CANNABINOID-MEDIATED REGULATION OF THE HYPOTHALAMO-PITUITARY-ADRENAL AXIS IN RATS: AGE DEPENDENT ROLE OF VASOPRESSIN

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Objective. Adaptation to stress is a fundamental component of life and the hypothalamo-pituitary-adrenocortical axis (HPA) plays a crucial role in it. The place of cannabinoid influence seems to be in the brain, especially where corticotropin releasing hormone and vasopressin (AVP) secreting neurons are located. The role of AVP is considered to be more important in young than in adult rats. Here we addressed the question if cannabinoid-mediated regulation of the HPA involves AVP and if there is any difference between young and adult rats in this process.

Methods. 10-day-old and adult AVP deficient Brattleboro rats were compared with their heterozygous littermates 1h after WIN 55,212-2 (6mg/kg i.p.) injection.

Results. In control animals the injection led to elevated adrenocorticotropin (ACTH) and corticosterone hormone levels at both ages without remarkable age difference in ACTH levels while all corticosterone levels of adults was approximately 10-times higher. The ACTH secretion of young AVP deficient rats failed to react to WIN 55,212-2 injection while their corticosterone levels were even higher than their littermates. In contrast in adult the role of AVP was diminished.

Conclusions. We can conclude that the peripheral administration of cannabinoids leads to HPA axis stimulation, which process involves AVP at least in the young rats. The discrepancy between ACTH and corticosterone levels in young rats suggests an alternative adrenal gland regulatory pathway, which might be present in all studied animals. However, it comes to the front just in AVP deficient pups.

Key words: WIN 55,212-2 – Vasopressin – Neonatal period – Adulthood – ACTH – Corticosterone

The cloning of the cannabinoid receptors (Matsuda et al. 1990; Munro et al. 1993) and the identification of endogenous cannabinoid ligands (Devane et al. 1992; Mechoulam et al. 1995) prompted a large interest in the functions of the endogenous cannabinoid system. The cannabinoid CB1 receptor is expressed in various regions of the brain and was shown to play important roles in locomotion, pain perception, memory, feeding, anxiety, etc. (Herkenham et al. 1991; Porter et al. 2001). In addition to their neural and behavioral effects, exogenous cannabinoids affect the production of various hormones, including the gonadal steroids,

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Abbreviations: ACTH = adrenocorticotropin; AVP = arginine vasopressin; CB1 = cannabinoid receptor 1 type; CRH = corticotropin releasing hormone; di/+ = heterozygous Brattleboro (AVP-deficient) animals; di/di = homozygous Brattleboro (AVP-deficient) rats; DMSO = dimethylsulfoxide; EDTA = ethylene diamine tetra-acetic acid; HPA = hypothalamo-pituitary-adrenal axis; i.p. = intraperitoneal; PVN = paraventricular hypothalamic nucleus; RIA = radioimmunoassay; THC = delta (9)-tetrahydrocannabinol; V = vehicle; WIN = WIN 55,212-2

growth hormone, prolactin, thyroid hormones and glucocorticoids (Brown et al. 2002). There are a series of studies suggesting that endogenous cannabinoid system also play a role in the control of the hypothalamo-pituitary-adrenal axis (HPA), e.g. Barna et al. (2004). Early studies showed that peripherally administered delta (9)-tetrahydrocannabinol (THC), as well as the widely used CB1 agonist WIN 55,212-2 (WIN) stimulates adrenocorticotropin (ACTH) secretion (Borcel et al. 2004; Puder et al. 1982). Later studies revealed that the cannabinoid receptors are expressed at the level of both the hypothalamus and pituitary, suggesting that the effects of cannabinoids on the HPA-axis are direct (i.e. mediated by their receptors) and not necessarily related to their sedative or cognitive effects (Herkenham et al. 1991). Despite their presence on the pituitary our previous work, as well as the study of Di et al. suggested that the main site of the cannabinoid-dependent HPA axis influence is rather the hypothalamus (Barna et al. 2004; Di et al. 2003).

In adult rats mainly two hypothalamic peptides, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) control the ACTH release from the anterior pituitary. Both are secreted into the hypophyseal portal circulation by neurons located in the parvocellular region of the paraventricular hypothalamic nucleus (PVN) and stimulate ACTH secretion (Sapolsky et al. 1986). ACTH reaches the adrenal gland through systemic circulation and the consequence is an increased secretion of glucocorticoids. The relative contributions of CRH and AVP to ACTH release seem to be stressor-specific, however there is a consensus that in adulthood the release is controlled mainly by CRH, while AVP is just support its effect (de Kloet 2003; Scott et al. 1998).

On the other hand, during perinatal period AVP seems to be the predominant secretagogue of the ACTH, while the glucocorticoid regulation of hypothalamic CRH gene expression is not matured (Avishai-Eliner et al. 1995; Grino et al. 1989; Levine 2002). In rats the HPA-axis is already functional in late gestation with high plasma corticosterone levels. However, plasma levels decrease dramatically during the first two postnatal days and remain low until postnatal day 14 (Walker et al. 1986). During this period the response of the HPA-axis to stressful stimuli (e.g. exposure to ether vapors or cold, endotoxin injection, maternal deprivation or electroshocks) is markedly reduced compared with the large responses seen in adult rats (Sapolsky et al. 1986). This stress hyporesponsive period was demonstrated in both rat pups and children and has major implications for the maturation of the HPA-axis (Gunnar et al. 2002; Sapolsky et al. 1986).

The aim of the present experiments was to investigate the role of AVP in the cannabinoid dependent HPA-axis regulation with special attention on age differences. To study the role of AVP the AVP-deficient Brattleboro rat is a good experimental model. This strain was discovered in mid 60‘s and shows an inherited single nucleotide deletion at the neurophysin II region of the AVP precursor molecule, which results in an abnormal AVP prohormone. This is not processed normally, consequently rats lack functional AVP and show diabetes insipidus (Evans et al. 2000).

Materials and Methods

Animals. The experiment was performed in 10-day-old and adult (8-10 weeks old) male Wistar (Charles River, Hungary) and Brattleboro rats (our colony with parent stocks from Harlan Laboratories; Indianapolis, IN, USA). The 0 day was determined when the offspring was found with the mother on the usual observation at around 9.00 h. Litter size was 3-12 and was not controlled. Only the male offspring was used for this experiment. Homozygous (di/di) AVP-deficient animals were compared to their heterozygous (di/+) littermates (Bohus et al. 1998; Zelena et al. 2003). Brattleboro breeding pairs consisted of a di/+ female and a di/di male. The rats were kept in a controlled environment (temperature: 23±1 ºC; relative humidity: 50-70 %) with 12/12h day/night schedule (lights on at 7.00 h) and were fed on a commercial rat chow (Charles River Laboratories, Hungary) and had free access to tap water.

Experiments were carried out between 9-12h a.m., in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine.

Experimental design. Experiment 1. In the first phase of the study, to get an adequate dose for the later experiment, we performed a pilot experiment on 10-day-old Wistar rats using 0-0.3-1-3 mg/kg WIN (Tocris; 1µl/g) dissolved in dimethylsulfoxide (DMSO, vehicle, V). The doses of the compound, the route of administration as well as the time were chosen on the basis of the literature and was not toxic, e.g. Borcel et al. (2004) and Martin-Calderon (1998). In fact, there were no data available about the effect of WIN

on the ACTH levels in 10-day-old rats, but it seemed that 60 min is needed for the development of nociceptive effect (BORCEL et al. 2004), since also MARTIN-CALDERON et al. (1998) used the same timing for studying the effect of another CB1 receptor agonist, HU-210. The pups got an intraperitoneal (i.p.) injection than they were returned back to their homecage/mother and decapitated 1h later. The trunk blood was collected on 20 µl 20 % K_2-EDTA (ethylene diamine tetra-acetic acid) containing ice-cold Eppendorf tubes. After centrifugation (3000 rpm/min for 20 min at -4 °C) plasma was frozen at -20 °C until hormone measurement (n=7-11).

Experiment 2 was done on 10-day-old Brattleboro pups. In each litter half of the pups was injected i.p. with WIN (6 mg/kg, 1 µl/g body weight; dissolved in V) while the other half was injected with the same amount of V. After injection all pups was returned to their dam and decapitated 1 h later. Trunk blood was collected as described above and whole pituitary was put in 100 µl 0.1 N HCl and stored there at -20 °C until homogenization for AVP measurement (n=14-18).

Experiment 3 was done on adult Brattleboro rats. The genotype of the rats was inferred upon their water consumption in their 4 week old age, which means they were separated for 3-4 days, than were kept 3-4 rats/cage until experiment. The animals were separated (one animal per cage) for 1 week and weighed on the day before the experiment. On the day of experiment rats were injected i.p. with 6 mg/kg WIN or V and 1 h later they were decapitated. Trunk blood was collected on K_2-EDTA into ice-cold tubes (n=15-18).

Experiment 4 was done in adult Wistar rats to study the strain dependent effect of WIN, as the WIN was able to induce only a small ACTH and corticosterone rise in adult Brattleboro rat. The animals were kept one/cage 1 week prior the experiment and were weighed the day before the experiment. V or 6 mg/kg WIN were injected i.p. and the animals were killed by decapitation 1 h later. Trunk blood was collected on ice-cold, K_2-EDTA containing tubes (n=9-10).

Hormone assays. Plasma ACTH was measured by radioimmunoassay (RIA) in 50 µl unextracted plasma as described earlier (BARNA et al. 2004; ZELENA et al. 1999). The ACTH antibody was raised in rabbit in the Institute of Experimental Medicine, Hungarian Academy of Sciences (Budapest, Hungary) and was directed against the middle part of the h-ACTH-1–39 molecule. The intra-assay coefficient of variation was 7.23 %. All the samples from a particular experiment were assessed in the same RIA.

Plasma corticosterone was measured in 10 µl unextracted plasma by a RIA using a specific antibody developed in our Institute as described earlier (ZELENA et al. 2003). The corticosterone antibody was raised in rabbits against B-carboximethyloxime bovine serum albumin. The intraassay coefficient of variation was 7.5 %. All the samples from a particular experiment were measured in one RIA.

AVP content was also measured by RIA. Pituitaries were collected on 100 µl 0.1 N HCl and were frozen to -20 °C. After thawing, pituitaries were boiled for 5 min, after which they were ultrasonically homogenized and refrozen. On the next day, samples were thawed and centrifuged for 20 min at 3000 rpm. The supernatant was stored at -20 °C till hormone measurements. The rabbit anti-AVP antiserum was obtained from dr. M. Vecsernyés (Szent-Györgyi Medical University, Szeged, Hungary). The intra- and inter-assay coefficients of variation were 10.7 and 17.7 %, respectively.

Statistical evaluation was performed by the STATISTICA 6.0 Software (Statistica Inc., Tulsa, USA). Data (expressed as mean±SEM) were analyzed by factorial ANOVA. Only statistically significant effects and interactions were mentioned in the results. The Newman-Keuls test was used for post-hoc comparisons. $P<0.05$ was considered statistically significant.

Results

The effect of WIN 55,212-2 in pups. The preliminary experiment on Wistar pups (Table 1) revealed that the smaller doses of WIN (0.3 or 1 mg/kg) did not in-

Table 1
Effect of WIN 55,212-2 in 10-day-old Wistar pups

	V	WIN 55,212-2		
		0.3mg/kg	1mg/kg	3mg/kg
ACTH (fmol/ml)	35.5±3.2	30.4±1.6	35.2±5.0	71.9±21.1*

*$p<0.05$ vs. vehicle treated control

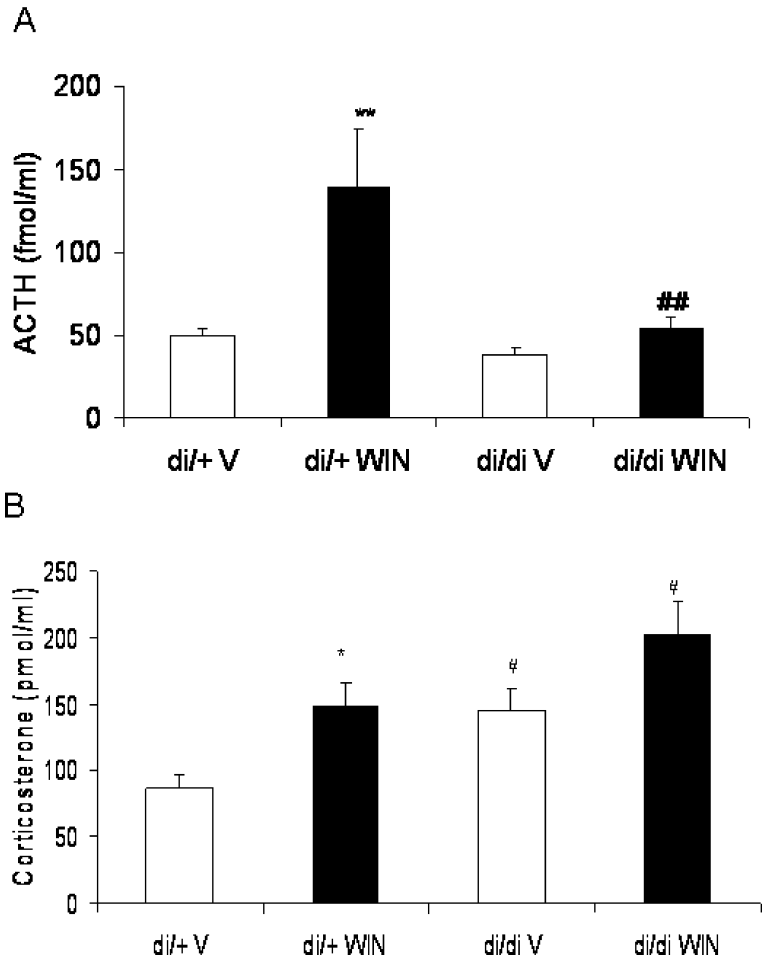

Fig. 1 (A) ACTH (fmol/ml) and (B) corticosterone (pmol/ml) plasma levels in 10-day-old Brattleboro pups 1 h after i.p. injection of 6mg/kg WIN 55,212-2. *p<0.05; **p<0.01 vs. vehicle treated control; #p<0.05; ##p<0.01 vs. di/+ pups of the same treatment

duce significant ACTH elevation 1 h after its i.p. administration, while the highest dose used (3 mg/kg) significantly elevated it (approx. 2-fold; p=0.04) [F(3,33)=2.7, p=0.06]. As we wanted to be sure that the ACTH elevating effect will be present we have chosen 6 mg/kg for later studies.

The genotype of the Brattleboro pups were determined at the end of the experiments upon their pituitary AVP content (di/+: 25.6±1.7 ng/pituitary; di/di: 0.44±0.07 ng/pituitary). The AVP levels of di/di rats were under the detection limit and were significantly lower than those for di/+ pups [F(1,65)=174.1, p<0.01].

The i.p. administration of WIN resulted in a significant ACTH elevation at 60 min (approx. 2.8-fold; Fig.1A) [treatment: F(1,65)=8.5, p<0.01]. The AVP-deficient pups had lower ACTH levels in all studied groups with no remarkable effect of WIN revealed by significant treatment*genotype interaction [genotype: F(1,65)=7.1, p<0.01; treatment*genotype: F(1,65)=4.1, p=0.047].

Surprisingly, the accompanied corticosterone levels showed different pattern (Fig.1B). Administration of WIN resulted in a significant elevation of the plasma corticosterone levels in both genotypes (di/+: 1.7-fold; di/di: 1.4-fold) without treatment*genotype interaction [treatment: F(2,97)=19.9, p<0.01]. However the vehicle treated AVP-deficient rats showed elevated corticosterone levels, which was similar to WIN-injected di/+ groups (about 1.5-fold increase in both case) [genotype: F(1,97)=14.6, p<0.01].

Effect of WIN 55,212-2 in adults. The genotype of the adult Brattleboro rats was inferred in 4-week-old

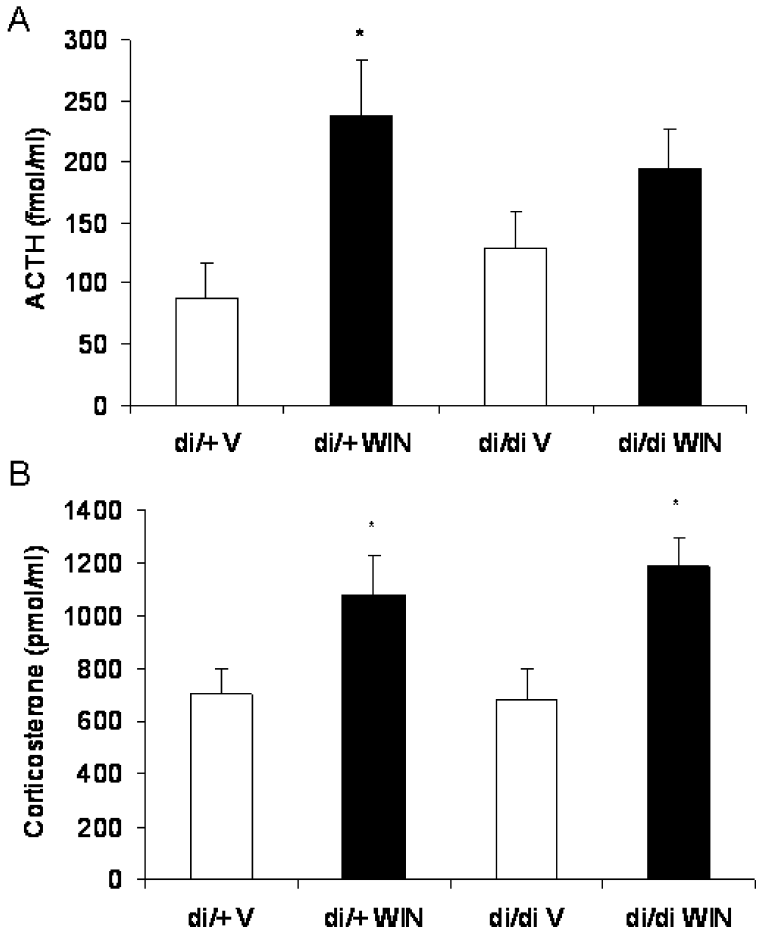

Fig. 2 (A) ACTH (fmol/ml) and (B) corticosterone (pmol/ml) plasma levels in adult Brattleboro rats 1 h after i.p. injection of 6mg/kg WIN 55,212-2. *p<0.05 vs. vehicle treated control

animals upon the water consumption, which was approximately 3-times more in di/di rats than in their heterozygous littermattes (di/+: 20.7±0.7ml/rat; di/di: 58.9±2.2ml/rat) [$F(1,65)=274.2$, $p<0.01$].

In Brattleboro strain the i.p. WIN injection was able to induce significant ACTH rise in both genotype without any effect of the AVP-deficiency (Fig. 2A) [treatment: $F(1,60)=9.7$, $p<0.01$; no further significance]. However in control, di/+ animals the ACTH elevation was 2.7-fold, while in di/di only 1.5-fold.

Similarly to the ACTH levels the WIN-induced corticosterone rises (approx. 1.5-fold) was also independent from the presence or absence of AVP (Fig. 2B) [treatment: $F(1,63)=14.5$, $p<0.01$].

In adult Wistar animals the i.p. injection of WIN elevated both the ACTH (14-fold) and corticosterone (3.6-fold) levels 60 min after its administration. (Fig. 3) [ACTH: $F(1,17)=76.1$, $p<0.01$; corticosterone: $F(1,18)=39.9$, $p<0.01$].

Discussion

The injection of the cannabinoid CB1 receptor agonist WIN was able to stimulate the HPA axis both in 10-day-old offspring and adult as could be seen on plasma ACTH and corticosterone levels. The mediator of this phenomenon seems to be the AVP during the perinatal period however its importance diminished in later life.

There is substantial evidence indicating that cannabinoid receptor agonists induce a CB1-receptor mediated activation of the HPA axis in both adult (MANZA-

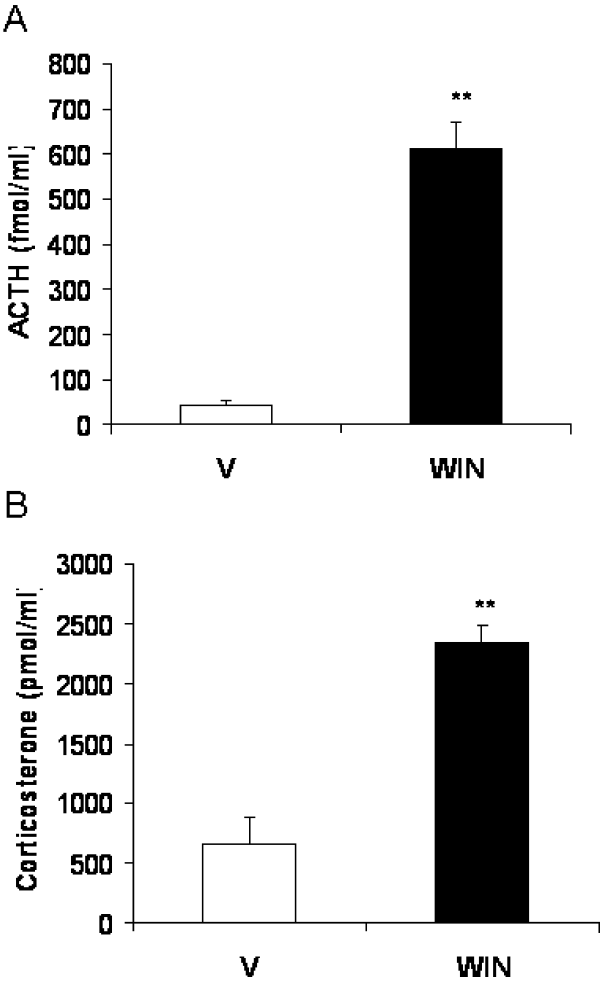

Fig. 3 (A) ACTH (fmol/ml) and (B) corticosterone (pmol/ml) plasma levels in adult Wistar rats 1 h after i.p. injection of 6mg/kg WIN 55,212-2.*p<0.05; **p<0.01 vs. vehicle treated control

NARES et al. 1999) and juvenile rats (ROMERO et al. 2002). However a previous work of BORCEL et al. (2004) indicated that at postnatal day 20 the cannabinoid system of the rat is immature and did not respond to 3 mg/kg WIN (BORCEL et al. 2004). This later finding is in contrast to our work as we were able to find a significant ACTH as well as corticosterone elevating effect of 3-6 mg/kg WIN in the 10-day-old rat. An explanation could be that BORCEL et al. (2004) measured the hormone levels at the end of an open field test, so the additional stress may confound the interpretation. Moreover they measured only corticosterone, which is know to react with smaller rises during perinatal period and we also demonstrated that the WIN-induced ACTH elevation was higher (pretty near 3-fold) than the corticosterone rise (approx. 1.5-fold). On the other hand, it was concluded with another CB1 receptor agonist (HU-210) that there were no functional CB1 receptors in the offspring up to 40 days of age, which could react with HPA axis changes to an exogenous cannabinoid (MARTIN-CALDERON et al. 1998). We could speculate that WIN might have CB1 receptor-independent function (HOWLETT 2002). CB2 has also been recently described in central nervous system and could account for some previously reported cannabinoid-mediated effects (ONAIVI et al. 2006). Increasing pharmacological evidence also suggest that other putative cannabinoid receptors coexist within CNS, e.g. the novel described GPR55 (that, however, do not bind WIN) (SAWZDARGO et al. 1999). Moreover, anandamide, the first described endocannabinoid, has been reported to activate HPA axis increasing both ACTH and corticosterone by an unknown cannabinoid receptor (WENGER et al. 2003). The known CB1 and the unidentified CBx receptors apparently share common activities in the brain, as e.g. regulating the CRH-erg cells in the nuclues paraventricularis hypothalami (KOFALVI et al. 2003, WENGER et al. 1997).

The central mediation of cannabinoid effects on HPA-axis is supported by several studies. In hypothalamic slice preparations WIN mimicked the HPA axis suppressing effects of glucocorticoids (DI et al. 2003), additionally in an in vitro system it was completely ineffective on the pituitary ACTH release as well as on the glucocorticoid feedback at the same level (BARNA et al. 2004). Our data add further detail as we were able to demonstrate that the AVP-erg neurons plays a crucial role in the cannabinoid mediated HPA axis regulation, which effect seems to be age dependent. There are several possible sites in the brain where AVP may interact with the cannabinoid receptors. As the PVN thought to be the main regulator of the HPA and has a large amount of AVP, it is the more thinkable nucleus (DI et al. 2003). Although the magnocellular part of the PVN contains larger amount AVP but the fewer AVP in the parvocellular part supposed to have a more prominent role in the HPA axis regulation (ANTONI 1993; WOTJAK et al. 2002). Supporting this theory it was already established, that systemic application of anandamide lead to c-fos elevation in the parvocellular PVN (WENGER et al. 1997). However we cannot close out the possibile involvement of other brain areas as well (e.g. septum or amygdala, (GERST-

BERGER et al. 1989; TSOU et al. 1998). Moreover one can hypothesize that endocannabinoid signalling in different brain structures plays different roles in the control of HPA axis.

It is known that the HPA axis function is changing with age. For example in aged rats the recovery after stress is delayed (SAPOLSKY et al. 1984). However in our hands both in young and adult Brattleboro rats the i.p. administration of 6mg/kg WIN resulted in similar HPA axis elevation (ACTH: 2.8-2.7-fold increase; Corticosterone: 1.7-1.5-fold increase). The contribution of different peptides in the HPA axis regulation can be also changed. It was demonstrated that the regulation of hypothalamic CRH gene expression was not mature during the perinatal period (GRINO et al. 1989), whereas the regulation of hypothalamic AVP gene expression matured very early (TRIBOLLET et al. 1991). In the offspring the insulin-induced hypoglycemia increased the ACTH and corticosterone secretion via AVP mediation (MURET et al. 1992). Our results support the theory that during the perinatal period the AVP is the predominant secretagogue of the ACTH secretion in the rat. In adult rat the relative importance of CRH and AVP in the regulation of the HPA activity is not so clear. Although several work suggested that the HPA axis regulatory role of AVP became more important during chronic stimuli, we failed to confirm this statement in our previous works (MAKARA et al. 2004, ZELENA et al. 2006). Moreover, we found that the role of AVP is more prominent, although not exclusive, in acute stress situations. With WIN we were able to demonstrate only a slight tendency for diminished ACTH secretion in AVP deficient adult animals. Therefore we conclude that the role of AVP in the cannabinoid-mediated HPA axis stimulation diminishes with age. However we have to take into consideration the development of compensatory mechanisms as well (eg. elevated CRH mRNA levels in PVN of adult Brattleboro rats (MLYNARIK et al. 2007)).

The different peptides may have species dependent effect as well. According to the general assumption the main ACTH secretagogue in adult rat is the CRH while in sheep the AVP (ANTONI 1993). As there are several studies confirming differences in the HPA axis of rat or mice strains, for instance that by REDEI et al. (1994), we assumed that there might be a difference between the cannabinoid sensitivity of generally used Wistar rats and the Brattleboro rats used in our study. Our result confirmed our hypothesis as we found that in Wistar rats the same amount of WIN induced approximately 5-fold higher ACTH elevation than in Brattleboro rat. The parallel corticosterone elevation was 2.5-fold higher in Wistars, probably as a consequence of the different time-course of the two hormone. The background of this phenomenon could be some differences in the number, affinity or distribution of the CB1 (or CBx) cannabinoid receptors in the two strain.

The discrepant ACTH and corticosterone levels in pups were also surprising and suggested a local, ACTH-independent regulation of the corticosterone secretion. It might be that these signals are present also among normal conditions, but are covered by the main regulator, ACTH. In the absence of the main signal, however, a minor cannabinoid-dependent regulation may come into highlight. It was already demonstrated, although in rabbit, that from whole isolated adrenal glands the in vitro adrenalin release was dose dependently decreased by WIN (NIEDERHOFFER et al. 2001). The strong correlation between stress-induced catecholamine and HPA axis regulation led us to hypothesize, that local cannabinoid-mediated regulation may be involved also in the regulation of corticosterone secretion, either directly or through regulation of the medullary adrenalin release. Actually there have been reports of CB1 expression in the adrenal gland, at least during embryonic stage (BUCKLEY et al. 1998). We cannot exclude a presence a yet unidentified cannabinoid receptor subtype, too (KOFALVI et al. 2003).

In the present study we were able to demonstrate an age and strain dependent cannabinoid-mediated HPA axis activation, which – at least in pups- strongly dependent on the presence of AVP. In the absence of ACTH a local cannabinoid-dependent regulation may come to the front to stimulate the glucocorticoid secretion from the adrenal gland.

Acknowledgements

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