Region-specific control of microglia by adenosine A_{2A} receptors: uncoupling anxiety and associated cognitive deficits in female rats

Running title: Microglia heterogeneity in the brain and anxiety

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Abstract

Epidemiologic studies have provided compelling evidence that prenatal stress, through excessive maternal glucocorticoids exposure, is associated with psychiatric disorders later in life. We have recently reported that anxiety associated with prenatal exposure to dexamethasone (DEX, a synthetic glucocorticoid) correlates with a gender-specific remodeling of microglia in the medial prefrontal cortex (mPFC), a core brain region in anxiety-related disorders. Gender differences in microglia morphology, the higher prevalence of anxiety in women and the negative impact of anxiety in cognition, led us to specifically evaluate cognitive behavior and associated circuits (namely mPFC-dorsal hippocampus, dHIP), as well as microglia morphology in female rats prenatally exposed to dexamethasone (*in utero* DEX, iuDEX). We report that iuDEX impaired recognition memory and deteriorated neuronal synchronization between mPFC and dHIP. These functional deficits are paralleled by microglia hyper-ramification in the dHIP and decreased ramification in the mPFC, showing a heterogeneous remodeling of microglia morphology both postnatally and at adulthood in different brain regions, that differently affect mood and cognition. The chronic blockade of adenosine A_{2A} receptors ($A_{2A}R$), which are core regulators of microglia morphology and physiology, ameliorated the cognitive deficits, but not the anxiety-like behavior. Notably, $A_{2A}R$ blockade rectified both microglia morphology in the dHIP and the lack of mPFC-dHIP synchronization, further heralding their role in cognitive function.

Significance Statement (120 words)

Immune cells of the brain - microglia - are affected in stress-related disorders, namely in anxiety, which is frequently associated with cognitive deficits. Women are particularly susceptible to these conditions. We report that microglia morphology in rodent females differs between two brain regions (pre-frontal cortex, PFC and hippocampus) implicated in anxiety. Furthermore, we show that the pharmacologic manipulaton of the adenosinergic molecular system (adenosine A_{2A} receptor) regulates anxiety and cognition by differential

modulation of microglia morphology in the PFC and in the hippocampus. This study correlates the morphology of microglia with different components of mood disorders, anxiety and cognition, and may be useful in the design of immune-based anxiolytic drugs targeting different components of psychiatric disorders.

Introduction

Exposure to high levels of glucocorticoids (GC) during neurodevelopment is relatively common, occurring in maternal stress situations and glucocorticoid-based therapies during pregnancy(1). During brain development, high levels of GC have been associated with psychiatric disorders, namely anxiety, later in life(1). The impact of GC on brain wiring and function, affecting neuronal migration(2), neuronal morphology(3-7) and spine density(5, 8, 9) in several brain regions, has been causally associated with their long-term consequences in terms of behavior(4, 5, 8, 10) and predisposition to brain disorders(1).

Besides neurons, GC also affect microglia during development(11). These immune cells colonize the brain early in development(12, 13) and indirectly sculpt neuronal circuits by controlling synapse formation(14-17), maturation(18) and elimination(19, 20). Microglia express functional glucocorticoid receptors (GR)(21) and their morphology is altered by the prenatal exposure to GC, an effect that persists until adulthood(11). In a developmental model of anxiety (prenatal exposure to GC), microglia undergo a complex process of morphologic plasticity in the medial prefrontal cortex (mPFC), that is different between males and females(11). This dimorphic effect is likely related with the function of adenosine A_{2A} receptors ($A_{2A}R$), which modulate microglia function and morphology(11, 22-25). In fact, $A_{2A}R$ blockade is anxiolytic in males(25, 26) and corrects morphologic changes in microglia(11, 27), whereas $A_{2A}R$ blockade in females fails to counteract both the anxious-like phenotype and the morphologic changes in microglia(11).

Since patients with anxiety disorders commonly display cognitive impairment(9) and $A_{2A}R$ blockade prevents cognitive deficits(22-25, 28-30), we now investigated if $A_{2A}R$ blockade recovers cognition in females with anxiety resistant to $A_{2A}R$ antagonism. We further assessed if $A_{2A}R$ in the dorsal hippocampus (dHIP) and in the mPFC differently control microglia morphologic remodeling in these two brain regions, which process anxiety and cognitive performance.

Results

Prenatal exposure to DEX leads to deficits in recognition memory in adult females

Females prenatally exposed to DEX (iuDEX, Figure 1A), as compared with saline-treated females (SAL), display a lower recognition index (SAL: 0.47±0.06, n=9; iuDEX: 0.21±0.03, n=10; $p<0.001$; Figure 1B). Confirming previous reports, iuDEX females also present anxiety-like behavior, evaluated by two independent tests, the EPM (SAL: 0.35±0.03, n=6; iuDEX: 0.20±0.04, n=7; $p<0.05$; Figure 1C) and the NSF (Supplementary Figure 1A). Moreover, iuDEX females presented altered serum levels of corticosterone at 8 a.m. (iuDEX: 78.9±5.90, n=7, $p<0.001$; Figure 1D). Also in line with previous results, iuDEX females did not display helplessness behavior, as assessed by the FST (Supplementary Figure 1B) or anhedonic behavior, as evaluated by the SDT (Supplementary Figure 1C). Moreover, iuDEX did not alter body weight (Supplementary Figure 1D) at adulthood, nor the distance travelled or the velocity in the OF test, indicating that iuDEX does not affect global locomotor function (Supplementary Figure 1E).

iuDEX decreases spectral coherence between the dHIP and mPFC

Hippocampal-prefrontal connectivity is related to anxiety, spatial learning and memory-related tasks(31), which requires an adequate synchronization between these regions (Figure 2A), measured as coherence. Compared to the control animals (SAL), iuDEX leads to a significant decrease of dHIP and mPFC coherence in different frequency bands, namely: delta (<4 Hz; SAL: 0.71±0.05, n=9; iuDEX: 0.47±0.08, n=7; $p<0.05$), theta (4-12 Hz; SAL: 0.79±0.04, n=9; iuDEX: 0.44±0.07, n=5; $p<0.001$), beta (12-20 Hz; SAL: 0.80±0.03, n=8; iuDEX: 0.58±0.04, n=5; $p<0.01$) and low gamma (20-40 Hz; SAL: 0.76±0.03, n=9; iuDEX: 0.60±0.06, n=6; $p<0.05$) (Figure 2 B,C). By contrast, the levels of neuronal activity in the mPFC (Supplementary Figure 2A,B) and in the dHIP (Supplementary Figure 2C,D) were not altered by iuDEX.

iuDEX induces short-term and long-lasting alterations in microglia morphology in the dHIP

We next evaluated the impact of iuDEX on microglia morphology in the dHIP of post-natal day 7 (PND 7) and of adult female rats (PND 90), focusing in the DG, a region associated with cognition(32, 33) and particularly involved in NOR performance(34). As previously found by our group in the mPFC(11), iuDEX induced a decrease in the total length of cellular ramifications, without affecting their number at PND 7 (Figure 3A,B, Supplementary Figure 3A and raw data and statistics in Supplementary Table 1). Microglia morphologic alterations are still observed at PND 90. At this time point, an increase of the number of microglia ramifications was observed compared with control animals (Figure 3C,D, Supplementary Figure 3B and raw data and statistics in Supplementary Table 2), but the total length of ramifications is not different from the control (Figure 3C,D, Supplementary Figure 3B and raw data and statistics in Supplementary Table 3).

The morphologic complexity of microglia is different between the dHIP and mPFC

The effect of iuDEX observed in the dHIP contrasts with its effect in the mPFC, where we previously reported a decrease in the number of microglia ramifications(11). Thus, we analyzed and compared microglia morphology under physiological conditions in both regions of adult female rats. Microglial cells in the mPFC exhibited a more complex morphology, with higher number of ramifications, which are longer, compared to microglial cells from the dHIP (Figure 3E,F, Supplementary Figure 3C and raw data and statistics in Supplementary Table 4).

A_{2A}R blockade rescues iuDEX-induced changes

Confirming our previous report(11), the chronic blockade of A_{2A}R with the selective A_{2A}R antagonist, SCH58261, administered for 21 days (0.1 mg/kg/day) before PND 90 (Figure 4A), exerted, *per se*, an anxiogenic effect (SCH58261: 0.16±0.06, n=7; SAL: 0.35±0.03, n=6; $p<0.05$; Figure 4B) and was not able to ameliorate iuDEX-induced anxiety-like behavior in

females (for the EPM, iuDEX+SCH58261: 0.19±0.04, n=10; iuDEX: 0.20±0.04, n=7; p>0.05; Figure 4B; for the NSF, see Supplementary Figure 1A). SCH58261 administered to control or iuDEX adult females did not interfere with the performance of the animals in tests for helplessness behavior (Supplementary Figure 1B) or anhedonia (Supplementary Figure 1C). Body weight and locomotor activity were also not affected by the treatment with SCH58261, alone or in combination with iuDEX (Supplementary Figure 1D,E). Regarding serum levels of CORT, we again confirm our previous data(11): SCH58261 per se did not alter these levels and was not able to normalize DEX-induced alterations (Figure 4D).

In contrast, the chronic treatment of adult females with the selective $A_{2A}R$ antagonist, SCH58261, improved cognition in iuDEX females (iuDEX + SCH58261: 0.35±0.02, n=7; iuDEX: 0.21±0.03, n=10; p<0.05; Figure 4C). However, iuDEX females treated with SCH58261 still presented a cognitive impairment in recognition memory, as compared with control animals (iuDEX + SCH58261: 0.35±0.02, n=7; SAL: 0.53±0.04, n=7; p<0.05; Figure 4C). This might be due to the surprising observation that, in contrast to the well established absence of effect on learning and memory tasks of $A_{2A}R$ antagonists in male rats(23, 35), the treatment with the $A_{2A}R$ antagonist, *per se*, deteriorated memory performance in female rats (SCH58261: 0.37±0.03, n=6; SAL: 0.53±0.04, n=7; p<0.05; Figure 4C).

In accordance with this effect of SCH58261 on memory performance, the chronic blockade of $A_{2A}R$ in adult females also decreased the coherence between the dHIP and the mPFC in most of the frequency ranges analyzed: delta (<4 Hz; SAL: 0.71±0.05, n=9; SCH58261: 0.54±0.11, n=7; p>0.05), theta (4-12 Hz; SAL: 0.79±0.04, n=9; SCH58261: 0.58±0.04, n=7; p<0.01), beta (12-20 Hz; SAL: 0.80±0.03, n=8; SCH58261: 0.44±0.06, n=7; p<0.001) and low gamma (20-40 Hz; SAL: 0.76±0.03, n=9; SCH58261: 0.52±0.04, n=7; p<0.001) (Figure 4 E,F), but most importantly, SCH58261 normalized the iuDEX-induced decrease in mPFC-dHIP coherence, an effect observed only for the theta frequency range: delta (<4 Hz; iuDEX+SCH58261: 0.59±0.08, n=10; p>0.05 as compared with iuDEX), theta (4-12 Hz; iuDEX+SCH58261: 0.67±0.06, n=8; p<0.05 as compared with iuDEX), beta (12-20 Hz; SAL:

iuDEX+SCH58261: 0.61±0.05, n=10; p>0.05, as compared with iuDEX) and low gamma (20-40 Hz; iuDEX+SCH58261: 0.52±0.06, n=10; p>0.05 as compared with iuDEX) (Figure 4E,F).

Regarding microglia morphology, in contrast to what happens in the mPFC, where $A_{2A}R$ blockade was unable to normalize iuDEX-induced changes in females(11), we now observed in the dHIP that iuDEX adult females treated with SCH58261 show a significant reduction of the number of ramifications compared with iuDEX females, which recovers to control levels (Figure 5A,E, Supplementary Figure 3B; Supplementary Table 2). Furthermore, the length of processes was also diminished in iuDEX adult females treated with SCH58261compared with iuDEX females (Figure 5C,E, Supplementary Figure 3B, Supplementary Table 3). Importantly, SCH58261 alone reduced the length of some microglial processes in the dHIP, compared with control animals (Figure 5D, Supplementary Figure 3B, Supplementary Table 3), although not affecting the number of processes (Figure 5B, Supplementary Figure 3B, Supplementary Table 2). Interestingly, SCH58261 also decreased the number of microglia ramifications in the mPFC, although it also decreased the total length of these ramifications(11).

Discussion

We now report that female rats prenatally exposed to DEX present memory impairments at adulthood together with a disruption of neuronal synchronization between the mPFC and the dHIP. This was accompanied by a brain region-specific regulation of microglia remodeling upon iuDEX (hyper-ramification in the dHIP and de-ramification in the mPFC). Our morphometric data revealed that besides regional differences in microglia remodeling associated with our model, iuDEX, the morphologic phenotype of these cells is heterogeneous comparing the mPFC and the dHIP in control conditions. We further observed that the chronic blockade of $A_{2A}R$ in iuDEX adult females normalized microglia morphology in the dHIP (but not in the mPFC), and rescued cognitive deficits and the lack of coherence between the mPFC and the dHIP. These results reinforce the robust ability of $A_{2A}R$ to control and rescue memory deterioration, now shown to be associated with a control of microglia morphology in a context of anxiety upon iuDEX.

In animal models, excessive stress/GC exposure at the prenatal period impairs brain development and results in abnormal behavior in adult offspring(2, 3). Our data further support this view: we now show that iuDEX causes the emergence in adulthood of both anxiety and cognitive deficits in association with altered synchronization between mPFC and dHIP. The most novel finding of our study was the observation that these iuDEX-induced cognitive deficits in the NOR test, which involves the hippocampal formation(36), were correlated with long-lasting changes in microglia morphology, already observable in the first post-natal week in the DG of the dHIP, a brain region enriched in and tightly affected by GR signaling(6).

These results suggest an impact of microglia in cognition that is in line with previous studies showing that microglia are crucial during development(37, 38). As an example, a study with a transient reduction in microglia during development leads to long-term deficits in synaptic transmission, functional brain connectivity, synaptic plasticity and cognitive deficits(39, 40). Indeed, a short-term depletion of microglia leads to memory deficits in normal animals(17,

41), but not in diseased animals(42-45). Therefore, conditions resulting in defective microglia during brain development can lead to impaired cognition(37, 38, 41). In fact, several studies demonstrated the contribution of glial signaling, namely astrocytes, for the modulation of the synchronization of cortical oscillations between the dHIP and the mPFC, which underlie an influence on cognitive performance(46-48). Knowing the correlation between morphology and functionality of microglia and the impact of microglia on synaptic formation and pruning(14-20), our results suggest that microglial regulation of synapse remodelling during neurodevelopment may be compromised in iuDEX females and that this malfunction may be the underlying cause of behavioral abnormalities, namely memory deficits.

This proposed association between the sustained alteration of microglia morphology in the dHIP and the cognitive deficits in iuDEX adult females is further supported by our observation that the treatment of adult iuDEX females with a selective $A_{2A}R$ antagonist reverted both the cognitive deficits and the decrease in coherence between the dHIP and the mPFC, as well as the alterations of microglia in the dHIP. Several studies have previously shown that $A_{2A}R$ blockade prevents memory deficits associated with chronic stress or depression(22, 25, 49, 50). These studies focused on the relation between synaptic dysfunction and memory deterioration and they have shown that neuronal $A_{2A}R$ were both necessary and sufficient to trigger memory deficits(30, 51, 52). However, we and others have also described the ability of $A_{2A}R$ to modulate microglia morphology(11, 53, 54), proliferation(55) and function(53). Our previous studies demonstrated that $A_{2A}R$ regulate microglia morphology in the mPFC in a gender-specific manner(11). The data now presented make it clear that there is also a marked heterogeneity of $A_{2A}R$-mediated microglia modulation according to the brain region considered. In fact, we now observed that selective $A_{2A}R$ antagonist was unable to correct microglia atrophy in the mPFC of females, but reverted microglia hyper-ramification in the dHIP of females. In parallel, $A_{2A}R$ blockade reverted memory impairment but not anxiety in these iuDEX females. Altogether, these results suggest that the functional uncoupling between anxiety and cognition in iuDEX

females that were treated with a selective $A_{2A}R$ antagonist may be, at least partially, explained by a differential regulation of microglia morphology by $A_{2A}R$ in different brain regions. The reasons underlying this region-specific response are still unexplored, but may be due to regional differences of $A_{2A}R$ function in the brain(26, 51, 56) or to differences in microglia function in different brain regions.

Worth of note is the surprising observation that the blockade of $A_{2A}R$ *per se* impaired recognition memory and neural coherence between mPFC and dHIP in adult female rats, which contrasts with the well-established absence of effect on learning and memory tasks of $A_{2A}R$ antagonists in adult male rats(23, 35). Similar observations regarding impairment of recognition memory in female rats come from studies in which rats were exposed to caffeine during development (pre- and postnatally) in doses expected to act via non-selective antagonism of adenosine receptors(57, 58). Our results now show that a selective $A_{2A}R$ antagonist reproduce the deleterious effect of caffeine on recognition memory in adult female rats, in parallel with the disruption in synchronization between mPFC and dHIP, a neural network essential for the performance of cognitive tasks. Given the therapeutic interest of these receptors in mood and memory-related disorders, our results highlight the need to take notice of gender differences in response to pharmacological treatments.

The present study provides direct evidence for differences of microglia morphologic dynamics in different brain regions. In fact, in iuDEX adult females, we observed a long-lasting hyper-ramification of microglial cells in the dHIP, that contrast with the opposite morphological profile observed in the mPFC(11) (de-ramification of microglia processes). Microglia morphology was also different in these two brain areas in physiological conditions, *i.e.* in control female rats, where microglia exhibited a higher degree of morphological complexity in the mPFC compared to the dHIP. These data are in line with previous studies reporting regional differences in microglia(59, 60), although most studies used rudimentary approaches rather than 3D reconstitutions to study microglia morphology. This regional heterogeneity of microglia is further heralded by distinct transcriptional identities of microglia

in the cortex and the hippocampus(61). However, further studies are required to categorize region-specific functionalities of microglia to determine how this influences microglia modulation for instance by purines, and the response of microglia to insults.

In conclusion, the data obtained show that iuDEX, in conditions mimicking the clinical use of GC in the early periods of brain development, induces alterations in microglia morphology in a region-specific manner with impact in behavior. Microglia morphology remodeling in the dHIP correlates with cognitive deficits observed in our animal model and a treatment with a selective $A_{2A}R$ receptor antagonist was able to revert behavioral changes, electrophysiological abnormalities and the alterations of microglia morphology in the dHIP. These observations reinforce the link between microglia function and the control of mood and memory, and emphasize the impact of gender and brain region evaluated to study novel therapeutic strategies targeting microglia homeostasis to manage brain disorders.

Materials and Methods

Animal handling and pharmacological treatment

Animals were handled according to the European Community guidelines on animal care and experimentation (2010/63/EU). The experimental protocols were approved by the Ethical committees of ICVS (Life and Health Sciences Research Institute, SECVS protocol #107/2015) and CNC (Center for Neuroscience and Cell Biology, Orbea 78/2013). All animals were housed under standard laboratory conditions (22 ^{o}C, light/dark cycle of 12 h; food and water *ad libitum*). Pregnant Wistar rats were administered with DEX (sc, subcutaneous, 1mg/kg) or saline on gestation days 18 and 19, as previously described(11). Females from the offspring were treated during 21 consecutive days before post-natal day (PND) 90 with saline or with the selective $A_{2A}R$ antagonist, SCH58261 (ip, intraperitoneal, 0.1 mg/kg/day), a dose displaying anxiolytic effects in adult male rodents subjected to stress protocols(11, 25).

Behavior evaluation

Open field (OF) test: The locomotor behavior was evaluated in the OF test. Each rat was placed in the center of the arena (white floor and transparent acrylic walls, 43.2 x 43.2 cm; Med Associates Inc., St. Albans, VT, USA) under bright white light. The locomotor activity was monitored using a two 16-beam infrared system for 5 min. Velocity and total distance travelled were analyzed using the Activity Monitor software (Med Associates, St Albans, VT, USA).

Forced swimming test (FST): Depressive-like behavior was evaluated in the FST. The test was conducted 24 h after a pre-test session (10 min), by placing the rats in glass cylinders (50 cm) filled with water (25 ºC) for 5 min. Trials were video recorded and the time spent immobile and latency to immobility were analyzed blindly.

Novelty suppressed feeding (NSF): Anxiety-like behavior was assessed using the NSF test. Following previously published protocols(62), rats were food-deprived (18 h) before being placed in a novel arena for 10 min, where a single food pellet was centrally placed. The latency to eat was measured, being an indicator of anxiety-like behavior. Rats were then transferred to their home cage and the amount of food consumed during 10 min was measured, as an indicator of appetite drive.

Elevated plus maze (EPM) test: Anxiety-like behavior was additionally assessed by the EPM test. Rats were placed in the center of a black polypropylene plus-shaped platform (Med Associates) with 2 open arms (50.8 x 10.2 cm) and 2 closed arms (50.8 x 10.2 x 40.6 cm), located 72.4 cm above the floor in a room illuminated with bright white light during 5 min. The time spent in the open arms and the number of total entries were measured with an infrared photo beam system and analyzed with a specific software (MedPCIV, MedAssociates, Georgia, VT, USA). The level of anxiety-like behavior was measured by the ratio of time spent in the open arms/total time and the number of entries into each arm of the maze.

Sweet drive test (SDT): Rats were allowed to freely explore the SDT arena, previously described(63), where regular (Mucedola 4RF21-GLP) or sweet pellets (Cheerios, Nestlé) were accessible, for 10 min. The preference for sweet pellets was calculated by the formula: sweet pellets consumed (g)/ total pellets consumed (g)(63) . The decreased preference for sweet pellets was used as an indicator of anhedonic behavior.

Novel object recognition (NOR) test: NOR test evaluates the ability to distinguish between a familiar and a novel object, a readout for recognition memory. Briefly, rats were placed in the arena in the presence of two identical objects in shape, color and size, during 10 min; 2 h later, one of the objects was replaced by a different object in color and shape. Trials were video recorded and the time spent in the novel and familiar objects was measured. The recognition index was measured by the quotient: time exploring the novel object/(time exploring the familiar object + time exploring the novel object).

Corticosterone (CORT) determination

Blood samples from adult female rats were collected (puncture of the tail vein) at 8:00 am (diurnal nadir) and 8:00 pm (diurnal zenith) in the day preceding the sacrifice and processed to isolate serum, where CORT levels were measured using the Corticosterone ELISA Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions.

Immunohistochemistry and tridimensional (3D) morphometric analysis of microglia

Rats were deeply anesthetized with an ip injection of sodium pentobarbital (60 mg/kg) and transcardially perfused with heparinized saline and 4% paraformaldehyde (PFA). Brains were fixed in 4% PFA overnight and transferred to 30% sucrose solution in phosphate buffered saline (PBS, 37 mM NaCl, 2.1 mM KCl, 1.8 mM KH2PO4 and 10 mM Na2HPO4.2H2O, pH 7.4) overnight at 4 ºC. After fixation, brains were cryopreserved at – 80 ^{o}C and sectioned (50 µm) in a cryostat. For immunodetection of microglia, free-floating coronal sections containing the dHIP (stereotactic coordinates of interaural 5.20 mm and bregma -3.80; Paxinos and Watson, 1998) (64) were incubated in a permeabilization and blocking solution of 5% BSA

(bovine serum albumin) and 0.1% Triton X-100 in PBS (2 h at room temperature, RT). Incubation with the primary antibody (rabbit anti-Iba-1, 1:1000, WAKO, Osaka, Japan) was performed for 48 h in the blocking solution at 4 ^{o}C. After washing, sections were incubated with the secondary antibody (donkey anti-rabbit, 1:1000, Invitrogen, Waltham, MA, USA) for 2 h at RT and with DAPI (1:5000) for 10 min at RT. Sections were mounted on gelatinized slices using glycergel (Dako mounting medium). Images of 10 random microglial cells from each animal were acquired in the dorsal dentate gyrus (DG) of the hippocampus with a laser scanning confocal microscope LSM 710 META connected to ZEN Black software (Zeiss Microscopy, Oberkochen, Germany), using a 63x objective lens (Plan-Apochromat 63x/1.40 Oil DIC M27).

Tridimensional reconstruction of microglial cells was obtained by manual reconstruction using the Neurolucida software (MBF Bioscience, Williston, VT, USA). Morphometric data (quantification of the number and the length of cellular processes) were extracted using Neurolucida explorer.

***In vivo* electrophysiology**

In vivo analysis of neural activity was performed as previously described, with minor changes(65, 66). Briefly, rats were anesthetized with 4% sevofluorane (SevoFlo, Abbott, USA) and placed in a stereotaxic frame (KOPF, USA) once they were deeply anesthetized. Concentric platinum/iridium electrodes (400 µm shaft diameter, Science Products, Germany) were implanted in the prelimbic area of the mPFC (3.3 mm anterior to bregma, 0.8 mm lateral and 4.0 mm below bregma) and in the CA1 of dHIP (3.8 mm anterior to bregma, 2 mm lateral and 2 mm below bregma) (Figure 2A)(64). Local field potential (LFP) signals were amplified, filtered (0.1-300 Hz, LP511 Grass Amplifier, Astro-Med, Germany), acquired (Micro 1401 mkII, CED, UK) and recorded by a dedicated software (Signal Software, CED, UK).

Coherence was calculated as a measure of phase and amplitude synchronization between mPFC-dHIP. This analysis was performed on the 2-channel 100 s long LFP signals and was

based on multi-taper Fourier analysis and calculated by custom-written MATLAB scripts, using the MATLAB toolbox Chronux. Coherence was calculated to reach 1s long segments and their mean was assessed for all frequencies: delta (<4Hz), theta (4-12 Hz), beta (12-20 Hz) and low gamma (20-40 Hz).

Data and statistics

Data are means ± SEM. Means were compared using the Student's *t*-test, when comparing two conditions or one-way analysis of variance (ANOVA), followed by a Turkey's *post hoc* test, when comparing more than two conditions. The level of significance was set at $p<0.05$.

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Figure legends

Figure 1. Effect of prenatal exposure to DEX on cognition, anxiety and HPA axis.

(A) Schematic view of the animal model and pharmacological treatment. Pregnant Wistar rats received DEX (1 mg/kg/day sc) on days 18 and 19 of gestation. (B) The recognition index (time spent in the novel object *per* total time spent in the novel and familiar objects) was calculated to evaluate cognitive deficits, using the NOR test. (C) Time spent in the open arms *per* total time of the EPM test, performed to evaluate anxiety-related behavior. (D) Diurnal and nocturnal quiescent levels of corticosterone were measured by ELISA, to evaluate the endogenous corticosterone levels. Results are presented as the mean ± SEM (n= 6-10animals); *p<0.05 and ***p<0.001, comparing with saline, calculated using an unpaired Student's *t* test.

Figure 2. Prenatal exposure to DEX decreases the coherence between the mPFC and the dHIP.

Pregnant Wistar rats were administered with DEX (1mg/kg/day, sc) at days 18 and 19 of gestation. *In vivo* electrophysiology was performed to evaluate the coherence between mPFC and dHIP. (A) Scheme of the *in vivo* electrophysiological recordings in dHIP and mPFC. (B) Group comparison of the coherence values between mPFC and dHIP for delta (<4 Hz), theta (4-12 Hz), beta (12-20 Hz) and low gamma (20-40 Hz) frequency bands. Results are presented as the mean ± SEM (n= 5-10 animals); *p<0.05, **p<0.01 and ***p<0.001, comparing with saline, calculated using an unpaired Student's *t* test. (C) Spectrograms of mPFC-dHIP coherence. Each horizontal line represents the spectrogram of an individual rat.

Figure 3. Effect of prenatal exposure to DEX on the number and length of dHIP microglial processes.

Pregnant Wistar rats received DEX (1 mg/kg/day, sc) at ED 18 and ED 19. Microglial cells of female brains were immunostained with Iba-1 at PND 7 and PND 90 and tridimensional reconstructions were performed using Neurolucida software. (A) Using the morphometric data extracted from the Neurolucida software, the number and length of microglial processes in the dHIP were assessed and compared between iuDEX-or saline-treated animals at PND 7. (B) Representative isolated manual reconstruction (skeleton) of microglial cells from the dHIP at PND 7 of females. (C) Number and length of microglial processes resulting from the morphometric analysis of reconstructed cells from dHIP, compared between iuDEX- or saline-treated animals at PND 90. (D) Representative isolated manual reconstruction (skeleton) of microglial cells from the dHIP at PND 90 of females. (E) Comparison of the number and length of microglial processes from mPFC and dHIP of control animals according to the respective branch order. (F) Representative isolated manual reconstruction (skeleton) of microglial cells from the dHIP and mPFC at PND 90 of females. Results are presented as the mean ± SEM (n= 3-6 animals); *p < 0.05, comparing with saline, calculated using a using an unpaired Student's *t* test.

Figure 4. Effect of chronic blockade of $A_{2A}R$ on the behavior and mPFC-dHIP coherence effects induced by prenatal exposure to DEX.

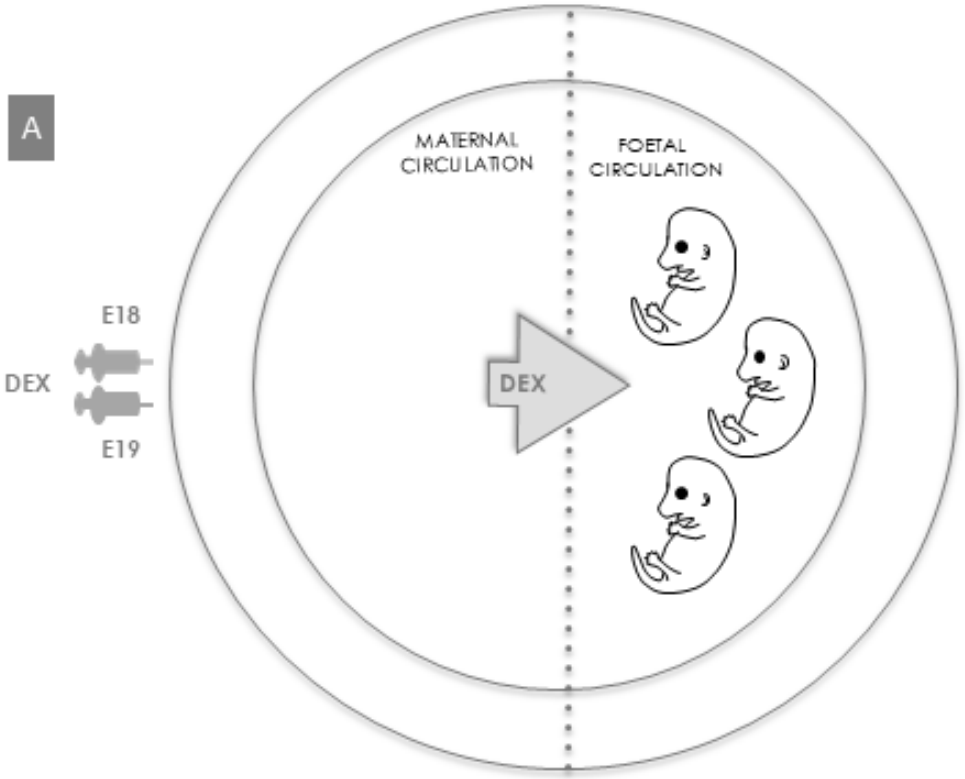
(A) Schematic drawing of the animal model and pharmacologic treatment: Animals from the offspring of saline- or DEX-treated pregnant rats were chronically treated with SCH58261 (0.1 mg/kg/day, ip) or saline for 21 consecutive days before PND 90. (B) Time spent in open arms *per* total time of the EPM test, performed to evaluate anxiety-related behavior. (C) The recognition index (time spent in the novel object *per* total time spent in the novel and familiar objects) was calculated to evaluate cognitive deficits, using the NOR test. (D) Diurnal and nocturnal quiescent levels of CORT were measured by ELISA, to evaluate the endogenous serum corticosterone levels. (E) Group comparison of the coherence values between mPFC and dHIP for delta (<4 Hz), theta (4-12 Hz), beta (12-20 Hz) and low gamma (20-40 Hz) frequency bands. (F) Spectrograms of mPFC-dHIP coherence. Each horizontal line represents the spectrogram of an individual rat. Results are presented as the mean ± SEM (n= 5-10 animals); *p<0.05, **p<0.01 and ***p<0.001, comparing with saline, $^{\$}p < 0.05$, comparing with iuDEX, calculated using a one-way ANOVA followed by a Turkey's multiple comparisons test.

Figure 5. Effect of $A_{2A}R$ chronic blockade on the morphologic alterations induced by prenatal exposure to DEX on the number and length of dHIP microglia processes.

Microglial cells of females at PND 90 were stained with Iba-1 and tridimensional reconstructions were performed using Neurolucida software. Using the morphometric data extracted from the Neurolucida software, the number (A, B) and length (C, D) of microglial processes in the dHIP was assessed and compared between treatments according to the respective branch order. (E) Representative isolated manual reconstruction of microglial cells. Results are presented as the mean ± SEM (n= 3-4 animals); *p < 0.05, comparing with saline,

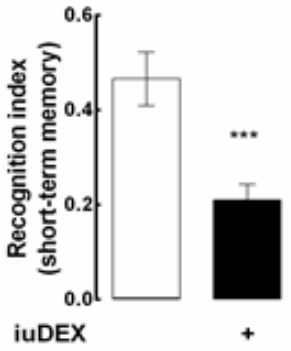
$^{\$}p < 0.05$, comparing with iuDEX, calculated using a one-way ANOVA followed by a Turkey's multiple comparisons test.

A



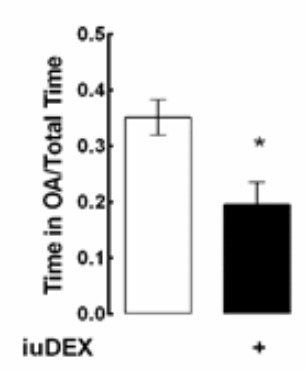
B

Novel **O**bject **R**ecognition



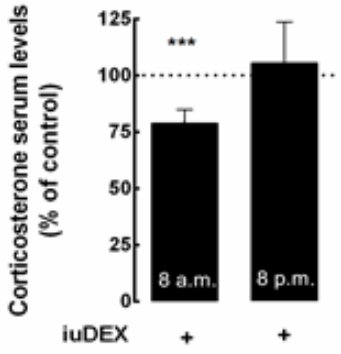
C

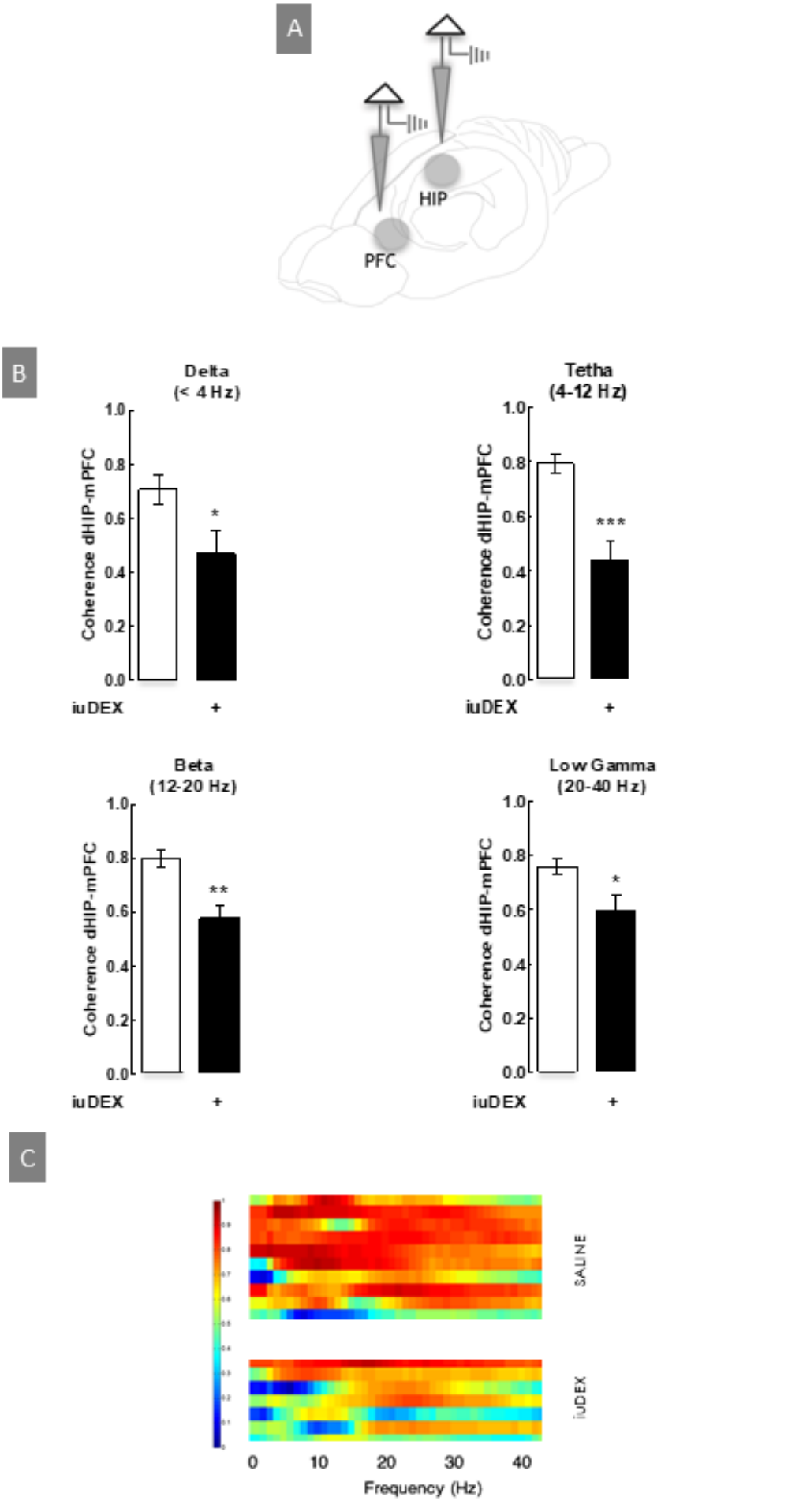
Elevated **P**lus **M**aze **T**est



D

Corticosterone




A
HIP
PFC
B
Delta
(< 4 Hz)
Coherence dHIP-mPFC
1.0
0.8
0.6
0.4
0.2
0.0
*
iuDEX
+
Tetha
(4-12 Hz)
***
Beta
(12-20 Hz)
**
Low Gamma
(20-40 Hz)
C
SALINE
iuDEX
0
10
20
30
40
Frequency (Hz)

A

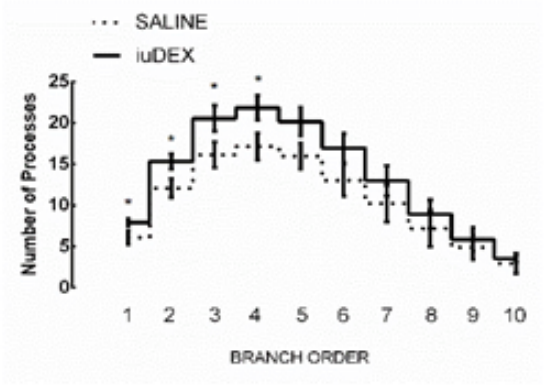

SALINE
iuDEX
Number of Processes
BRANCH ORDER

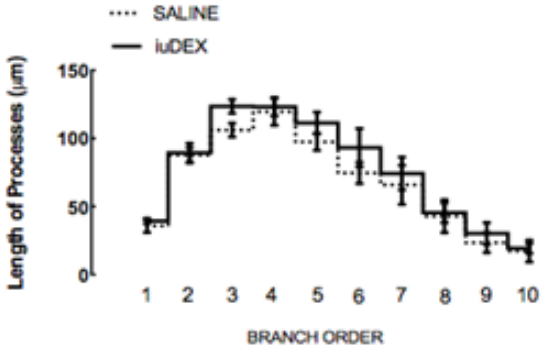

SALINE
iuDEX
Length of Processes (µm)
BRANCH ORDER

B

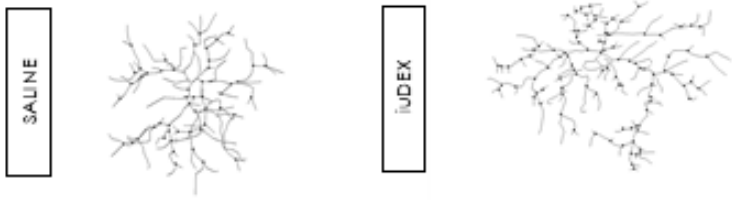

SALINE
iuDEX

C


SALINE
iuDEX
Number of Processes
BRANCH ORDER

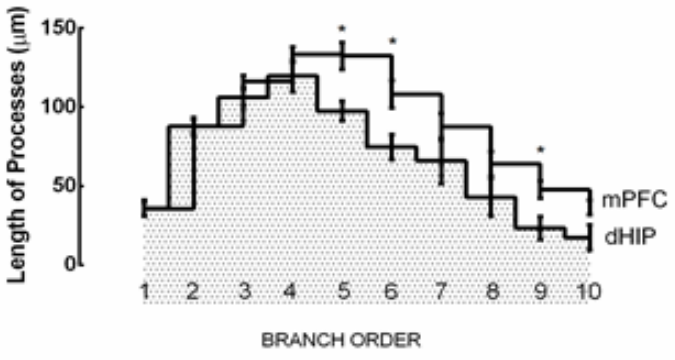

SALINE
iuDEX
Length of Processes (µm)
BRANCH ORDER

D

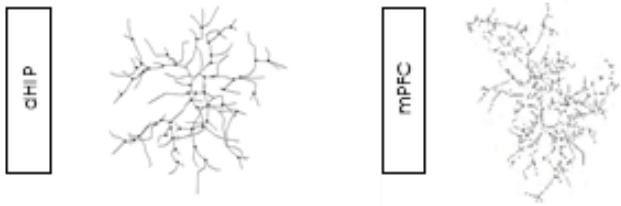

SALINE
iuDEX

E

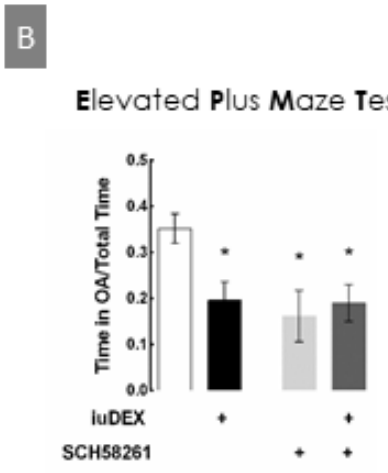

Number of Processes
mPFC
dHIP
BRANCH ORDER

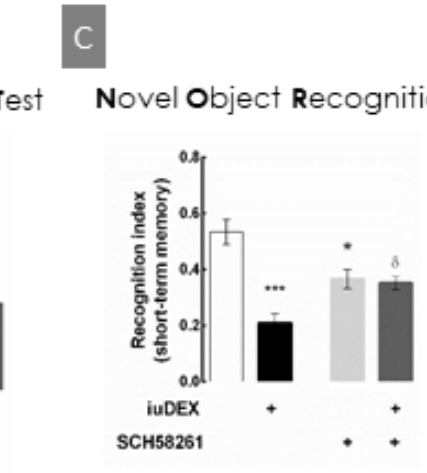

Length of Processes (µm)
mPFC
dHIP
BRANCH ORDER

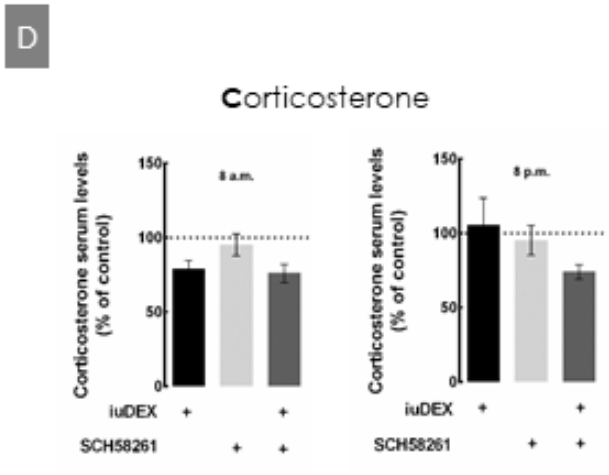
F

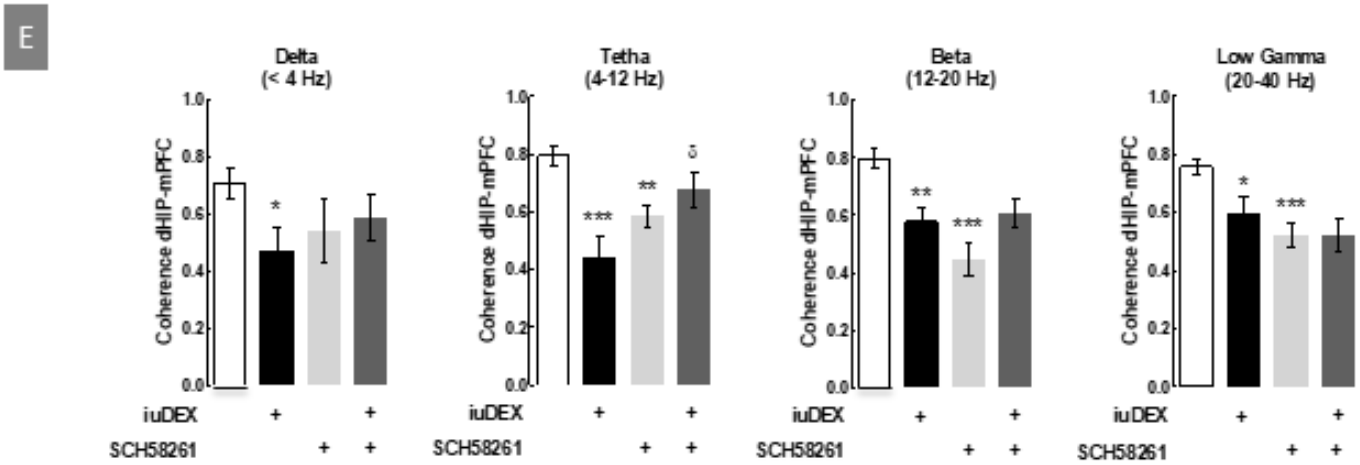

dHIP
mPFC

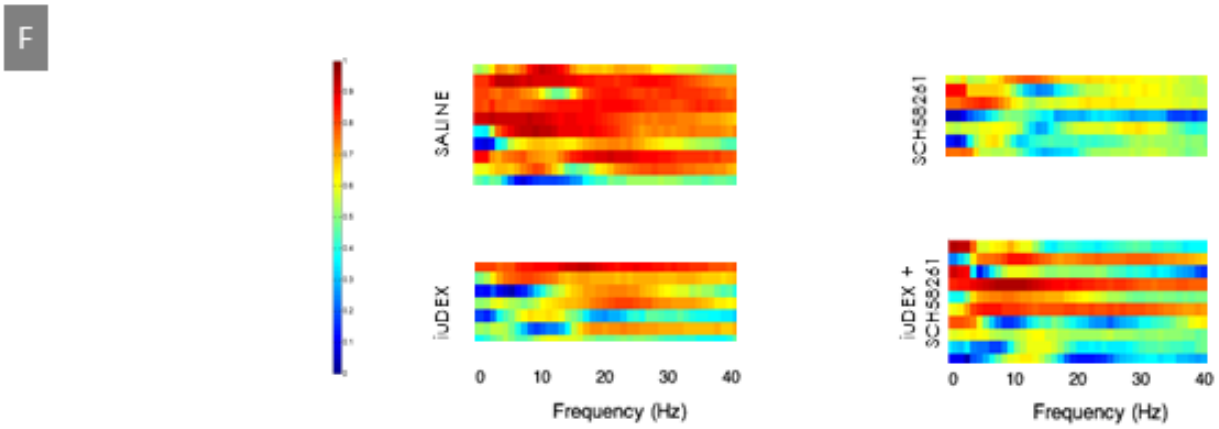

A
SCH58261
BIRTH
PND 90
female progeny


B
Elevated Plus Maze Test
Time in OA/Total Time
iuDEX
SCH58261

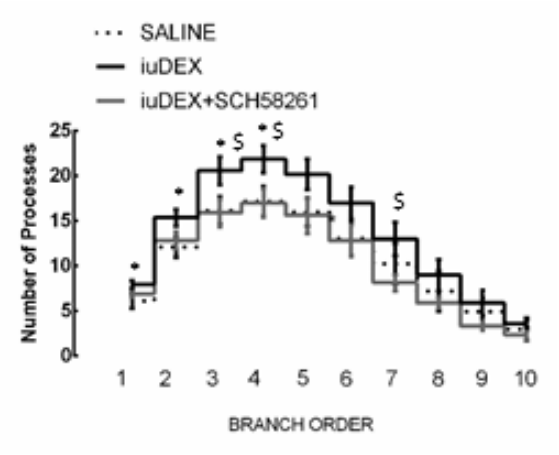

C
Novel Object Recognition
Recognition index (short-term memory)
iuDEX
SCH58261


D
Corticosterone
Corticosterone serum levels (% of control)
8 a.m.
8 p.m.
iuDEX
SCH58261

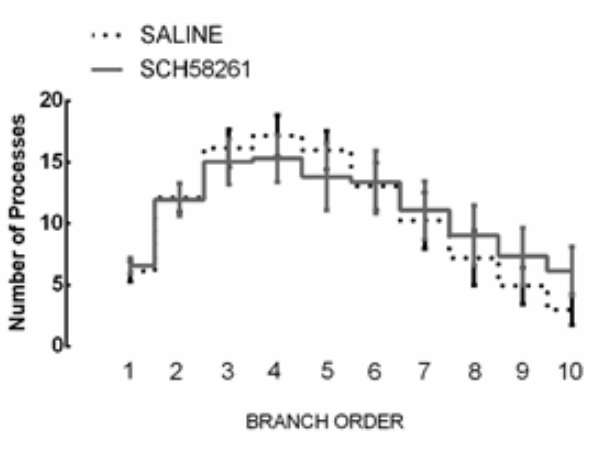

E
Delta (< 4 Hz)
Tetha (4-12 Hz)
Beta (12-20 Hz)
Low Gamma (20-40 Hz)
Coherence dHIP-mPFC
iuDEX
SCH58261


F
SALINE
iuDEX
SCH58261
iuDEX + SCH58261
Frequency (Hz)

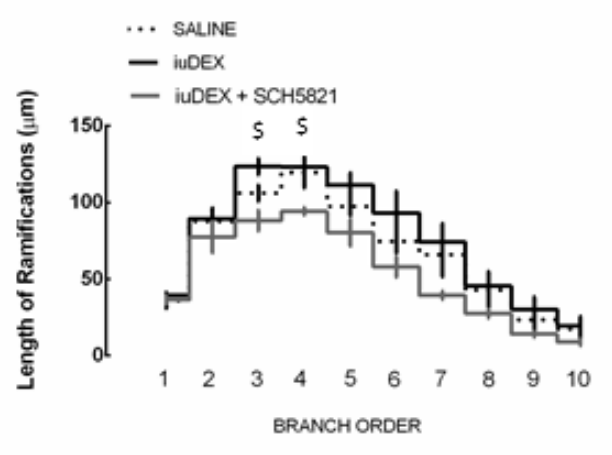
A


SALINE
iuDEX
iuDEX+SCH58261
Number of Processes
BRANCH ORDER

B


SALINE
SCH58261
Number of Processes
BRANCH ORDER

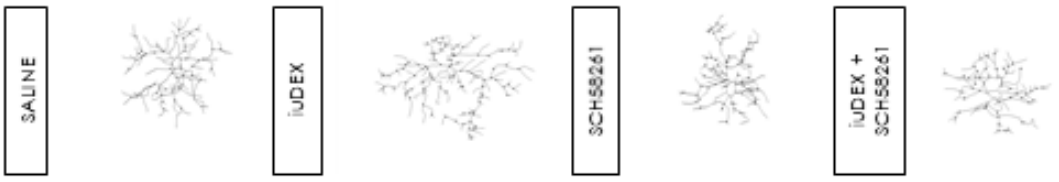
C


SALINE
iuDEX
iuDEX + SCH5821
Length of Ramifications (µm)
BRANCH ORDER

D


SALINE
SCH5821
Length of Processes (µm)
BRANCH ORDER

E


SALINE
iuDEX
SCH58261
iuDEX + SCH58261