the case of CMS, the stressors application was following: CR, AP, WB, CR, PS, FS, AP, WB, CR, PS, FS, AP, WB, CR, PS, WB, AP, FS, CR, and PS. Except the FS, which lasted 10 min, the time exposure to other stressor lasted 24 h (from 8:30 a.m. of the one day to 8:30 a.m. of the next day). After cessation of the FS, the animals were returned to their home cages and exposed to warming lamp until get dry.

On the 21st day of the experiment, after cessation of the 20 days-lasting HDL and CMS preconditioning, the rats were free of the stressor exposure. On the 22nd day, approximately 24 h after the last HDL and CMS exposures, both groups of animals were acutely intra-

peritoneally injected with vehicle (4% DMSO (Sigma-Aldrich, Germany) in saline, injected 0.1 ml/100 g) or a particular antipsychotic. All antipsychotics were purchased from Sigma-Aldrich (Germany). Antipsychotics were administered in the following doses: olanzapine 5 mg/kg, amisulpride 20 mg/kg, aripiprazole 10 mg/kg, and quetiapine 15 mg/kg of the body weight (b.w.). All antipsychotics were dissolved in vehicle. The antipsychotics doses were selected based on the literature (Kiss et al. 2019).

Ninety min after the treatments, the rats were anesthetized by a combination of Zoletil (30 mg/kg, Virbac, Carros, France) and Xylarium (15 mg/kg, Riemser, Germany) in the volumes 0.1 ml and 0.24 ml/300 g b.w., respectively and sacrificed by a transcardial perfusion with 60 ml of saline containing 450 μl of heparin (5000 IU/l, Zentiva, Slovakia) followed by 250 ml of fixative containing 4% paraformaldehyde (Sigma-Aldrich, Germany) in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed from the rats and postfixed in a fresh fixative overnight. Thereafter, the brains were washed two times in 0.1 M PB, infiltrated with 30% sucrose for 2 days at 4°C , cut into 30 μm thick coronal sections using Reichert-Jung, cryo-cut E (Austria), and collected in a cryoprotectant solution at -20°C until used.

Brain areas investigated. Three forebrain areas including the septal ventrolateral (seVL) nucleus, the striatal dorsolateral (stDL) area, and the shell of the nucleus accumbens (shell) were selected based on the atlas of the rat brain (Paxinos and Watson 2007) corresponding to the following brain stereotaxic coordinates: seVL – Bregma: 1.00–0.70 mm, stDL: 1.00–0.48 mm, shell: 1.20–1.60 mm (Figure 1).

Figure 1. Schematic drawing depicting the localization of the investigated forebrain structures including the ventrolateral septal nucleus (1), the dorsolateral striatum (2), and the shell of the accumbens nucleus (3).

Quantitative evaluation (counting) of Δ FosB and c-Fos colocalizations was performed on fluorescence photomicrographs captured on Axio-Imager A1 fluorescence microscope (Carl Zeiss) coupled to a video camera and monitor. The fluorescence photomicrographs containing Δ FosB (green, Alexa488) and c-Fos (red Alexa596) profiles were converted into a gray tone and transformed by threshold into the black and white picture. Then the black and white pictures were returned into RGB and both Δ FosB and c-Fos profiles adjusted by a channel mixer into basic blue color. Using hue/saturation adjustment, the green Δ FosB and red c-Fos colors were restored and the green Δ FosB and red c-Fos containing sections were merged, whereas the overlapping green Δ FosB and red c-Fos profiles turned yellow (Figure 2). The final ratio of yellow (colocalized) to green (Δ FosB) and red (c-Fos) colored profiles was expressed in percentages (percentage of the cells colocalizing c-Fos and FosB from the total amount of cells expressing colocalized c-Fos and FosB plus cells expressing only FosB or only c-Fos).

Fos immunohistochemistry. Forebrain free-floating sections were repeatedly washed in cold 0.05

M PB and preincubated in a blocking solution of 0.3% H_2O_2 in 0.05 M PB (Fisher Scientific, Fair Lawn, NJ, USA) for 20 min at room temperature (RT). Then the sections were rinsed 3×10 min in 0.05 M PB and incubated with a monoclonal mouse Δ FosB antiserum diluted 1:1000 in 0.05 M PB containing 4% normal goat serum (Gibco, Grand Island, NY, USA), 0.5% Triton X-100 (Koch-Light Lab. Ltd., Colnbrook Berks, England), and 0.05% sodium azide (Sigma Chemical Ltd. St. Louis MO, USA) at 4°C for 48 h. After several rinsing in PB, the sections were exposed to biotinylated goat anti-mouse IgG conjugated with Alexa Fluor 488 (1:250) in 0.05 M PB containing 0.5% Triton X-100 at 4°C overnight. After several rinsing in PB, the sections were exposed to a rabbit polyclonal c-Fos antiserum (12-5) diluted 1:1500 in 0.05 M PB (containing the same components as described above for Δ FosB) at 4°C for 48 h. After several rinsing in PB, the sections were exposed to the rabbit polyclonal c-Fos antiserum at 4°C for 48 h. After several washings in PB, the sections were exposed to biotinylated donkey anti-rabbit IgG conjugated with Alexa Fluor 594 (1:250) in 0.051 M PB containing 0.5% Triton X-100 at 4°C overnight. Following several rinsing

Figure 2. Microphotograph demonstrating the Δ FosB and c-Fos merging procedure. By overlapping the Δ FosB - green (A) and c-Fos - red (B) sections, merged Fos profiles (yellow) emerged (C). By removing the histological background of the overlapped sections, pure colored circles showed up (D) ready for the quantification process (for details see Methods).

of the sections in 0.05 M PB, they were mounted on positive charged adhesive slides, dried at RT for 20 min, coverslipped with Pertex (Stockholm, Sweden), and stored in dark histological boxes.

Antibodies. The primary polyclonal rabbit anti-c-Fos antibody was a gift (see Acknowledgement). The primary monoclonal mouse anti-FosB/ Δ FosB (ab11959), secondary goat anti-mouse Alexa Fluor 488 (ab150113), and donkey anti-rabbit Alexa Fluor 594 (ab150076) antibodies were purchased from Abcam (Cambridge, UK).

Statistical analysis. Statistical analysis was carried out by Student's t-test. Statistical comparisons were performed individually only between Δ FosB and c-Fos groups in each forebrain area in each group of the animals. All the data were expressed as mean $\pm$ SEM. The value of $p < 0.05$ was considered as statistically significant. The outliers were excluded if the data point more than 1.5 interquartile ranges below the first quartile or above the third quartile.

Results

Both the HDL and CMS preconditioning induced a distinct Δ FosB protein expression in each of the brain areas investigated, i.e. in the seVL, the stDL, and the shell. However, VEH injection did not induce substantial activation of c-Fos expression in either HDL or CMS exposed group in any of the above mentioned forebrain areas (data are not shown).

In the HDL and CMS preconditioned rats, the OLA distinctly activated c-Fos expression in all the three investigated areas, i.e. the seVL, the stDL, and the shell, whereas all of them revealed around 60% of colocalizations with Δ FosB (Figure 3, top, bottom). The maximal level of c-Fos with Δ FosB colocalizations exceeded 80% in the stDL after both HDL and CMS preconditioning (Figure 3, top, bottom). On the other hand, the maximal level of Δ FosB colocalizations was observed in the seVL of HDL preconditioned rats (Figure 3, top).

In the HDL and CMS preconditioned rats, the AMI treated rats distinctly activated c-Fos expression only in two from the three investigated areas, including the seVL and the shell, whereas both of them revealed more than 50% of colocalizations with Δ FosB (Figure 4, top, bottom). More than 80% of c-Fos colocalizations with Δ FosB were seen in the seVL area in CMS stimulated rats (Figure 4, top). In the stDL area, the AMI did not evoke any Δ FosB or c-Fos protein expression (Figure 4, top, bottom). The Δ FosB colocalizations occurred between 28 and 52% (Figure 4, top, bottom).

Figure 3. Colocalization levels of the Δ FosB and c-Fos labeled profiles in repeatedly handled (HDL, top) or exposed to a sequence of heterogeneous mild stressors (CMS, bottom) after administration of a single dose of olanzapine. Except the seVL in both stress challenges, c-Fos revealed significantly higher level of colocalization than Δ FosB in both experimental groups [top (HDL) seVL $p < 0.001$, stDL $p = 0.005$, shell $p = 0.002$; bottom (CMS) seVL $p = 0.063$, stDL $p < 0.001$, shell $p = 0.027$].

In the HDL and CMS preconditioned rats, the ARI also distinctly activated c-Fos expression in all the three investigated areas, whereas all of them, except the seVL of HD and also CMS animals, revealed more than 60% of colocalizations with Δ FosB (Figure 5, top, bottom). More than 80% of Δ FosB colocalizations with c-Fos profiles were seen in the seVL in HDL as well as CMS stimulated rats (Figure 5, top, bottom). And over 40% of Δ FosB colocalizations with c-Fos protein were observed in the stDL and shell of HD and CMS exposed animals (Figure 5, top, bottom).

In the HDL and CMS preconditioned rats, the QUE also distinctly activated c-Fos expression in all the three investigated areas, including the seVL, the stDL, and the shell, whereas all of them revealed around 70% of colocalizations with Δ FosB (Figure 6, top, bottom). More than 80% of c-Fos colocaliza-

tions with Δ FosB profiles were seen in the stDL in both HDL and CMS preconditioned rats (Figure 5, top, bottom). Except the seVL of HDL group, all other groups showed less than 50% of Δ FosB colocalizations (Figure 6, top, bottom).

Discussion

The data of the present study clearly demonstrate that Δ FosB and c-Fos colocalizations occurred in each of the three areas investigated, i.e. seVL, stDL, and shell, in both homogeneously with HDL as well as heterogeneously with CMS preconditioned rats. The levels of Δ FosB and c-Fos colocalizations varied in the individual forebrain areas studied, but neither c-Fos nor Δ FosB reached 100% level.

The extent of antipsychotics anatomical actions and their functional consequences might be related to the

fact whether they stimulate only short-lasting c-Fos or also long-lasting Δ FosB transcriptional factors expression in the targeted structures. As it has been documented, the functional or anatomical relationships between Δ FosB and c-Fos can be studied after an acute treatment with antipsychotics (Dragunow et al. 1990; Madsen et al. 2006) or after combination of long lasting physical or emotional pretreatment with acute treatment with antipsychotics (Hiroi and Graybiel 1996). In our study, we have applied the second variant, i.e. anatomical relationship between the above mentioned transcriptional factors, whereas long-lasting HDL and CMS preconditionings were used as repeated external physical/psychological impulses for the Δ FosB protein induction, while the c-Fos expression was induced by an acute treatment with one of four antipsychotics, i.e. by OLA, AMI, ARI, or QUE.

Figure 4. Colocalization levels of the Δ FosB and c-Fos labeled profiles in repeatedly handled (HDL, top) or exposed to a sequence of heterogeneous mild stressors (CMS, bottom) after administration of a single dose of amisulpride. Notice the similar trend in response of Δ FosB and c-Fos in seVL as well as shell areas, whereas in all four cases c-Fos revealed significantly higher level of colocalization than Δ FosB [top (HDL) seVL $p=0.001$, shell $p=0.084$; bottom (CMS) seVL $p=0.004$, shell $p=0.007$].

Figure 5. Colocalization levels of the Δ FosB and c-Fos labeled profiles in repeatedly handled (HDL, top) or exposed to a sequence of heterogeneous mild stressors (CMS, bottom) after administration of a single dose of aripiprazole. Except the seVL in both stress challenges, c-Fos revealed significantly higher level of colocalization than Δ FosB in stDL and shell areas in both experimental groups [top (HDL) seVL $p<0.001$, stDL $p=0.238$, shell $p<0.001$; bottom (CMS) seVL $p<0.001$, stDL $p=0.007$, shell $p<0.001$].

In the present study, by employing dual immunohistochemistry, the presence of single labeled c-Fos protein, single labeled Δ FosB protein, and colocalized c-Fos with Δ FosB has regularly been detected in all three areas investigated, i.e. seVL, stDL, and shell, in both HDL and CMS preconditioned rats. The Δ FosB presence in different brain areas speaks out for persistent activation of various brain regulatory circuits involved in the different functions. Actually, the implication of Δ FosB has been demonstrated in a number of different molecular and behavioral pathways usually activated by repeated physical challenges (Greenwood et al. 2011; Kovacs et al. 2019), drug exposures (Bing et al. 1997; Perez-Otano et al. 1998; Renthal et al. 2008; Lafragette et al. 2017), and various external stimuli (Tulchinsky 2000), whereas the formation of Δ FosB in multiple brain regions can be directly linked to the structural, physiological, and behavioral changes (Lopes et al. 2018). It has also been reported that Δ FosB plays an important role also in regulating behavioral responses to natural rewards, such as palatable food (Teegarden et al. 2008), sex (Hedges et al. 2009), exercise, and stress (Greenwood et al. 2011; Lobo et al. 2013). In the present study, the HDL and CMS preconditionings represented a sequence of repeated homogenous and heterogeneous mild stressor challenges, respectively (Kovacs et al. 2019). In contrast to Δ FosB, the c-Fos transcription factor has generally been accepted as a functional marker (Mahner et al. 2008) to delineate individual neurons responsivity to a wide variety of acute external stimuli (Tulchinsky 2000; Werme et al. 2002).

The attempt of the present study was to find out whether the acute treatment with OLA, AMI, ARI, or QUE, indicated by c-Fos presence, will affect already stimulated neurons, indicated by the Δ FosB presence, by HDL or CMS preconditionings or it will activate only a population of silent neurons. The study is one of the first ones dealing with the Δ FosB and c-Fos colocalization in the neurons of the seVL, stDL, and shell after acute treatment with antipsychotics after exposing the animals to repeated homogenous and heterogeneous mild physical/psychological challenges. The data of this investigation demonstrate that Δ FosB and c-Fos colocalizations occurred in each of the three areas investigated, i.e. seVL, stDL, and shell, in both homogeneously with HDL as well as heterogeneously with CMS preconditioned rats. The levels of Δ FosB and c-Fos colocalizations varied in the individual forebrain areas studied. From the total 22 areas measured, c-Fos expression prevailed over Δ FosB in 18 ones. However, neither c-Fos nor Δ FosB reached

Figure 6. Colocalization levels of the Δ FosB and c-Fos labeled profiles in repeatedly handled (HDL, top) or exposed to a sequence of heterogeneous mild stressors (CMS, bottom) after administration of a single dose of quetiapine. Except the seVL in the HDL rats, c-Fos in other areas of both HDL and CMS rats revealed significantly higher colocalization level than Δ FosB [top (HDL) seVL $p=0.087$, stDL $p<0.001$, shell $p=0.009$; bottom (CMS) seVL $p<0.001$, stDL $p<0.001$, shell $p<0.001$].

100% level of colocalization in the forebrain areas investigated. However, whether the simultaneous occurrence of c-Fos and Δ FosB in the same neuron might not only anatomically but also functionally be associated is impossible to amenably anticipate from the present study. Supposing that the latter situation would be the case, then the functional competence of neurons with Δ FosB and c-Fos colocalization might be different of those labeled with only the single c-Fos or c-Fos co-expressed with Δ FosB, which might speak out for a possible dual, i.e. short- and long-lasting, functions of acutely administered antipsychotics.

The question of the dual functionality of neurons, has also been queried by Marin-Blasco et al. (2018), who have investigated whether neurons preconditioned by a prolonged immobilization may further respond to a novel stressor. They have shown that the response of the stress preconditioned neurons to a novel stressor

may be different, depending on the fact, whether they are located in a high- or low-hierarchy processing brain area (Marin-Blasco et al. 2018). However, in the present study the action of antipsychotics is more specific than the above mentioned nonspecific novel stressor and therefore, the above mentioned responsiveness hierarchy system is probably not applicable to the present data. In spite of the fact that we had no intent to establish the exact quantitative ratio between these three types of Fos proteins in the individual structures, we can only estimate from the random quantifications that Δ FosB and c-Fos colocalization vs a single c-Fos occurrence may considerably vary per section in each structure. In addition, no exact topographic distribution with respect to the functional significance of colocalized c-Fos and Δ FosB

transcriptional factors within the individual structures investigated can be established from the present study. Actually, more studies have to be performed for the answering the functional significance of activated neurons by antipsychotics with respect to their phenotype character as well as their involvement in the stress-related brain circuitries.

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