

## Original Article

# Effect of Asenapine on the Activity of Hypocretin Neurons in Normal and Unpredictable Mild Stress Preconditioned Rats

(asenapine / hypocretin / hypothalamus / stress / rat)

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**Abstract.** Asenapine (ASE) is a novel atypical antipsychotic used in schizophrenia treatment. Here, the effect of ASE on Fos expression in hypocretin (Hcrt) neurons in medial and lateral portions of the lateral hypothalamus (LH) and the effect of chronic unpredictable variable mild stress (CMS) preconditioning were studied. CMS consisted of restraint, social isolation, crowding, swimming, and cold and lasted 21 days. The rats were sacrificed on day 22, 90 min after a single injection of vehicle (saline 300 µl/rat subcutaneously – s.c.) or ASE (0.3 mg/kg s.c.). Control (CON), ASE, CMS, and CMS + ASE groups were used. Fos protein was visualized by the avidin biotin peroxidase technique, while Hcrt perikarya by fluorescent dye. Fos/Hcrt co-localizations were evaluated under parallel light and fluorescent illuminations. In the single Fos expression assessment, the Fos number was significantly higher in the medial in comparison with the lateral LH portion in each group. No differences in Fos amount were observed between the individual groups within the medial and lateral LH portions. In the Fos/Hcrt co-localization assessments, ASE significantly reduced the number of Fos/Hcrt neurons in the medial, but not lateral, LH portion in ASE and CMS + ASE groups. CMS only slightly contributed to the inhibitory effect of ASE in the CMS + ASE groups. The present data show as the first that ASE may reduce the activity of Hcrt cells in the medial LH portion, which might correspond with

the relatively low weight gain liability of ASE. CMS preconditioning did not significantly interfere with this impact of ASE.

## Introduction

The hypocretin/orexin (Hcrt) family of neuropeptides, which was simultaneously described by two independent groups of researchers (de Lecea et al., 1998; Sakurai et al., 1998), was located in neurons of the lateral hypothalamus and perifornical area with widespread projections within the central nervous system (Ebrahim et al., 2002). As a neurotransmitter, Hcrt plays an important role in the regulation of energy balance, appetite, and weight gain (Sutcliffe and de Lecea, 2000; Karnani et al., 2011). Literature data have indicated that antipsychotics may influence the activity of Hcrt neurons. However, not all antipsychotics may interact with these neurons equally. In Hcrt neurons, increased Fos expression has been shown to be induced by clozapine, olanzapine, and risperidone, but not ziprasidone, haloperidol, and fluphenazine in the lateral hypothalamus and the perifornical area (Fadel et al., 2002).

Asenapine (ASE) is a novel psychopharmacologic agent, an atypical antipsychotic being developed for treatment of schizophrenia and bipolar disorder (Szegedi et al., 2011; Stoner 2012; Fagiolini, 2013; Leucht et al., 2013). It is characteristic with a lower incidence of extrapyramidal side effects (Boyda et al., 2013). ASE shows high affinity for numerous receptors, including the serotonergic, adrenergic, dopaminergic, histaminergic, and cholinergic ones. At the 5-HT<sub>1A</sub> receptors ASE acts as a partial agonist while at all other targets it acts as an antagonist (Shahid et al., 2009). Many antipsychotic drugs induce significant weight gain (Allison et al., 1999; Taylor and McAskill, 2000). However, ASE seems to have a relatively low weight gain liability for an atypical antipsychotic (which are notorious for their metabolic side effects) and according to a recent meta-analysis it produces significantly less weight gain. In rats, a weight loss has been observed five weeks after ASE treatment as well as combined treatment of ASE with hypercaloric diet (de Brito and Ghedini, 2014). In

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Received March 5, 2015, Accepted April 20, 2015.

Financial supports are from BrainCentrum and VEGA (2/0069/12) grants of the Slovak Academy of Sciences.

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Abbreviations: ASE – asenapine, CMS – chronic unpredictable mild stress, CO – cold, CON – control, CR – crowding, Hcrt – hypocretin, LH – lateral hypothalamus, RE – restraint, RT – room temperature, SAB – sodium acetate buffer, SI – social isolation, SW – swimming.

mice with experimentally destroyed Hcrt cells, a significant increase in body weight has been demonstrated (Hara et al., 2001).

Ordinarily occurring daily stress episodes may not only influence the body homeostasis but may also be accompanied by different psychical disturbances, and even the effect of drugs' therapeutic potency might be influenced. It is believed that a complex of mild events used in animal models may mimic the human natural stressful environment (Nyuyki et al., 2012). Therefore, to know the anatomical substrates of antipsychotic action and their response to stress episodes may be a useful knowledge leading to better understanding the antipsychotic brain actions. Moreover, a link between chronic or repeated stress and emotional and physiological dysfunctions has been demonstrated in rodents (Willner, 1990; Amat et al., 2005). Finally, the Fos protein detection has become one of the well-accepted and broadly utilized approaches in studies regarding the impact of different antipsychotics on the activity of brain structures (Dragunow and Faull, 1989).

The aim of the present study was to extend our previous investigations about ASE acute impact on the activity of forebrain neurons (Majercikova et al., 2014) and to find out whether acute ASE treatment may also have an impact on the Hcrt cells, which play an important role in the food intake regulation. We were interested: 1) to delineate the quantitative profile of the ASE effect on Fos expression in the Hcrt neurons in medial and lateral portions of the lateral hypothalamus (LH), and 2) to find out whether chronic unpredictable variable mild stress (CMS) preconditioning may modify the effect of acute ASE treatment.

## Material and Methods

### *Animals*

Adult male Wistar rats (Charles River, Germany) (N = 24) weighing 240–250 g were used. They were housed two per cage in a room with controlled temperature (22 ± 1 °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 Animal Care Committee of the Institute of Experimental Endocrinology, Slovak Academy of Sciences, Slovak Republic. The investigation conditions are 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 four groups: CON (N = 6), ASE (N = 6), CMS (N = 6), and CMS + ASE (N = 6). The following stressors were used: restraint (RE, plastic restrainers), social isolation (SI), crowding (CR), cold (CO, 4 °C cold room), and swimming (SW, 30 cm high

× 20 cm wide glass cylinders). The sequence of the stressors and the time of exposure were the 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 stressor predictability, the particular stressor was applied each day at a different time.

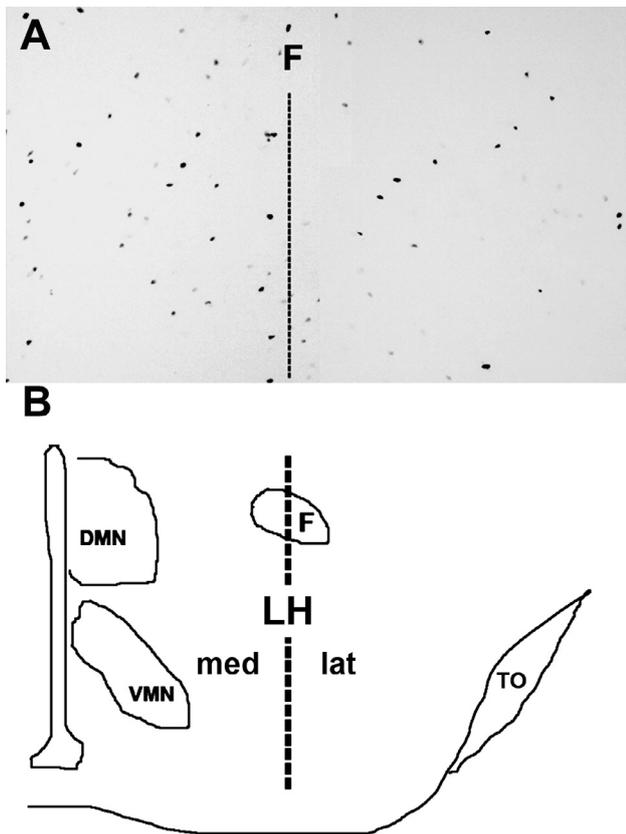
The CMS and CMS + ASE groups of animals were exposed to stress for 21 days. On the 22<sup>nd</sup> day, after the last exposure to stressor, the CMS and CMS + ASE groups were s.c. injected (between 8:00 to 9:00 a.m.) with vehicle (saline 300 µl/kg) or a single dose of ASE (0.3 mg/kg), respectively. On the same day, CON and ASE groups of animals were subcutaneously injected (between 9:00 to 10:00 a.m.) with vehicle (saline 300 µl/kg) and a single dose of ASE (0.3 mg/kg), respectively. The animals were sacrificed by transcardial perfusion with fixative 90 min after the single vehicle or ASE injection. The sacrificing time was selected based on the data of a pilot study in which Fos expression extensiveness was tested 90, 120, and 150 min post ASE treatment.

### *Brain areas and cell counting*

The number of single-labelled Fos profiles, Hcrt perikarya, and combined Fos/Hcrt cells was counted separately in the medial and lateral portions of the LH. The borderline between these two portions was defined by an arbitrary vertical line leading across the middle of the fornix according to Fadel et al. (2002). For the counting, four representative sections/rat were selected (bregma -2.56 and -3.30) (Fig. 1A,B), using Paxinos and Watson (2007) rat brain atlas coordinates. Each part of LH (together 4 parts/section bilaterally) was separately captured and evaluated. Together, 16 LH parts/rat (8 medial and 8 lateral) were analysed on photomicrographs. All the counting was performed manually, using Adobe Photoshop 7.1, from photomicrographs captured in Axio-Imager A1 light microscope (Carl Zeiss, Jena, Germany) coupled to a video camera and monitor. Fos/Hcrt co-localizations were counted from photomicrographs captured simultaneously under light and fluorescent illuminations (Fig. 2) (Majercikova et al., 2012). The number of single Fos profiles was expressed per LH portion and Fos/Hcrt co-localizations were expressed in percentage by adjusting the real number of Fos/Hcrt cells to 100 Hcrt perikarya.

### *Fos immunohistochemistry*

The animals were anesthetized with sodium pentobarbital (50 mg/kg of body weight, intraperitoneally) and perfused with 50 ml of cold isotonic saline contain-



### Bregma from -2.56 to -3.30

Fig. 1. Schematic drawing demonstrating the medial and lateral portions of LH at the level of bregma (from -2.56 to -3.30) delineated by an arbitrary vertical line led across the middle of the fornix (B). The DAB-nickel stained section demonstrating the distribution of Fos profiles (black dots) evenly distributed over the medial and lateral portions of the LH (A).

DMN – hypothalamic dorsomedial nucleus, F – fornix, lat – lateral LH portion, med – medial LH portion, TO – tractus opticus, VMN – hypothalamic ventromedial nucleus

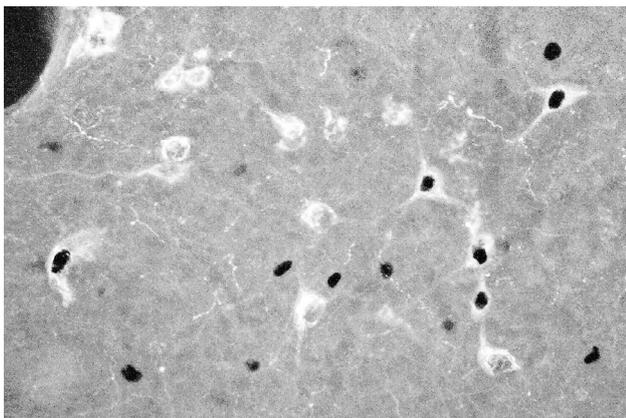


Fig. 2. Representative photomicrograph used for the quantitative evaluation of single Fos profiles (black dots), Hcrt perikarya (white cells), and Fos/Hcrt co-localizations (white cells with black dots) in the same histological section visualized by simultaneous light and fluorescent microscopic illuminations

ing 450  $\mu$ l of heparin (5000 IU/l) followed by 250 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were post-fixed in a fresh fixative overnight. Then, the brains were two times washed in 0.1 M PB at 4 °C overnight, infiltrated with 30% sucrose for two days, and cut into 30  $\mu$ m thick coronal sections. Free floating sections were repeatedly washed in cold 0.1 M PB and pre-incubated with 0.3 %  $H_2O_2$  in 0.1 M PB (Fisher Scientific, Fair Lawn, NJ) for 10 min at room temperature (RT). Then the sections were rinsed 3  $\times$  10 min in 0.1 M PB and incubated with a rabbit polyclonal Fos protein antiserum (1 : 3000, No 12-2) diluted in 0.1 M PB containing 4% normal goat serum (Gibco, Grand Island, NY), 0.5% Triton X-100 (Koch-Light Lab. Ltd., Colnbrook Berks, England), and 0.1 % sodium azide (Sigma-Aldrich Ltd., St. Louis, MO) for 48 h at 4 °C. After several rinsing rounds in PB, the sections were incubated with biotinylated goat anti-rabbit IgG (1 : 500, VectorStain Elite ABC Kit, Vector Lab., Burlingame, CA) 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), Fos 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 % hydrogen peroxide. Developing time was 6–10 min. The heavy metal intensification of DAB yielded to black staining in the Fos-labelled nuclei. The sections were then exposed to a rabbit polyclonal Hcrt antiserum (No 99010, 1 : 1000) for 48 h at 4 °C. After several washings in PB, the sections were incubated with goat anti-rabbit IgG labelled with Alexa555 (Abcam, Cambridge, UK) fluorescent dye (diluted 1 : 500 in PB containing 0.1 % Triton X-100), overnight. After several washings in PB, the sections were mounted on microscopic slides and coverslipped with Pertex (Histolab AB, Göteborg, Sweden).

### Statistical analysis

Statistical analysis for the single Fos expression was carried out by one way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison *post hoc* test. Fos/Hcrt co-localization response to ASE and CMS was analysed by ANOVA 2-factor with replication. The value of  $P < 0.05$  was considered as statistically significant. All the data were expressed as mean  $\pm$  SEM.

### Results

In all four groups of animals investigated, the DAB-nickel stained sections revealed the presence of Fos profiles over the entire extent of the lateral hypothalamus (Fig. 1A). The number of Fos profiles in the medial, in comparison with the lateral LH portion, was significantly higher ( $P < 0.001$ ) in each group of animals investigated (Fig. 3). However, neither of the conditions, i.e. CMS preconditioning, single ASE treatment, or combi-

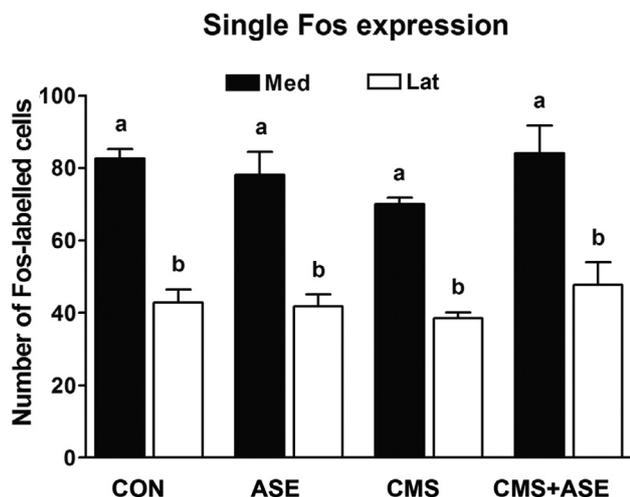


Fig. 3. Graph demonstrating the number of Fos profiles in the medial (Med) and lateral (Lat) portions of LH in individual groups of animals investigated. Different superscripts designate statistically significant differences ( $P < 0.001$ ).

CMS – stress preconditioned

nation of the ASE treatment with the CMS preconditioning, significantly affected the number of the Fos profiles in the medial or lateral portions of LH in comparison with the CON group of animals (Fig. 2).

The Hcrt-immunolabelled neurons visualized by a fluorescent dye under combined light and fluorescent microscopic illuminations (Fig. 2) revealed an extensive pattern of Hcrt perikarya distribution in the LH in CON, i.e., only with vehicle-treated animals. The Hcrt neurons exhibited immunostaining over the whole LH, including its medial and lateral portions and the perifornical area. In the medial portion of the LH, distinctly immunolabelled Hcrt cells were present mainly along the lateral margin of the dorsomedial nucleus and the medial half of the perifornical area. In the medial portion of the LH in CON and CMS preconditioned animals, approximately one third of the Hcrt perikarya displayed Fos presence as revealed by the Fos number analysed on photomicrographs captured under combined light and fluorescent microscopic illuminations (Fig. 4). The ASE administration itself as well as its combination with CMS preconditioning statistically significantly reduced the number of Fos-positive Hcrt neurons in comparison with the CON and CMS preconditioned animals (Fig. 4). Two-way ANOVA with factor treatment (ASE) and CMS did not reveal involvement of the stress in the ASE inhibitory action ( $F = 1.554471$ ,  $P = 0.215427$ ). A decreasing tendency in the number of activated Hcrt neurons was also seen in the CMS group of rats in comparison with the CON (Fig. 4). On the other hand, the quantity of activated Hcrt neurons was lower, approximately by half, in the lateral portion in comparison with the medial portion of the LH (Fig. 4). However, in the lateral portion of LH, no distinct differences in the amount of Fos-labelled cells were seen between the individual groups of rats investigated (Fig. 4).

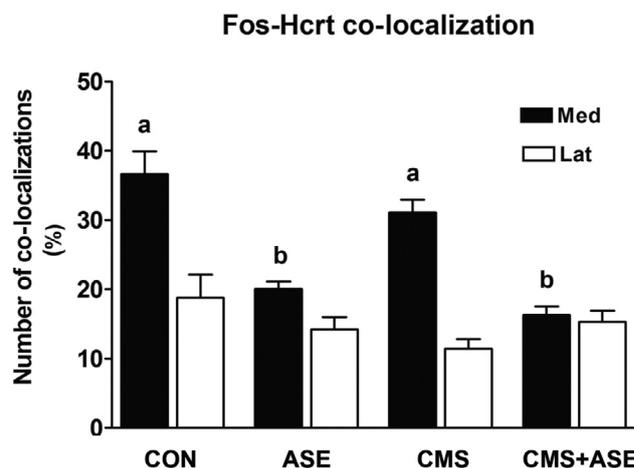


Fig. 4. Graph demonstrating the number of Fos-Hcrt co-localizations in the medial (Med) and lateral (Lat) portions of LH in individual groups of animals investigated. Different superscripts designate statistically significant differences ( $P < 0.05$ ).

CMS – stress preconditioned

## Discussion

The data of the present study revealed that in the single Fos expression assessment, the Fos number was significantly higher in the medial in comparison with the lateral LH portion in each group of rats, and in the Fos/Hcrt co-localization assessments, ASE and CMS + ASE treatments significantly reduced the number of activated Hcrt neurons only in the medial portion of the LH, whereas CMS preconditioning did not significantly interfere with the inhibitory impact.

It has been well documented that treatment with many antipsychotic drugs may lead to a significant weight gain (Allison et al., 1999; Wetterling and Mussigbrodt, 1999; Taylor and McAskill, 2000; Allison and Casey, 2001). In preliminary observations, Taylor and Askill (2000) have reported that all atypical antipsychotic drugs studied, with the exception of ziprasidone, have been associated with weight gain. According to the data of Taylor and Askill (2000), clozapine seems to have the highest risk of weight gain, followed by olanzapine and quetiapine. There is probably a lower risk with risperidone, sertindole, and zotepine and still a lower risk with amisulpride (Taylor and Askill, 2000). Separate meta-analyses have confirmed that of the atypical antipsychotic drugs, clozapine and olanzapine have the highest propensity to cause weight gain and that ziprasidone, fluphenazine, and aripiprazole have the lowest risk (Allison et al., 1999). In C57BL/6J mice, for example, risperidone-induced weight gain has been shown to be associated with hyperphagia and a reduction in locomotor activity (Li et al., 2013). Based on the literature data, however, ASE, used in the present study, seems to have a relatively low weight gain liability and according to a recent meta-analysis, it produces only low weight gain. In rats, a weight loss was observed both five weeks after ASE treatment and after combined treatment of ASE with hypercaloric diet (de Brito and Ghedini, 2014).

Fadel et al. (2002) have shown that weight gain is associated with activation of a distinct population of Hcrt neurons. Although Hcrt has been described to be involved in the regulation of many physiological and pathophysiological processes, including arousal and wakefulness, Hcrt is critically involved in the body weight regulation (Fadel et al., 2002). For example, intracerebral administration of Hcrt has been shown to increase the food intake (Edwards et al., 1999). In the brain, LH is the only area where Hcrt perikarya occur (Peyron et al., 1998; Date et al., 1999). Actually, from the functional point of view, the Hcrt neurons represent a heterogeneous agglomerations of neurons. However, no study is available at present which might deal with the anatomical delineation of functionally equivalent groups of Hcrt-producing neurons. Therefore, in the present study, a model of an arbitrary separation of the medial portion from the lateral one of LH by a vertical line leading across the middle of the fornix was used (Fadel et al., 2002).

Actually, there are not many studies available dealing with the effect of antipsychotics on the activity of Hcrt neurons. This is a relatively new area and we have found only one study evaluating the effect of antipsychotics on the activity of Hcrt neurons (Fadel et al., 2002). These authors investigated the effect of several antipsychotic drugs on the activity of Hcrt neurons and found out that some of them (clozapine, olanzapine, risperidone, and chlorpromazine) may increase, while some others (ziprasidone, haloperidol, and fluphenazine) may not increase Fos expression in the Hcrt neurons. In the present study, the effect of ASE is in concordance with those antipsychotics that do not elevate the number of stimulated Hcrt cells. Some of the psychoactive drugs, including amphetamine (a psychostimulant drug), may induce activation of Hcrt neurons separately, i.e., only in one portion of LH (Fadel et al., 2002). In our case, the number of activated neurons was significantly lower in the lateral in comparison with medial portion of the LH, and only those located in the medial LH portion were affected with ASE treatment. Although this result indicates that the ASE effect is more pronounced in the medial than in the lateral portion of LH, it is not clear whether the ASE-influenced neurons are represented functionally by a homogeneous or heterogeneous group of cells. Finally, it has to be taken into consideration that Hcrt neurons in the control of the food intake and weight gain processes represent only a part of a complex of hypothalamic and extrahypothalamic structures, where a number of antipsychotics may also act. Indeed, it has been reported that subchronic exposure to olanzapine increases not only the expression of orexigenic neuropeptides, but also up-regulates neuropeptide Y and agouti-related protein and down-regulates proopiomelanocortin in the arcuate nucleus of the hypothalamus (Ferno et al., 2011).

In summary, the present data show as the first that ASE has a potential to reduce the activity of Hcrt cells in the LHA (lateral hypothalamic area), which generally

might correspond with the relatively low weight gain liability of ASE. The inhibitory effect of ASE on Hcrt expression was mainly efficient in the medial portion of LH. CMS preconditioning lasting for 21 days did not significantly affect the ASE impact on the activity of Hcrt neurons.

### Acknowledgements

The authors are grateful to Jan Pecenek M.D. (Department of Psychiatry, Faculty of Medicine Comenius University, Bratislava Slovakia) for thoughtful comments on this article and Dr. J. Osacka, Dr. L. Horvathova, and Alena Cernackova for their expert technical assistance. We are also grateful for the primary antibodies Hcrt (99010) and Fos (12-2), provided as a gift by Dr. Jens Mikkelsen, Denmark.

The authors declare no conflict of interest.

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