LONG-TERM ALTERATIONS IN THE ENDOCANNABINOID SIGNALING SYSTEM IN BRAINS OF ADULT RATS SUBJECTED TO SOCIAL ISOLATION AND HANDLING

by

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(Under the Direction of Andrea G. Hohmann)

ABSTRACT

Social isolation, in rodents, produces psychopathological features that are reversed by handling. However, the neurochemical mechanisms underlying isolation rearing and handling remain poorly understood. Moreover, whether handling alters the endocannabinoid system (eCBS) is unknown. Therefore, we examined whether isolation rearing and handling alter the eCBS. We also evaluated whether handling would reverse isolation-induced eCBS perturbation. At weaning, rats were isolation or group reared and concomitantly handled or non-handled daily until adulthood. Cannabinoid receptor densities and endocannabinoid content were measured in brains from these rats using [^{3}H]CP55,940 binding and quantitative autoradiography and lipid analysis. Isolation rearing altered cannabinoid receptor densities and endocannabinoid content. Handling altered both cannabinoid receptor densities and content in regions that control emotional expression compared to non-handling. Unlike group-, isolation-reared rats failed to exhibit handling-induced increases in both receptor densities and content. These data further implicate a pivotal role for the endocannabinoid system in adaptation to stress.

INDEX WORDS: Endocannabinoid, Social Isolation, Handling, Anxiety, Stress, Anandamide, 2-Arachidonoylglycerol

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DEDICATION

To BAW and JDP, thank you for your unfaltering belief in me.

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CHAPTER 1: INTRODUCTION

Rearing rats in isolation is an animal model of social deprivation that recapitulates features of limbic-based psychopathology in humans. Post-weaning social isolation models aspects of anxiety disorders (Haller and Halasz, 1999, Lukkes et al., 2009), substance abuse (Hall et al., 1997, Howes et al., 2000, Advani et al., 2007), and schizophrenia (Varty et al., 1999a, Schubert et al., 2009). Rodents reared in deprivation of social contact exhibit an abnormal behavioral phenotype that includes hyperlocomotion in response to a novel environment (Sahakian et al., 1982, Hall et al., 1998a), altered habituation (Einon and Morgan, 1976, Gentsch et al., 1982), and disrupted exploratory behaviors (Paulus et al., 2000, Varty et al., 2000).

Isolation rearing produces schizophrenia-like features, including deficits in sensorimotor gating (Geyer et al., 1993, Powell et al., 2002) and increases in aggression (Wongwitdecha and Marsden, 1996, Toth et al., 2008) and avoidance (Petkov and Rousseva, 1984, Del Arco et al., 2004). Brains derived from schizophrenia patients show altered cytoarchitectural and volumetric changes in the hippocampus and prefrontal cortex (for review see McGlashan and Hoffman, 2000), cortical regions that regulate social cognition (Gur et al., 2000, Venkatasubramanian et al., 2008). These anatomical changes are mirrored in isolation-reared rats. Brains derived from socially-isolated animals exhibit reductions in dendritic spine densities in the prefrontal cortex and hippocampus (Silva-Gomez et al., 2003). Isolates also exhibit lower levels of the synaptic marker synaptophysin in the dentate gyrus (Varty et al., 1999b) and reduced

brain volume in the prefrontal cortex (Schubert et al., 2009). Isolation-reared rats exhibit increased dopamine D_2 receptor binding in rat striatum (Guisado et al., 1980, King et al., 2009), nucleus accumbens, amygdala, and substantia nigra pars compacta (Djouma et al., 2006). These observations are in line with increased striatal dopamine and D_2 receptors in schizophrenics (Abi-Dargham et al., 2000).

Isolation rearing alters dopaminergic activity (see also Bardo and Hammer, 1991, Del Arco et al., 2004, Malone et al., 2008). Isolates display hypodopaminergia in the mesocortical dopamine system, as evidenced by decreased dopamine metabolite 3,4-dihydroxyphenylacetic acid to dopamine ratio (Heidbreder et al., 2000, Miura et al., 2002). However, treatment with atypical, but not typical, antipsychotics increases dopamine responsiveness in the medial prefrontal cortex in isolated rats (Heidbreder et al., 2001). Isolates also display hyperdopaminergia in the mesolimbic dopamine system (Hall et al., 1998b), as indicated by heightened response to both natural rewards and drugs of abuse (Hall et al., 1998a, Brenes and Fornaguera, 2008) and faster conditioning of appetitive stimuli, relative to controls (Phillips et al., 2002).

Endocannabinoids are lipid-derived neuromodulatory substances that regulate signaling at the interface between dopaminergic, glutamatergic, and gamma-aminobutyric acid (GABA)-ergic transmission (for review see Katona and Freund, 2008). The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) bind to presynaptic CB_1 receptors to modulate synaptic strength. CB_1 receptors moderate synaptic transmission by curbing GABA and glutamate release (Misner and Sullivan, 1999, Ohno-Shosaku et al., 2001, Straiker and Mackie, 2005). The endocannabinoid signaling system thus regulates dopamine transmission both *indirectly*, by preventing glutamate and

GABA release onto dopaminergic neurons, and *directly* by potentially forming CB_1-D_2 receptor heteromers (for review see Ferre et al., 2009).

Dysregulation of the endocannabinoid signaling system – comprised of cannabinoid receptors, their endogenous ligands, and endocannabinoid-metabolizing proteins – is implicated in disturbances of emotion and stressor responsiveness (Hill et al., 2005, Eisenstein et al., 2009). Schizophrenics exhibit increased cannabinoid receptor densities in the dorsolateral prefrontal cortex (Dean et al., 2001) and anterior and posterior cingulate gyrus (Zavitsanou et al., 2004, Newell et al., 2006), but not in the superior temporal gyrus (Deng et al., 2007). Furthermore, acute schizophrenics demonstrate elevated anandamide levels in cerebrospinal fluid (Leweke et al., 1999, Giuffrida et al., 2004) and blood (De Marchi et al., 2003). Cannabinoid receptor activation induces behavioral deficits in sensorimotor gating and deficits in the medial prefrontal cortex and hippocampus (Fernandez-Espejo and Galan-Rodriguez, 2004, Ballmaier et al., 2007, Dissanayake et al., 2008); These effects are reversed by CB_1 receptor antagonists (see also Martin et al., 2003, Malone et al., 2004). These data suggest that the endocannabinoid signaling system is perturbed in humans that suffer from the developmental disorder schizophrenia as well as other affect-related disturbances.

The effects of early life adversity on the endocannabinoid signaling system in the brain remain poorly understood. In the present study, we evaluated whether this signaling system undergoes long-term alterations in brains of adult rats subjected to post-weaning social isolation rearing. In socially deprived rodents, chronic daily experimenter handling attenuates anxiety-like behavior (Gentsch et al., 1988), induces stress-protective

effects (Plotsky and Meaney, 1993, Krebs-Thomson et al., 2001), and parallels brain changes produced by environmental enrichment (Szeligo and Leblond, 1977). However, whether handling treatment changes endocannabinoid levels and cannabinoid receptor densities is unknown.

CHAPTER 2: MATERIALS AND METHODS

Post-weaning social isolation and chronic handling

Subjects were 32 male Sprague Dawley rats. Subjects were derived from timed pregnant female Sprague Dawley rats (Charles River Laboratories, INC, Wilmington, MA) that were received 14 days post-gestation. Dams were monitored daily to determine the date of parturition (University of California, Irvine, CA). Pups remained undisturbed and co-housed with their dam and siblings from birth until weaning. On postnatal day 21, male offspring were used for experimentation and were transferred to single (1 rat/42 x 22 x 20 cm cage) or group (5 rats/52 x 28 x 20 cm cage) housing until brain extraction 7 weeks later. Rats were given *ad libitum* access to food and water and cages were cleaned and refilled with sawdust bedding weekly. All rats were kept in a shared holding room under a 12:12 light:dark cycle to ensure similar sensory experience (i.e., shared visual, auditory, and some olfactory cues). Single housing prevented, while group housing permitted cohort-induced tactile stimulation. Half of the single- and group-reared rats were subjected to experimenter-induced tactile stimulation by exposure to 5 minutes of daily handling on postnatal days 21 – 70.

Tissue preparation

Isolation- and group-reared rats that were either handled or non-handled (n = 8/group) were decapitated on postnatal day 70. Brains were rapidly dissected, and snap frozen in precooled isopentane (-30°C). Brains were stored at low temperature (-30°C and -80°C) until use. One hemisphere was used to measure cannabinoid receptor

densities and distribution using [^{3}H]CP55,940 binding and quantitative autoradiography and the other hemisphere was used to obtain tissue punches for endocannabinoid quantification using liquid chromatography/mass spectrometry (LC/MS).

Receptor binding and autoradiography

Coronal brain sections (14 µM thickness) were cryostat cut and mounted four sections per slide. Cannabinoid receptor binding was performed using [^{3}H]CP55,940 (specific activity 139.6 Ci/mmol; Research Triangle Institute, Research Triangle Park, NC, USA) as described previously (Herkenham et al., 1991, Hohmann and Herkenham, 1998, Hohmann et al., 1999). Nonspecific binding was determined in the presence of 10 µM CP55,940. Briefly, binding was performed in cytomailers (3 h at 37 ˚C) in 50 mM Tris-HCl (pH 7.4) containing 5% bovine serum albumin and either 4.6 or 3.3 nM [^{3}H]CP55,940. Binding assays were performed by neuroanatomical level of section, so that all animals in all four experimental groups were processed concurrently in the same assay. Slides were washed (4 h at 0 ˚C) in the same buffer containing 1% bovine serum albumin, fixed in 0.5% formalin in 50 mM Tris-HCl (pH 7.4 at 25 ˚C) and blown dry. Sections were apposed to [^{3}H]-sensitive film (Amersham Hyperfilm, GE Healthcare LifeSciences, Piscataway, NJ) together with [^{3}H] standards ([^{3}H] microscales, Amersham, Arlington Heights, IL, USA) for 8 weeks for levels incubated in 4.6 nM [^{3}H]CP55,940 and 9 weeks for levels incubated in 3.3 nM [^{3}H]CP55,940. Images were captured using a ScanMaker 9800XL scanner (Microtek, Cerritos, CA, USA).

Densitometry

Densitometry was performed using the public domain NIH Image software (U.S. National Institutes of Health, http://rsb.info.nih.gov/nih-image/) using a Macintosh

computer (Macintosh, Cupertino, CA, USA). The mean densities for relevant brain regions of the scanned tissue images were calculated and converted to nCi/mg tissue wet weight based upon a best-fit 3rd degree polynomial calibration formula that incorporates tissue equivalent values provided by Amersham. Brain areas were outlined using the rat brain atlas (Paxinos and Watson, 1998). The nCi/mg values for tissue sections for each animal were calculated. Densitometry measurements were determined separately for total and nonspecific binding in each rat by averaging values obtained from 3-4 near adjacent sections. Nonspecific binding (determined in sections adjacent to total binding sections) was subtracted from total binding values to obtain specific binding values used in data analysis. Receptor densities were calculated within groups by averaging specific binding values across rats.

Lipid extractions

Punches derived from single-hemisphere frozen brains were homogenized in methanol (0.3 mL) containing [$^2H_4$]-anandamide and [$^2H_8$]-2-AG (Cayman Chemicals, Ann Arbor, MI, USA) as internal standards. Protein concentration was determined in the homogenate to normalize samples using the bicinchinonic acid protein assay (Pierce, Rockford, IL). Tissue was punched according to distance from bregma using the rat brain atlas of Paxinos and Watson (1998) as a guide. The level of section and dimensions of punches collected for selected structures of interest were as follows: hippocampus, (-2.3 mm anterior-posterior (AP), +1 mm medial-lateral (ML), -4 mm dorsal-ventral (DV); 2 mm x 2 mm); piriform cortex, (+1.7 mm AP, +4.5 mm ML, -7 mm DV; 2 mm x 1 mm); prefrontal cortex, (+1.7 mm AP, +0.5 mm ML, -3 mm DV; 2 mm x 2 mm; adapted from Marsicano et al. (2002); and nucleus accumbens (+1.7 mm AP, +1 mm ML, -3 mm DV;

2 mm x 2 mm). Protein content in punches derived from hippocampus, prefrontal cortex, and nucleus accumbens averaged 25-30 µg per sample. Punches derived from the piriform cortex averaged 10-15 µg protein per sample. Lipids were extracted with chloroform (2 vol) and washed with water (1 vol). Endocannabinoids were fractionated by open-bed silica gel column chromatography, as previously described (Moise et al., 2008, Astarita and Piomelli, 2009). Lipids were briefly reconstituted in chloroform, loaded onto small glass columns packed with Silica Gel G (60-Å 230-400 Mesh ASTM; Whatman, Clifton, NJ, USA), and washed with 2 ml of chloroform. Anandamide and 2-AG were eluted with 1 ml of chloroform/methanol (9:1, vol/vol). Eluates were dried under N_2 and reconstituted in 50 µL of methanol for LC/MS analyses.

LC/MS analyses

An 1100-LC system coupled to a 1946A-MS detector (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with an electrospray ionization interface was used to measure anandamide and 2-AG levels in each punch sample. Lipids were separated using a XDB Eclipse C18 column (50 x 4.6 mm i.d., 1.8 µm, Zorbax), eluted with a gradient of methanol in water (from 75% to 85% in 2.5 min, to 90% in 7.5 min, to 100% in 14 min, and to 75% in 20 min) at a flow rate of 1.0 ml/min. Column temperature was kept at 40˚C. MS detection was in the positive ionization mode, capillary voltage was at 3 kV, and fragmentor voltage varied from 120V. N_2 was used as drying gas at a flow rate of 13 liters/min and a temperature of 350 ˚C. Nebulizer pressure was set at 60 PSI. Quantifications were conducted using an isotope-dilution method (Moise et al., 2008, Astarita and Piomelli, 2009) by monitoring Na^+ adducts of the molecular ions ($[M+Na]^+$)

in the selected ion-monitoring mode. Quantification limits were 0.08 pmol for anandamide and 0.4 pmol for 2-AG.

Statistical Analysis

Homogeneity of variance and group normality were validated using the Levene and Kolmorgov-Smirnoff statistics, respectively. A separate two-way (Rearing x Handling) independent analysis of variance was performed on each structure for LC/MS and densitometry data analysis. Tukey post hoc tests were performed to identify the source of significant interactions. Planned comparisons (two-tailed, independent samples *t*-tests) were used to compare the effects of rearing on endocannabinoid content in non-handled and handled rats separately. Planned comparisons that did not meet the equal variance assumption were corrected for by fractional adjustment of the degrees of freedom. Classic eta squared (η^2) effect size calculations were performed to additionally gauge the amount of variance that our manipulations accounted for in dependent measures that were evaluated. Using Cohen's standards, eta squared values above 0.0099, 0.058 , and 0.1379 can be considered small, medium, and large effects, respectively (Cohen, 1998), although limitations of these stated criteria (e.g., overestimation of population association, dependence upon sample size) must also be acknowledged (Levine and Hullett, 2002, Pierce et al., 2004). Eta squared calculations were calculated using the formula $\eta^2 = SS_{\text{factor}}/SS_{\text{total.}}$ All other analyses were performed using SPSS statistical software (version 16.0; SPSS Incorporated, Chicago, IL, USA).

CHAPTER 3: RESULTS

Isolation rearing alters cannabinoid receptor densities

Percent specific binding, averaged across films, was 94% (S.D. ± 1.35), documenting the high sensitivity of the binding and autoradiographic methods employed here. Post-weaning social isolation differentially altered cannabinoid receptor densities in the rostral caudate putamen and thalamus. Isolation rearing increased cannabinoid receptor densities in the dorsomedial (by 19%) ($F_{1,28} = 4.24$, $P < 0.05$; $\eta^2 = 0.010$; Fig. 1a) and ventrolateral (by 24%) caudate putamen ($F_{1,28} = 4.21$, $P = 0.05$; $\eta^2 = 0.016$; Fig. 1b) compared to group rearing (see also Fig. 1c – f). However, isolation rearing decreased cannabinoid receptor densities in the supraoptic hypothalamic nucleus (33%) ($F_{1,21} = 5.89$, $P < 0.05$; $\eta^2 = 0.016$; Fig. 2a) and ventrolateral thalamic nuclei (19%) ($F_{1,24} = 4.56$, $P < 0.05$; $\eta^2 = 0.007$; Fig. 2b) compared to group rearing (see also Figs. 5 – 6).

Handling alters cannabinoid receptor densities

Daily handling produced site-specific changes in cannabinoid receptor densities within the limbic input-output loop of the basal ganglia. Handling increased cannabinoid receptor densities in basal ganglia output nuclei (lateral globus pallidus) as well as allocortical areas (cingulate and piriform cortex) of the limbic loop, but decreased receptor densities in archicortex (hippocampus), relative to non-handling (see Figs. 5 – 6). Handled rats exhibited increased cannabinoid receptor densities in the lateral globus pallidus (by 21%) ($F_{1,23} = 6.57$, $P < 0.05$; $\eta^2 = 0.012$; Fig. 3a), cingulate cortex (by 24%) ($F_{1,23} = 10.06$, $P < 0.005$; $\eta^2 = 0.018$; Fig. 3b), and piriform cortex (by 21%) ($F_{1,23} = 4.79$,

$P < 0.05$; $\eta^2 = 0.012$; Fig. 3c) relative to non-handled controls. By contrast, handling decreased cannabinoid receptor densities in molecular regions CA1 – 3 and the dentate gyrus of the hippocampus (by 14%) ($F_{1,24} = 4.38$, $P < 0.05$; $\eta^2 = 0.004$; Fig. 3d). Subsequent analysis of hippocampal regions revealed that handled rats exhibited decreased cannabinoid receptor binding densities in the CA2 region (by 20%) ($F_{1,24} = 5.95$, $P < 0.05$; $\eta^2 = 0.008$) and, to a lesser extent, in the CA1 – 3 regions (by 14%) ($F_{1,24} = 4.10$, $P = 0.05$; $\eta^2 = 0.004$), compared to non-handled rats.

Handling altered cannabinoid receptor binding densities at caudal levels of the caudate putamen ($F_{1,23} = 8.59$, $P < 0.01$; $\eta^2 = 0.015$; Fig. 4a), specifically in the dorsal caudate putamen ($F_{1,23} = 4.80$, $P < 0.05$; $\eta^2 = 0.014$), as well as in the anterior thalamus ($F_{1,23} = 7.15$, $P = 0.01$; $\eta^2 = 0.005$; Fig. 4b) in a manner that was dependent on rearing conditions. Handling increased cannabinoid receptor binding densities in these regions in group-reared rats (by 40%, 35%, and 26% in caudal caudate putamen, caudal dorsal caudate putamen, and anterior thalamus, respectively). By contrast, handling failed to alter cannabinoid receptor binding densities in these same brain regions in isolation-reared rats ($P > 0.05$ for all comparisons; Fig. 4). No change in [^{3}H]CP55,940 binding densities were observed in any other structures examined (see Table 1).

Isolation rearing increases endocannabinoid content in a region and ligand specific manner

Isolation rearing produced regionally-restricted changes in brain endocannabinoid levels that were dependent on prior handling treatment (Fig. 7a – l). In non-handled rats, isolation rearing increased 2-AG ($t_{8,24} = -3.71$, $P = 0.006$; Fig. 7b), but not anandamide ($P > 0.05$; Fig. 7c), levels in the prefrontal cortex compared to group rearing. However,

isolation rearing did not alter 2-AG levels (t_{13} = -3.71, P = 0.413; Fig. 7b) in the prefrontal cortex in animals subjected to daily handling, relative to group rearing. In non-handled rats, isolation rearing also increased both 2-AG ($t_{8.06}$ = -2.48, P < 0.05; Fig. 7h) and anandamide (t_{14} = -2.20, P < 0.05; Fig. 7i) levels in the piriform cortex compared to group rearing. However, isolation rearing did not alter endocannabinoid levels in the nucleus accumbens (P > 0.05; Fig. 7d – f) or the hippocampus (P > 0.05; Fig. 7j – l).

Handling alters endocannabinoid content in a region and ligand specific manner

In the prefrontal cortex, handling markedly increased 2-AG levels ($F_{1,26}$ = 22.51, P = 0.00; η^2 = 0.175; Fig. 7b) in both isolation- and group-reared rats, but did not reliably alter anandamide levels (P = 0.43; Fig. 7c), compared to non-handling. Handling also increased anandamide levels in the nucleus accumbens ($F_{1,28}$ = 5.03, P < 0.05; η^2 = 0.081; Fig. 7f), without altering levels of 2-AG (P = 0.11; Fig. 7e), relative to non-handling. However, handling decreased anandamide levels in the piriform cortex ($F_{1,28}$ = 4.83, P < 0.05; η^2 = 0.021; Fig. 7i) relative to non-handling. In the piriform cortex, the ability of handling to increase 2-AG levels was dependent upon rearing condition ($F_{1,28}$ = 13.13, P = 0.001; η^2 = 0.025; Fig. 7h). Handling increased 2-AG levels in the piriform cortex in group- (P < 0.05), but not isolation-reared rats (P > 0.05), relative to non-handling. By contrast, endocannabinoid levels in the hippocampus were not affected by handling manipulations (P > 0.05; Fig. 7k – l).

Table 1. [^{3}H]CP55,940 binding to cannabinoid receptors in the adult rat brain after long-term post-weaning rearing and handling manipulations. Data are mean ± S.E.M. (n = 8). caudate putatmen, CPu.

Brain Region	Group-Reared Non-Handled	Group-Reared Handled	Isolation Reared Non-Handled	Isolation Reared Handled
Basal Ganglia/Striatum				
Caudal CPu	13.71 ± 0.87^{++}	22.68 ± 1.90	15.73 ± 1.44^{+}	15.99 ± 1.66^{+}
Caudal dorsal CPu	12.83 ± 0.80^{+}	19.64 ± 2.21	14.27 ± 0.77^{++}	15.08 ± 0.97
Caudal ventral CPu	13.06 ± 0.87	21.13 ± 2.24	16.10 ± 2.28	16.40 ± 3.18
Rostral CPu	16.28 ± 0.91	12.63 ± 1.91	18.42 ± 2.63	17.48 ± 1.42
Rostral dorsolateral CPu	19.35 ± 1.17	15.34 ± 2.36	21.20 ± 3.04	20.64 ± 1.75
Rostral dorsomedial CPu	14.10 ± 0.69	10.60 ± 1.50	16.01 ± 2.15*	14.63 ± 0.99*
Rostral ventrolateral CPu	17.32 ± 0.93	13.93 ± 2.48	20.42 ± 3.26*	20.67 ± 2.31*
Rostral ventromedial CPu	14.56 ± 0.71	11.12 ± 1.65	16.48 ± 2.62	15.64 ± 1.53
Lateral globus pallidus	34.72 ± 4.24	48.72 ± 2.32	35.78 ± 4.25*	39.95 ± 2.20*
Nucleus accumbens core	12.16 ± 0.72	9.13 ± 1.25	13.54 ± 2.49	13.11 ± 1.38
Nucleus accumbens shell	11.39 ± 0.74	8.59 ± 1.39	12.21 ± 2.19	12.65 ± 1.41
Olfactory tubercle	7.57 ± 0.41	5.20 ± 0.71	8.17 ± 1.67	8.48 ± 1.00
Cerebral Cortex				
Cingulate cortex	9.00 ± 0.58	13.09 ± 1.38$^{\#\#}$	9.01 ± 0.47	10.74 ± 0.88$^{\#\#}$
Motor cortex	9.76 ± 0.85	7.55 ± 0.95	10.30 ± 1.58	10.13 ± 0.92
Piriform cortex	6.95 ± 0.47	9.57 ± 1.16$^{\#}$	7.51 ± 0.49	8.62 ± 1.08$^{\#}$
Septum				
Lateral septum	12.47 ± 1.04	9.30 ± 1.56	10.27 ± 1.54	13.48 ± 1.68
Limbic diagonal band nuclei	13.66 ± 0.95	10.56 ± 1.13	12.71 ± 1.23	11.63 ± 1.51
Vertical limbic diagonal band	16.08 ± 0.89	12.67 ± 1.60	14.21 ± 1.52	12.57 ± 1.60
Amygdala				
Basolateral amygdala nuclei	9.08 ± 0.70	9.93 ± 1.02	9.28 ± 1.29	8.12 ± 0.88
Central amygdaloid nuclei	12.04 ± 0.60	11.11 ± 1.19	10.29 ± 1.06	10.07 ± 1.10
Thalamus				
Anterior thalamic nuclei	4.19 ± 0.17^{++}	5.66 ± .047	4.34 ± 0.22^{+}	4.20 ± 0.23^{++}
Arcuate nuclei	5.92 ± 0.47	6.63 ± 1.15	6.01 ± 0.78	6.33 ± 1.67
Medial preoptic area	6.99 ± 0.60	8.44 ± 0.92	7.15 ± 0.87	7.42 ± 0.56
Superoptic nucleus	8.80 ± 0.66	11.25 ± 1.31	7.26 ± 1.02*	7.86 ± 0.77*
Ventrolateral thalamic nuclei	7.16 ± 0.40	6.70 ± 0.58	6.14 ± 0.56*	5.53 ± 0.47*
Hippocampal Formation				
CA1 - CA3 & dentate gyrus	13.74 ± 0.70	10.85 ± 1.02$^{\#}$	12.31 ± 0.74	11.95 ± 0.66$^{\#}$
CA1 - CA3	14.82 ± 0.66	11.67 ± 1.02$^{\#}$	14.07 ± 0.91	13.58 ± 0.96$^{\#}$
CA1	17.39 ± 1.05	12.59 ± 1.54	16.80 ± 1.47	16.76 ± 1.33
CA2	17.91 ± 0.82	13.00 ± 1.85$^{\#}$	15.95 ± 1.21	15.14 ± 0.62$^{\#}$
CA3	18.50 ± 0.79	13.61 ± 2.18	16.37 ± 1.10	17.02 ± 1.45
Dentate gyrus	16.50 ± 0.85	13.50 ± 1.32	13.73 ± 1.25	13.77 ± 0.91

$^{*}P < 0.05$ vs. Group-reared; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. Non-handled; $^{++}P < 0.01$, $^{+}P < 0.05$ vs. Group-reared/handled.

Table 2. Cannabinoid ligand content in the adult rat brain after long-term post-weaning rearing and handling manipulations. Data are mean ± S.E.M. (pmol/mg protein), where n = 8.

Brain Region	Group Reared Non-Handled	Group Reared Handled	Isolation Reared Non-Handled	Isolation Reared Handled
Anandamide				
Hippocampus	4.79 ± 0.54	4.41 ± 0.83	3.64 ± 0.87	3.28 ± 0.60
Piriform cortex	35.07 ± 5.53	32.79 ± 6.50$^{\#}$	51.34 ± 4.91	30.17 ± 4.09$^{\#}$
Prefrontal cortex	14.00 ± 3.80	12.93 ± 7.29	21.09 ± 6.80	11.11 ± 3.02
Nucleus accumbens	6.73 ± 1.22	15.04 ± 5.66$^{\#}$	4.45 ± 0.95	12.41 ± 4.28$^{\#}$
2-Arachidonoylglycerol (2-AG)				
Hippocampus	537.03 ± 45.17	530.75 ± 35.72	619.12 ± 72.90	534.79 ± 77.10
Piriform cortex	257.01 ± 11.27	372.62 ± 28.81^{+}	361.97 ± 40.82	270.18 ± 25.57
Prefrontal cortex	177.72 ± 36.29	1468.36 ± 276.03$^{\#\#\#}$	646.72 ± 121.11	1307.76 ± 271.80$^{\#\#\#}$
Nucleus accumbens	819.73 ± 168.96	628.56 ± 67.52	745.99 ± 84.53	588.29 ± 64.62

*$P < 0.05$ vs. Group Reared; $^{\#\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. Non-Handled; $^{+}P < 0.05$ vs. Group Reared/Handled

Figure 1. Isolation-reared rats show increased [^{3}H]CP55,940 binding to cannabinoid receptors in the (a) dorsomedial and (b) ventrolateral caudate putamen compared to group-reared rats. Representative photomicrographs show [^{3}H]CP55,940 binding in brains of adult rats that were either (top: c, e) group or (bottom: d, f) isolation reared and concomitantly (right: e, f) handled or (left: c, d) not handled daily post-weaning. Sections were collected +1.70 mm from bregma. The rostral caudate putamen was divided into quadrants as previously reported (Hohmann and Herkenham, 2000). dm, dorsomedial; dl, dorsolateral; vm, ventromedial; vl, ventrolateral. The scale bar equals 1 mm. Data are mean ± S.E.M. *$P < 0.05$ vs. Group Reared (ANOVA).

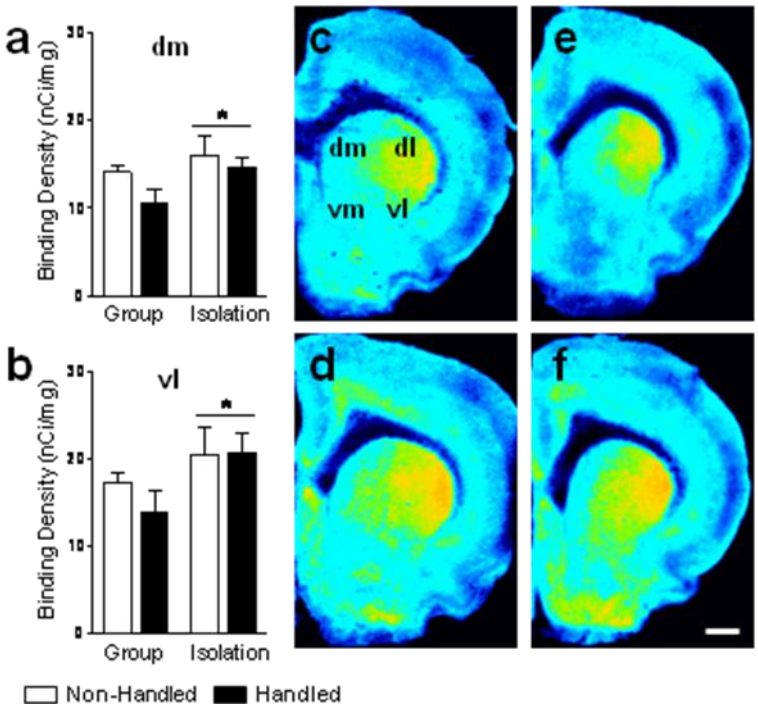

Figure 1. Isolation rearing increases cannabinoid receptor densities in regions of the anterior caudate putamen.

Figure 2. Isolation-reared rats exhibit lower [^{3}H]CP55,940 binding densities in the supraoptic nucleus of the (a) hypothalamus and (b) ventrolateral thalamus compared to group-reared rats. Data are mean ± S.E.M. *$P < 0.05$ vs. Group Reared (ANOVA). Representative photomicrographs are shown in Figures 5 – 6. SO, supraoptic nucleus; vlTN, ventrolateral thalamic nuclei.

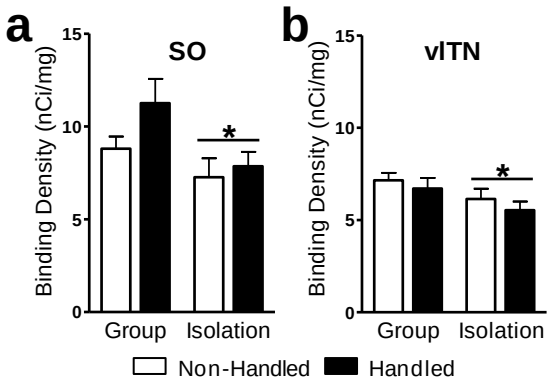

Figure 2: Isolation rearing decreases cannabinoid receptor densities in the supraoptic hypothalamus and venterolateral thalamus

Figure 3. Handled rats show altered [^{3}H]CP55,940 binding in the limbic loop of the basal ganglia. Handled rats exhibit increased binding densities in the (a) lateral globus pallidus, (b) cingulate cortex, and (c) piriform cortex compared to non-handled rats. Handled rats also exhibit decreased binding densities in the (d) hippocampus relative to non-handled rats. Data are mean ± S.E.M. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. Non-Handled (ANOVA). Representative photomicrographs are shown in Figures 5 – 6. Cg, cingulate cortex; Hippo, hippocampus CA 1 – 3 and dentate gyrus; lGP, lateral globus pallidus; Pir, piriform cortex.

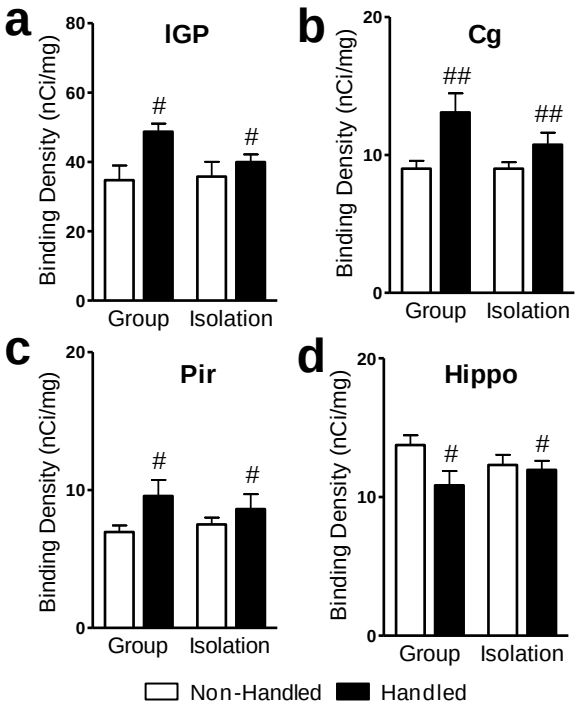

Figure 3: Handled rats show altered [^{3}H]CP55,940 binding in the limbic loop of the basal ganglia.

Figure 4. Isolation-reared rats fail to exhibit handling-induced increases in [^{3}H]CP55,940 binding exhibited by their group-reared counterparts in the (a) caudal caudate putamen and (b) anterior thalamic nuclei. Data are mean ± S.E.M. $^{++}P < 0.01$, $^{+}P < 0.05$ vs. Group Reared/Handled (ANOVA). Representative photomicrographs are shown in Figures 5 – 6. aTN, anterior thalamic nuclei; cCPu, caudal caudate putamen.

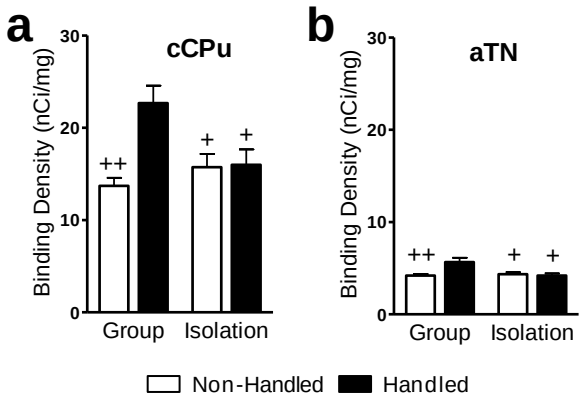

Figure 4: Isolation-reared rats fail to exhibit handling-induced increases in cannabinoid receptor densities in the caudal caudate putamen and anterior thalamus.

Figure 5. Representative photomicrographs showing [^{3}H]CP55,940 binding in brains derived from adult rats that were either (top: a, c) group or (bottom: b, d) isolation reared and concomitantly (right: c, d) handled or (left: a, b) not handled daily post-weaning. Sections were collected -1.30 mm from bregma. aTN, anterior thalamic nuclei; Cg, cingulate cortex; lGP, lateral globus pallidus; Pir, piriform cortex; cCPu, caudal caudate putamen; SO, supraoptic nucleus. The scale bar equals 1mm.

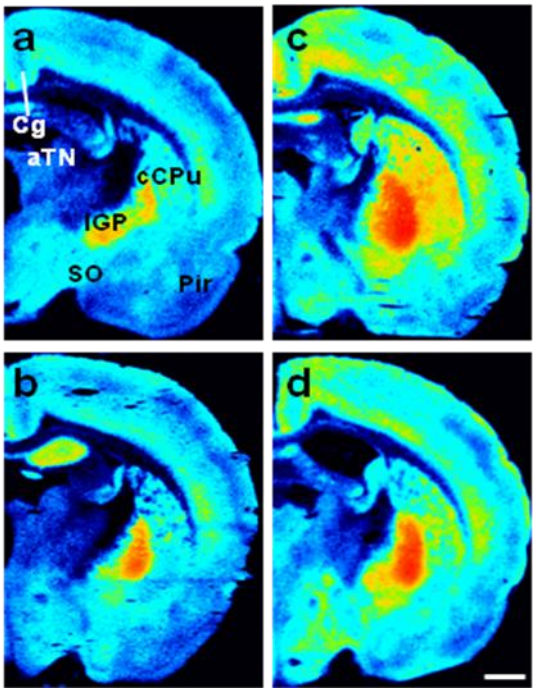

Figure 5: Representative photomicrographs of [^{3}H]CP55,940 binding in brain regions -1.30 mm from bregma in adult rats with manipulated rearing and handling experience.

Figure 6. Representative photomicrographs showing [^{3}H]CP55,940 binding in brains derived from adult rats that were either (top: a, b) group or (bottom: c, d) isolation reared and concomitantly (right: b, d) handled or (left: a, c) not handled daily post-weaning. Sections were collected -2.30 mm from bregma. CA 1 – 3, molecular layers of hippocampus CA 1 – 3; DG, dentate gyrus; vlTN, ventrolateral thalamic nuclei. The scale bar equals 1mm.

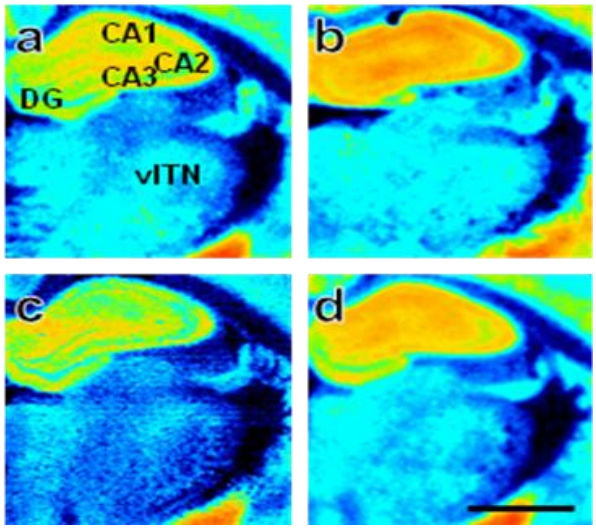

Figure 6: Representative photomicrographs of [^{3}H]CP55,940 binding in brain regions -2.30 mm from bregma in adult rats with manipulated rearing and handling experience.

Figure 7. Endocannabinoid content in brain punches derived from adult rats with a history of post-weaning rearing and handling manipulations. Single hemisphere punches were obtained at the level of the (a – c) prefrontal cortex, (d – f) nucleus accumbens, (g – i) piriform cortex, and (j – l) hippocampus as outlined (a, d, g, j) and were assayed for 2-arachidonoylglycerol (2-AG) and anandamide (AEA). (a – c) In the prefrontal cortex, isolation rearing increased (b) 2-AG, but not (c) AEA, levels in non-handled rats. Handling increased 2-AG, but not AEA, levels in the prefrontal cortex (b, c), compared to non-handled rats. (d – f) In the nucleus accumbens, handling increased (f) AEA, but not (e) 2-AG, relative to non-handled rats. (g – i) In the piriform cortex, isolation rearing increased both (h) 2-AG and (i) AEA levels of non-handled rats. Handling decreased AEA in the piriform cortex relative to non-handled rats. Handling increased (h) 2-AG in the piriform cortex in group- but not in isolation-reared rats relative to non-handling. (j – l) Rearing and handling manipulations did not alter endocannabinoid content in the hippocampus. Data are mean ± S.E.M. $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ vs. Group Reared/Non-Handled (*t*-test, two-tailed); $^{\#\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. Non-handled (ANOVA); $^{+}P < 0.05$ vs. Group reared/Handled (ANOVA, Tukey post hoc, two-tailed). The scale bar equals 1 mm.

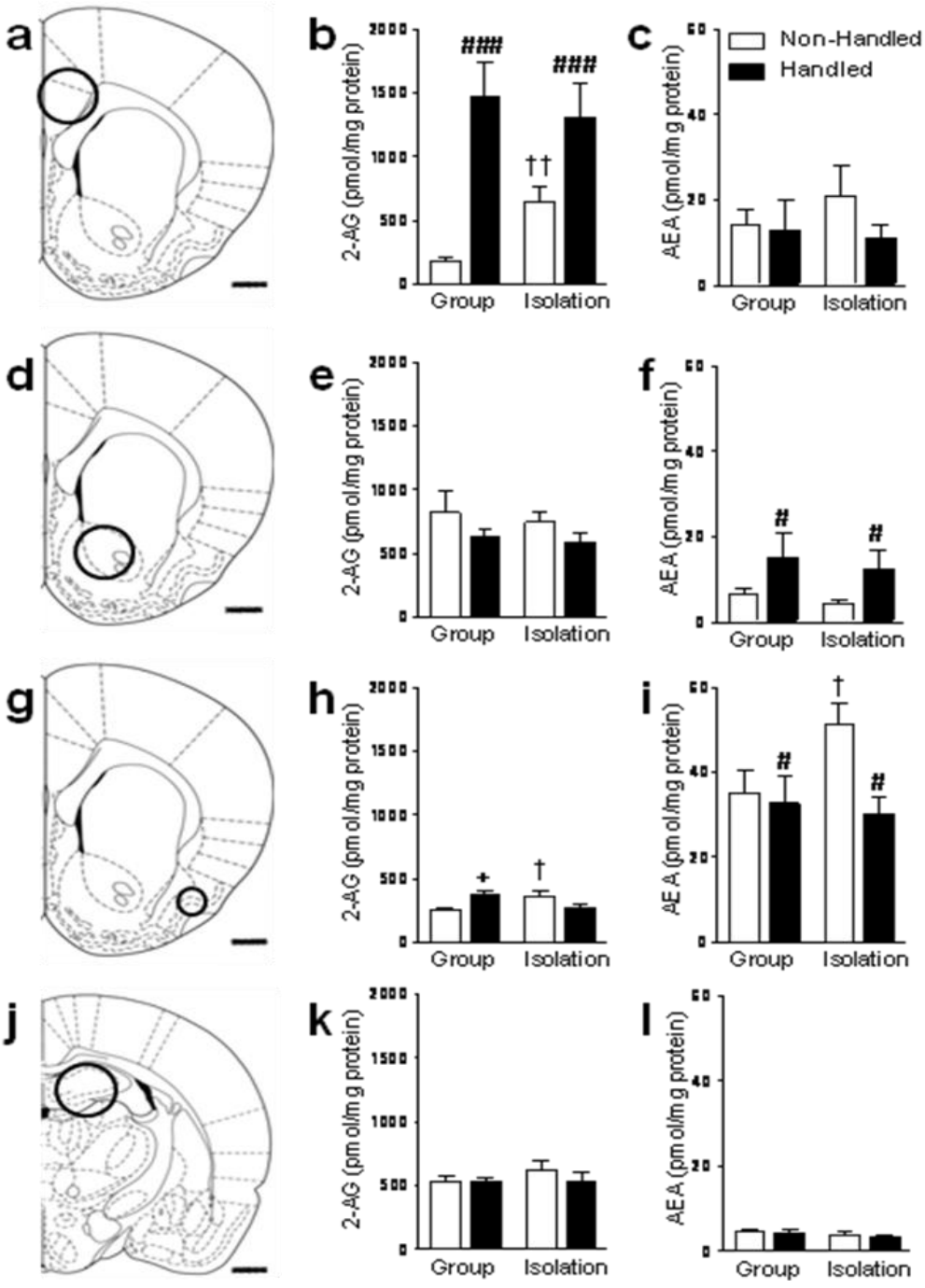

Figure 7: Endocannabinoid content in brain punches derived from adult rats with a history of post-weaning rearing and handling manipulation.

Figure 8. Neuroanatomical circuitry altered by post-weaning rearing and handling manipulations alone and in interaction. Key indicates the manipulated variables that produced the designated changes in cannabinoid receptor densities and endocannabinoid levels. Thick arrows connect structures of Papez circuitry of emotion whereas thin arrows connect structures of input-output loops through the basal ganglia. AEA, anandamide; 2-AG, 2-arachidonoylglycerol; CB_R, cannabinoid receptor.

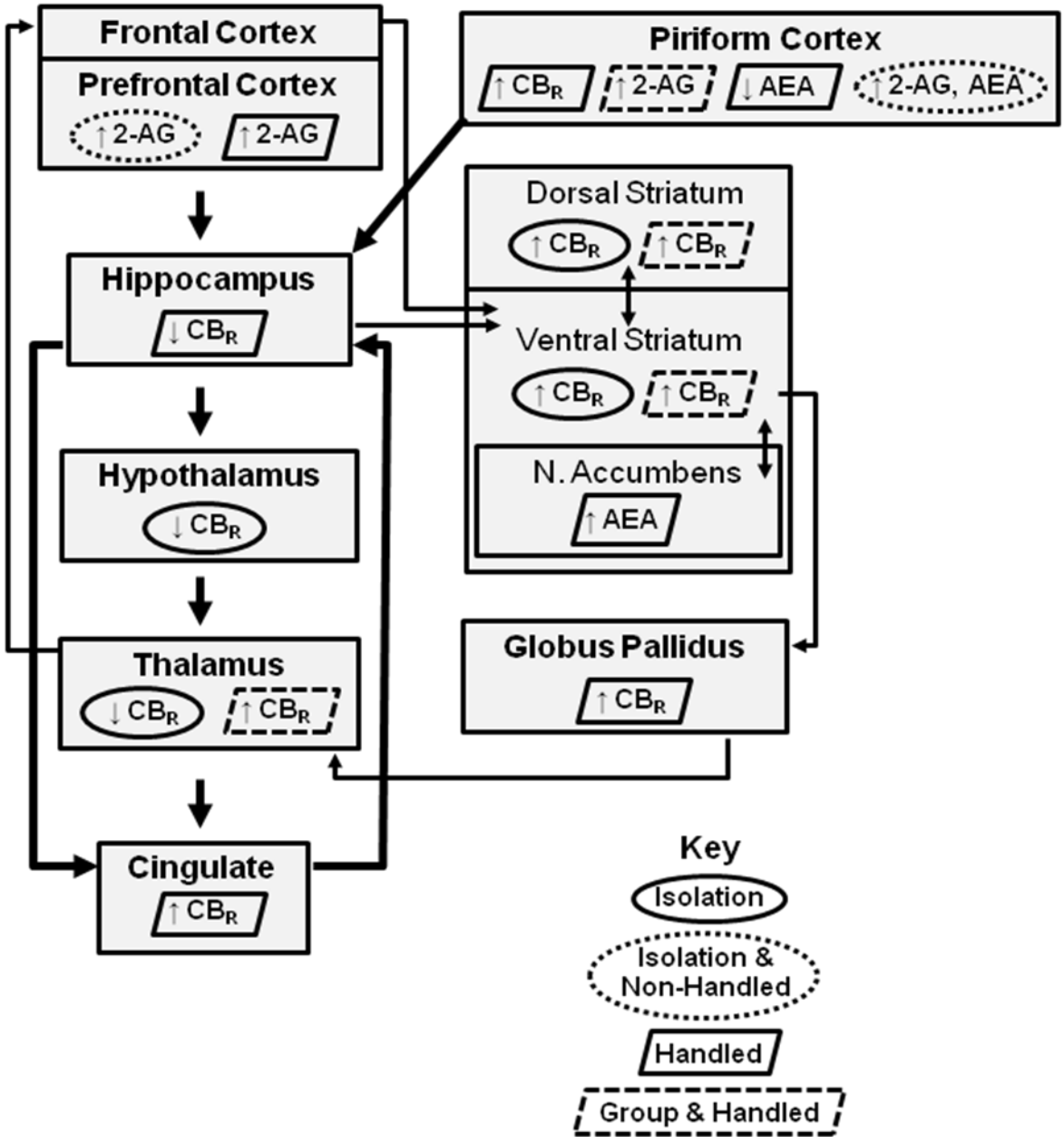

Figure 8: Neuroanatomical circuitry altered by post-weaning rearing and handling manipulations alone and in interaction.

CHAPTER 4: DISSCUSSION

Deprivation of social contact during development reproduces, in rodents, key features of limbic-founded human disorders. The present study characterized the long-term effects of post-weaning social isolation and chronic daily handling on key features of the endocannabinoid signaling system of the adult rat brain: cannabinoid receptor densities and endocannabinoid levels. Chronic daily experimenter handling was also evaluated for the ability to *i)* alter components of the endocannabinoid system and *ii)* interrupt the effects of isolation rearing on this system. The findings of the present report are schematically summarized in Figure 8.

Post-weaning social isolation

Isolation-rearing produced long-term alterations in cannabinoid receptor densities in somatosensory thalamic relay nuclei and the supraoptic nucleus of the hypothalamus. The ventrolateral thalamic nucleus, like the anterior thalamic nucleus, receives input from basal ganglia structures that bear presynaptic CB_1 receptors (e.g. substantia nigra pars reticulata and globus pallidus). In the supraoptic nucleus, isolation rearing decreased cannabinoid receptor densities compared to group rearing. The supraoptic nucleus, a brain region implicated in social behaviors, is part of the magnocellular neurosecretory system of the hypothalamus (Martin, 2003). Input to oxytocin synthesizing neurons of the supraoptic nucleus is modulated by endocannabinoids that act at CB_1 receptors (McDonald et al., 2008). It is interesting to speculate that the downregulation of cannabinoid receptors observed here in socially isolated rats could be associated with

increased endocannabinoid signaling; such changes would be expected to modify GABA-ergic and glutamatergic inputs to oxytocin neurons in the supraoptic nucleus, and ultimately regulate oxytocin release. Long-lasting dysregulation in the endocannabinoid signaling system in the supraoptic nucleus may, therefore, contribute to the perturbed social behavior of isolates (if the loss of these receptors are on inhibitory neurons) or represent a compensatory mechanism (if the loss of these receptors are on excitatory neurons) exhibited in the brains of isolates.

In the caudate putamen, isolation rearing increased cannabinoid receptor densities relative to group rearing. Likewise, cannabinoid receptor densities are increased in the caudate putamen in a model of schizophrenia induced by neonatal basolateral amygdala lesions (Bouwmeester et al., 2007). However, Malone and colleagues (2008) recently reported decreases in CB_1 immunoreactivity in the caudate putamen of isolation-reared rats using immunohistochemical methods. There may be several explanations for the discrepancy between our study and that of Malone et al. (2008). First, differences in techniques used to measure receptors (immunofluorescence vs. binding density) exist between the two studies. In our work, [^{3}H]CP55,940 binding would be expected to label all populations of cannabinoid receptors, whereas the C-terminal antibody used by Malone et al. (2008) may preferentially label specific subpopulations of cannabinoid receptors. Malone et al. (2008) used a CB_1 antibody, raised in goat, that was directed against residues 401 – 473 of the C-terminal of CB_1. This antibody is thought to preferentially label CB_1 receptors on GABAergic, but not glutamatergic, neurons (Katona et al., 2006, Kawamura et al., 2006, Nyilas et al., 2009). By contrast, a recently described highly sensitive second-generation CB_1 antibody, raised in guinea pig, detects CB_1

receptors on glutamatergic axons in the hippocampus and spinal cord (Katona et al., 2006, Kawamura et al., 2006, Nyilas et al., 2009) that were unrecognized by earlier generations of CB_1 antibodies (Katona et al., 1999, Egertova and Elphick, 2000, Farquhar-Smith et al., 2000, Salio et al., 2002). Second, immunoreactive labeling by C-terminal antibodies may be masked by the presence of C-terminal interacting proteins (e.g. CRIP1a) that were recently shown to modulate CB_1 receptor activity (Niehaus et al., 2007). Third, sensitivity of immunostaining may vary with the level of tissue fixation and receptor internalization (Hohmann, 2002). Fourth, the anatomical divisions of the caudate putamen evaluated also differed between the two studies (rostral dorsal in the previous study vs. rostral, dorsal and ventral measurements in the present study). One or all of these factors may contribute to differences observed between the previous (Malone et al., 2008) and present reports.

Changes in cannabinoid receptor densities were not observed in cingulate and prefrontal cortices after isolation rearing. These observations are consistent with the results of immunohistochemical data published by Malone et al. (2008). In both the present study and that of Malone et al. (2008), transient changes in cannabinoid receptor densities may have resulted during development, but normalized in adulthood when cannabinoid receptor densities were measured. In contrast to isolation-reared rats, brains derived from schizophrenia patients exhibit increased cannabinoid receptors in these cortices compared to controls (Dean et al., 2001, Zavitsanou et al., 2004, Newell et al., 2006). Thus, the isolation rearing model may not recapitulate all features of schizophrenia. Further investigation of alterations in the endocannabinoid signaling system at different developmental stages following isolation rearing is warranted. In

addition, the impact of the observed changes in the endocannabinoid signaling system on the abnormal behavioral phenotype of isolates remains to be elucidated.

Our findings demonstrate, for the first time, that the early-life stress of isolation rearing can alter endocannabinoid content. Isolation-reared rats exhibited increases in 2-AG, but not anandamide, levels in the prefrontal cortex and increases in both 2-AG and anandamide levels in the piriform cortex relative to group-reared/non-handled counterparts. In line with our findings, repeated restraint stress also increases 2-AG, but not anandamide, in the forebrain (Patel et al., 2005). In isolates, altered endocannabinoid content in the piriform cortex may result from deprivation of informative olfactory cues from littermates. Isolation stress produces deficits in a conditioned odor association compared to controls (Zimmerberg et al., 2009). Isolation rearing also increases cholinergic and serotonergic fiber densities in brain structures involved in smell, including the olfactory bulb and piriform cortex (Lehmann and Lehmann, 2007). These findings are congruent with an emerging body of literature that suggests that endocannabinoids are mobilized after exposure to a stressor (Hohmann et al., 2005, Rademacher et al., 2008, Rossi et al., 2008).

Chronic daily handling

Daily handling produced stable changes in the endocannabinoid signaling system in both Papez circuitry and input-output loops of the basal ganglia (see Figure 8). We demonstrate, for the first time, that chronic daily handling alters both cannabinoid receptor densities and endocannabinoid content within brain structures that control emotional expression (i.e., lateral globus pallidus, prefrontal, piriform, and cingulate cortices, hippocampus, nucleus accumbens). In line with the present findings, the

anxiolytic effect of URB597, an inhibitor of the anandamide-degrading enzyme fatty-acid amide hydrolase, was altered after handling (Haller et al., 2009). We found that, in the prefrontal cortex, handling increased 2-AG without reliably altering anandamide levels, compared to non-handling. Moreover, the effect of handling accounts for approximately one fifth of the total variance in 2-AG content in the prefrontal cortex; this represents a relatively large effect based upon effect size criteria outlined by Cohen (1998). In the nucleus accumbens, handling increased anandamide levels without altering levels of 2-AG, compared to non-handling. The antipsychotic-like properties of handling, in terms of changes in endocannabinoid levels reported here and in alterations of sensorimotor gating reported elsewhere (Krebs-Thomson et al., 2001), support the use of handling as a research tool to manipulate endocannabinoid levels in animal models. Chronic daily handling may facilitate habituation to stressors in an endocannabinoid-dependent manner, as demonstrated by the fact that handling itself eventually loses its aversive quality after chronic exposure.

Handling increased cannabinoid receptor densities in the limbic loop of the basal ganglia, including basal ganglia output structures (lateral globus pallidus) and allocortical areas (cingulate and piriform cortex), but decreased cannabinoid receptor densities in archicortex (hippocampus), relative to non-handling. Handling, like subchronic treatment with the antipsychotic haloperidol (Andersson et al., 2005), also increased [^{3}H]CP55,940 binding to cannabinoid receptors in the globus pallidus. Moreover, handling modified endocannabinoid levels in the piriform cortex by decreasing anandamide levels, relative to non-handling. It is possible that decreases in endocannabinoid levels are associated with an upregulation of cannabinoid receptors in the same allocortical regions. In

archicortex, downregulation of cannabinoid receptors may affect hippocampal gating processes by increasing glutamate release in the hippocampus and facilitating long term potentiation. Previous data suggests that handling is sufficient to increase the amplitude of hippocampal long-term potentiation (Wilson et al., 1986). Together, these data implicate a role for the endocannabinoid system in the ability of handling to alter behavior and neuronal physiology.

In isolation-reared rats, daily experimenter handling was not sufficient to modify cannabinoid receptor densities in the caudate putamen and thalamus; handling increased cannabinoid receptor densities in these same structures in group-reared rats only. We also found that handling increased 2-AG levels in the piriform cortex in group-reared rats only. Handling has been shown to attenuate many isolation-induced behaviors, including hyperlocomotor activity (Holson et al., 1991), hypoalgesia (Gentsch et al., 1982), and deficits in sensorimotor gating (Krebs-Thomson et al., 2001). However, it is, perhaps, unsurprising that handling isolates for 5 minutes per day did not reverse isolation-rearing induced changes in the endocannabinoid system. Handling has been shown to preferentially reverse corticosterone levels in mice bred for low aggressiveness, but failed to reverse corticosterone levels in mice bred for high aggressiveness (Gariépy et al., 2002). Overall, we interpret our data to suggest that handling alone produces regulatory changes in the endocannabinoid system, but the effectiveness of this manipulation is diminished in rats reared in social deprivation.

The present findings document the existence of long-term alterations in cannabinoid receptors and endocannabinoid content following post weaning social isolation and chronic handling treatments. Perturbations in the endocannabinoid

signaling system may contribute to the abnormal behavioral phenotype of isolation-reared rats. The results further suggest that handling alone cannot reverse the long-term effects of social deprivation on cannabinoid receptor densities or endocannabinoid levels. Our study validates the use of chronic daily handling to alter both endocannabinoid levels and cannabinoid receptor densities. These observations provide additional evidence for a pivotal role for the endocannabinoid signaling system in adaptation to stressful life events (for review see Finn, 2009, Rossi et al., 2009).

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