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Evidence for a key role of the peripheral kynurenine pathway in the modulation of anxiety- and depression-like behaviours in mice: Focus on individual differences

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ABSTRACT

We previously reported that confronting mice to the Unpredictable Chronic Mild Stress procedure (UCMS) resulted in peripheral and cerebral alterations of the kynurenine pathway (KP). The present study tested whether KP disturbances are associated with differences in anxiety- and depressive-like behaviors in both naïve and UCMS mice. Non-stressed and UCMS mice were subjected to the elevated plus maze test and to the forced swim test. Mice were then sacrificed for quantification of tryptophan (TRP)-serotonin (5-HT) and TRP-kynurenine (KYN) metabolites in two corticolimbic structures involved in the regulation of mood (cingulate cortex = CC; amygdala = AMY). We showed that an elevated peripheral (lung) KYN/TRP ratio is correlated to the magnitude of anxiodepressive-like phenotypes only in UCMS mice. We also observed, in UCMS mice, that a high peripheral (lung) KYN/TRP ratio is associated with an increased metabolism of 5-HT in CC and a reduced level of kynurenic acid (KYNA) in AMY. Our results suggest that elevated peripheral KP might underlie cerebral biochemical changes and might consequently be involved in the modulation of behavior but only in UCMS mice. These findings make more complete the comprehension of the KP involvement in behavioral changes induced by chronic stress and suggest that it could play a crucial role in the modulation of emotional states.

1. Introduction

Since 1970, the major route of tryptophan (TRP) metabolism – the so-called kynurenine pathway (KP) – is suspected to be related to the pathophysiology of depressive disorders (Curzon and Bridges, 1970). In this work, Curzon and Bridges reported that patients with major depression presented high levels of urinary kynurenine (KYN) and other KP metabolites like 3-hydroxykynurenine (3HK) (see Fig. 1 for a description of the metabolic pathways of tryptophan). This phenomenon was widely attributed to a glucocorticoid-induced activation of tryptophan-2,3-dioxygenase (TDO), one of the two enzymes converting TRP to KYN. Glucocorticoids are chemical mediators secreted in response to stress through activation of the Hypothalamus–Pituitary–Adrenal (HPA) axis. Stress is a key etiological factor in anxiety and major depressive disorders (Caspi et al., 2003) and it is now largely acknowledged that HPA axis abnormalities are one of the major features of depressive disorders leading to high levels of circulating cortisol (Barden, 2004). Later on, it was proposed that depressive states could also be associated with immune system dysregulations leading to an increased production of proinflammatory cytokines (Myint et al., 2005; Renault et al., 1987; Smith, 1991). Such a phenomenon also occurs in response to stress (Steptoe et al., 2007). Interestingly, it was found that the second enzyme converting TRP to KYN, the indoleamine-2,3-dioxygenase (IDO), was activated by proinflammatory cytokines (O'Connor et al., 2009a). Therefore, two depression-related mechanisms, the neuroendocrine response to stress and inflammatory processes, might activate the KP.

More recently, a body of evidence has considerably strengthened the hypothesis of a crucial involvement of the KP in the pathophysiology of depression and other stress-related disorders since the KYN/TRP ratio was found to be increased in the blood of patients suffering from major depression (Maes et al., 2002; Myint et al., 2006). An increased KYN/TRP ratio was also observed in patients treated with interferon α (IFNα) experiencing depressive symptoms (Capuron et al., 2002; Vignau et al., 2009; Wichers et al., 2005). In line with this are the data from the group of Fuchs et al. on the evidence of an association between neuropsychiatric symptoms induced by innate

Abbreviations: UCMS, unpredictable chronic mild stress; TRP, tryptophan; KYN, kynurenine, 5-HT, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; KP, kynurenine pathway; 3HK, 3-hydroxykynurenine; QUIN, quinolinic acid; KYNA, kynurenic acid; IDO, indoleamine-2,3-dioxygenase; TDO, tryptophan-2,3-dioxygenase; IFN, interferon; CC, cingulate cortex; HIPPO, hippocampus; AMY, amygdala; HPLC, high performance liquid chromatography.

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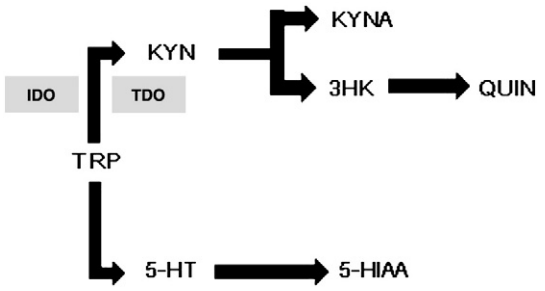


Fig. 1. Simplified overview of the tryptophan metabolism. TRP = tryptophan; 5-HT = serotonin; 5-HIAA = 5-hydroxyindoleacetic acid; KYN = kynurenine; TDO = tryptophan-2,3-dioxygenase; IDO = indoleamine-2,3-dioxygenase; 3HK = 3-hydroxykynurenine; QUIN = quinolinic acid, KYNA = Kynurenic acid.

immune system and activation of the KP [see for review (Widner et al., 2002)]. Animal studies have also strongly contributed to support this assumption as it was found that an elevated peripheral KP (measured by the blood KYN/TRP ratio) was related to expression of depression-like behaviors in the forced swim test (FST) while its inhibition resulted in a reduction of these behaviors (O'Connor et al., 2008). In addition, we have recently demonstrated that confronting mice to the Unpredictable Chronic Mild Stress (UCMS) – an animal model of stress-induced depression – promoted an increase of the KYN/TRP ratio at the peripheral level (lung) (Laugeray et al., 2010).

Peripheral KYN can be taken-up from blood to brain, where it is further metabolized along two distinct pathways (see Fig. 1). On one side, it is transformed to quinolinic acid (QUIN) whereas on the other side kynurenic acid (KYNA) is synthesized. These compounds are interesting as modulators of emotional states since they respectively increase and decrease the extracellular level of glutamate (Tavares et al., 2005; Wu et al., 2009) which has been shown to be involved in anxiety and stress-related disorders (Cortese et al., 2010; Cortese and Phan, 2005). We have shown that UCMS promotes a corticolimbic region-specific imbalance between QUIN and KYNA pathways suggesting that glutamatergic homeostasis could be differentially modulated by KP changes in these structures (Laugeray et al., 2010).

The aim of our study was first to verify whether peripheral KP is related to stress-related behaviors in mice confronted to the UCMS. As patients with the highest KYN/TRP ratios are also those having the most intensive depressive symptoms (Maes et al., 2002; Wichers et al., 2005), we expected to obtain a similar link between behavior and peripheral (lung) KP activity measured by the KYN/TRP ratio in mice. Secondly, given that serotoninergic metabolism has long been implicated in the regulation of emotional states, we measured serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels to check if peripheral KP could be related to changes in 5-HT neurochemistry. Cerebral QUIN and KYNA contents were also evaluated because of their modulating role on glutamatergic neurotransmission that is also importantly involved in emotional reactivity (Cortese and Phan, 2005; Kugaya and Sanacora, 2005). All analyses were also performed in a group of non-stressed mice. Measurements have been carried out in two corticolimbic structures known to be importantly involved in the regulation of emotional states (cingulate cortex = CC; amygdala = AMY) (Mayberg, 1997).

It is now widely accepted that mice of the same strain may have different behavioral/biochemical response to stress, even if genetically identical (Koolhaas, 2008; Lathe, 2004), and that confronting mice to aversive situations may lead to inconsistent results if individual differences are not taken into account. Thus, we used correlations rather than classical intergroup comparisons in order to bring more informative data on the extent to which peripheral KP is linked to the modulation of stress-related behaviors and brain chemistry in both normal and chronic stress conditions.

2. Materials and methods

2.1. Animals

Male BALB/c mice aged of 2 months were purchased from *Centre d'Élevage Janvier* (Le Genest Saint Isle, France). Animals were group-housed (n = 5 per cage) until the beginning of the experiments and maintained under standard laboratory conditions (12 h/12 h light-dark cycle – on at 8:00/off at 20:00 – 22 ± 1 °C, food and water *ad libitum*). All animal care and treatment were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All the mice used in this study are from the same group that have already been tested by the authors in the previous article (Laugeray et al., 2010).

2.2. General procedure

On their arrival, mice were kept in the laboratory for two weeks before the onset of the experiments. A 6-week UCMS procedure was then conducted on half of the mice (n = 14). UCMS exposed mice were isolated in small individual cages (24 cm × 11 cm × 12 cm), while non-stressed control mice (n = 15) were housed in groups of 5 in standard laboratory cages (42 cm × 28 cm × 18 cm). UCMS and control mice were housed in two different rooms. The day after the end of UCMS, mice behavior was assessed as follow: elevated plus maze test (EPM) on the first day and forced swim test (FST) on the second day. One day after the last behavioral assay, mice were sacrificed and relevant peripheral/brain tissues were collected. Then, neurochemical analyses were performed. The experimental design is illustrated in Fig. 2A.

2.3. Unpredictable Chronic Mild Stress (UCMS)

It is a variant of the chronic mild stress procedure described by Willner in rats (Willner, 1997) and then adapted to mice (Monleon et al., 1995). Briefly, mice were recurrently subjected to various psychogenic stressors according to a "random" schedule for a total period of 6 weeks. The stress schedule is detailed on Fig. 2B. The stress procedure did not involve any food or water deprivation. The stressors were: altered bedding (change or removal of sawdust, damp sawdust, substitution of sawdust with 21 °C water), cage exchange (mice were placed in the empty cage of another male), altered length and time of light/dark cycle.

2.4. Behavioral procedures

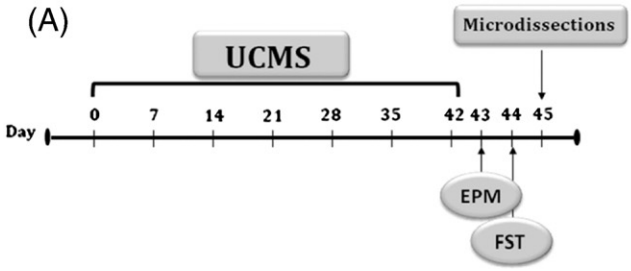
2.4.1. The elevated plus maze (EPM)

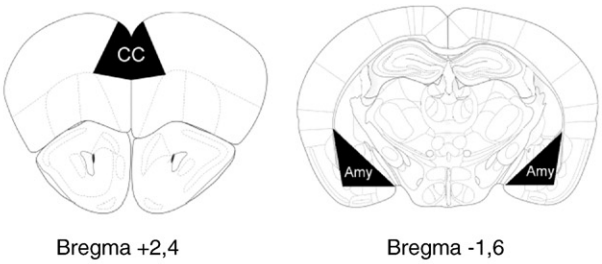
The EPM was used because of its documented ability to readily detect the variability of anxiety-like behaviors in rodents (Pellow et al., 1985; Yee et al., 2007). The apparatus was composed of a central part (5 × 5 cm), two opposing open arms (27 × 5 cm) and two opposing enclosed arms (27 × 5 × 15 cm). The maze was made of black Plexiglas, elevated at a height of 40 cm and the open arms were illuminated by two 20-W white bulbs providing a 50-lx illumination on their extremities. The test lasted 5 min and began with the placement of mice in the center of the maze, facing an enclosed arm. A video camera mounted above the maze was used to record each trial and to allow a later analysis. The time spent in anxiogenic open arms was used as a conventional spatiotemporal measure of open-arm avoidance. However, given that BALB/c mice are known to strongly express avoidance of open spaces (Anisman et al., 2001) we also assessed ethological measures of risk assessment (time spent in stretched-attend postures + time spent in flat-back approach) also reported to be relevant as an index of anxiety (Rodgers et al., 1997; Rodgers and Johnson, 1995).

2.4.2. The forced swim test (FST)

The FST was performed according to standard published procedures with minor modifications (Ducottet et al., 2003; Porsolt et al.,

(A)





(B)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Week 1	Coat state (10h) Damp sawdust (12h30-14h30) Without sawdust (15h30-18h30)	Three changes of sawdust (10h - 11h - Cat fur (15h30-17h)	Bath (10h-11h) Sounds of predators (15h30-16h) Without sawdust (16h-18h)	Restraint (9h30-10h30) 3 changes with rat sawdust (15h - 16h -	Without sawdust (10h-12h30) Reversal of the light-dark cycle	Short succession of light-dark cycles - every 1h (10h-14h)	Without sawdust (12h-14h) Restraint (14h-16h) New sawdust (18h)
Week 2	45° tilt cages (9h-10h) Coat state (11h) Bath (12h30-13h30) Mice, food weighing (15h) Damp sawdust (15h30h-16h30)	Food weighing (24h consumption; 9h) Old mouse sawdust (9h30-11h30) Sounds of predators (12h-12h30) 10sec in water (20°C; 13h) Cat fur (15h-16h)	Reversal of the light-dark cycle Social stress (10h) Souds of predator (11h-11h30) Social stress (14h) Rat sawdust (16h-18h)	Without sawdust (9h-10h30) Restraint (10h30-11h30) New sawdust (14h)	Short succession of light-dark cycles - every 1h (14h-18h)	Short succession of light-dark cycles - every 1h (10h-14h)	Reversal of the light-dark cycle
Week 3	Coat state (10h) Damp sawdust (10h30-12h30) Mice, food weighing (15h) Restraint (15h16h)	Food weighing (24h consumption; 9h) 45° tilt cages (11h-14h) Potent light (17h-18h)	Without sawdust (13h) Social stress (15h)	Reversal of the light-dark cycle	New sawdust (10h) Social stress (15h)	Short succession of light-dark cycles - every 1h (9h-18h)	Cat fur (12h-14h) Social stress (14h) Damp sawdust (15h-18h)
Week 4	Coat state (10h) Rat defecations in cages (10h30) Cat fur (14h) Damp sawdust (15h17h)	Food weighing (24h consumption; 9h) New sawdust (9h) 45° tilt cages (11h-14h) Old rat sawdust (14h)	New sawdust (9h) Bath (10h-11h)	45 ° tilt cages (10h-13h) Sounds of predators (14h-14h30) Damp sawdust (15h30-16h30) Social stress+new sawdust (16h)	Short succession of light-dark cycles - every 1h (9h-18h)	Reversal of the light-dark cycle	45° tilt cages (11h-13h) Without sawdust (13h-15h) Social stress (15h)
Week 5	Coat state (10h) New sawdust (14h) Rat defecations in cages (16h30) Social stress (17h30)	Food weighing (24h consumption; 9h) 45° tilt cages (14h-15h30) Social stress (15h) Restraint (15h30-17h30)	10sec in water (20°C; 11h) Restraint (12h30-13h30) Cat fur (13h30) Damp sawdust (14h30-16h)	New sawdust (9h) New sawdust (11h30)	Social stress (14h) Damp sawdust (15h-16h) Social stress (16h) New sawdust (16h30)	Reversal of the light-dark cycle	Short succession of light-dark cycles - every 1h (9h-18h)
Week 6	Coat state (10h) Social stress (14h) 45° tilt cages (14h30-17h30)	Food weighing (24h consumption; 9h) 45° tilt cages (10h-12h30) Without sawdust (15h)	New sawdust (9h) Potent light (9h-12h) Bath (12h-14) Restraint (14h-16h)	Reversal of the light-dark cycle	Social stress (14h) 45° tilt cages (14h-18h)	Short succession of light-dark cycles - every 1h (9h-18h)	45° tilt cages (14h-16h) Restraint (17h-18h)

Fig. 2. Experimental design. (A) 14 mice were confronted to the *Unpredictable Chronic Mild Stress* (UCMS) regimen during 6 weeks. One day after the last stressor, mice were tested in the elevated plus maze test (EPM) and the forced swim test (FST). Cingulate cortex (CC) and amygdala (AMY) were collected from all the mice for biochemical analyses, one day after the last behavioral test. Representative figures of the brain regions dissected are adapted from Paxinos and Franklin (2001). (B) Detailed schedule of the UCMS.

1977). Mice were placed into a glass cylinder (12 cm diameter, 25 cm tall) filled to a depth of 10 cm with water (23 °C). A 6 min test session was conducted and videotaped for a latter analysis. The time of immobility was measured during the last 4 min of test. A mouse was considered immobile when floating passively performing only slight movements of its limbs and tail without any gross head movements that could be judged as searching. "immobility" included slow drifting on the water surface without any initiation of swimming.

2.5. Tissue sampling

2.5.1. Brain tissues

Brain structures were microdissected by a single investigator as previously described (Surget et al., 2009). Brains were rapidly removed from CO_2-killed mice and placed in ice-cold slurry of 0.9% NaCl. Rostro-caudal sections (2 mm) were quickly obtained on a brain tissue blocker with slots that served as guides for razor blades. These provided a series of four coronal brain sections from Bregma +2.4 to - 3.1 that were then microdissected (Fig. 2A). Using the mouse brain atlas of Paxinos and Franklin (2001), CC was dissected from the first section and included prelimbic and cingulate cortices. AMY was obtained from the third section. All samples were immediately frozen and stored at −80 °C.

2.5.2. Peripheral tissues

Lungs were removed just after the brain was extracted from the mice skull (by another investigator) and immediately frozen and stored at −80 °C.

2.6. Biochemical measurements of TRP metabolites

TRP, 5-HT and 5-HIAA were determined by high-performance liquid chromatography (HPLC) using the method of Kema et al. (Kema

et al., 1993). KYN was measured using HPLC as described by Fujigaki et al (Fujigaki et al., 1998). KYN/TRP ratio was calculated from absolute concentrations of KYN and TRP.

KYNA was purified as previously described in Moroni et al., 1988 (Moroni et al., 1988) and quantified using HPLC with a post column derivatization method (Swartz et al., 1990). Separation was obtained with a reverse phase column (S10 ODS2) and a mobile phase containing 50 mM sodium acetate buffer (pH 6.20) and 4% acetonitrile at a flow rate of 1 ml:min. The post column derivatizating agent was 0.5 M zinc acetate at a flow rate of 0.6 ml:min. Quantification was obtained using a Perkin Elmer (mod. LC 240) fluorimeter with an excitation wavelength of 344 nm and an emission of 398 nm.

QUIN was measured in tissue homogenates and in incubation media using mass-fragmentography and a modification of procedures previously reported (Heyes and Markey, 1988; Moroni et al., 1984). Briefly, after the addition of 13C-QUIN as an internal standard, each sample was dried and derivatized at 80 °C for 1 h with 100 ml of hexafluor-2-propanol and 100 ml of trifluoroacetylimidazole. Water (100 ml) and heptane (100 ml) were added to the derivatized samples which were vigorously mixed and then frozen at −80 °C. The heptane fraction was collected and injected into a GC: MS system (HP6890, GC: HP5973MS) equipped with an automatic injector. The chromatographic column used was a HP 5MS 30 m_ 0.25 mm_0.25 mm. The carrier gas was helium at a constant flow of 1.2 ml:min. The oven temperature was, 1 min at 80 °C, raised at a rate of 10 °C:min to 135 °C and then at a rate of 25 °C:min to 300 °C. Injector and transfer line temperatures were 230 and 270 °C, respectively. The mass spectrometry detector operated at 70 eV in the selected ion monitoring mode. Six ions (272.1:300.1:448.1 for QUIN and 278.1:307.1:455.1 for 13C-QUIN) were recorded; the dwell time was 50 ms for each ion. Sensitivity of the method was 0.11 nM (1.9 pg/sample); Intra-assay CV was 3.34%; Inter-assay CVwas 5.72%.

2.7. Statistics

Because our goal is not to make comparisons between non-stressed and UCMS mice but rather to explore the extent to which peripheral (lung) KP could be related to both emotional reactivity and brain chemistry in each group. Consequently, Spearman rank correlation coefficients were used to characterize the link between emotional reactivity in the EPM/FST and peripheral KP on the one hand and between neurochemical parameters and peripheral KP on the other. Given that calculating numerous correlations increases the risk of a type I error, i.e., to erroneously conclude the presence of a significant correlation, all reported p-value were Bonferroni-corrected (Curtin and Schulz, 1998).

3. Results

3.1. Link between peripheral (lung) KP and anxiety/depression-like behaviors (Fig. 3)

3.1.1. In UCMS mice

The Spearman correlation test indicates that peripheral KP activity, measured by the KYN/TRP ratio, is positively correlated with time spent in risk-assessment in the EPM ($r=0.57$; $p=0.032$) and with immobility time in the FST ($r=0.66$; $p=0.014$). By contrast, no correlation was observed between KYN/TRP and the time spent in open arms of the EPM ($r=-0.20$; $p=0.48$).

3.1.2. In non-stressed mice

The Spearman correlation test revealed no correlations between peripheral KYN/TRP ratio and any behavioral parameters in the EPM and the FST: time in open arms of the EPM ($r=-0.10$; $p=0.73$), time in risk assessment ($r=0.03$;$p=0.92$), immobility time in the FST ($r=0.07$;$p=0.80$).

(A)

	UCMS			NON STRESSED		
	EPM		FST	EPM		FST
	Time in open arms (s)	Time in risk assessment (s)	Immobility time (s)	Time in open arms (s)	Time in risk assessment (s)	Immobility time (s)
Peripheral KP activity (KYN/TRP ratio)	r=-0.20 p=0.48	**r=0.57 p=0.032**	**r=0.66 p=0.014**	r=-0.10 p=0.73	r=0.03 p=0.92	p=0.80

(B)

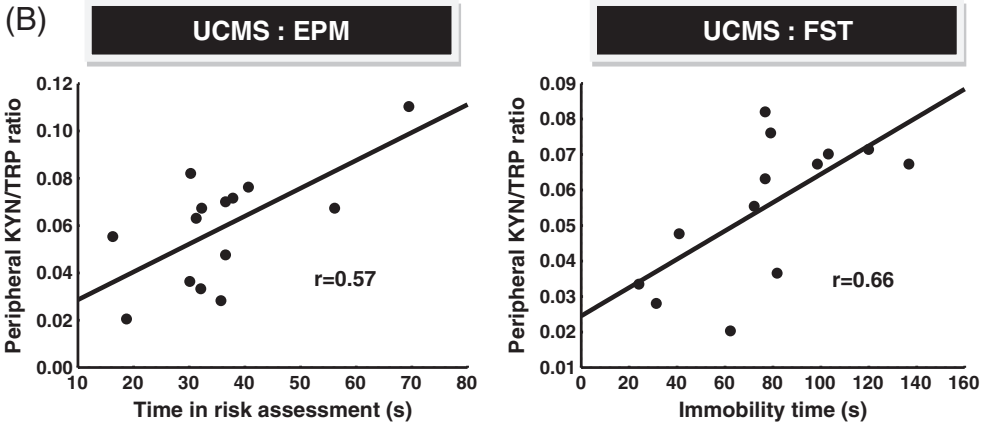


Fig. 3. Correlations between peripheral (lung) kynurenine pathway and anxiety/depression-like behaviors in both Unpredictable Chronic Mild Stress and non-stressed mice. Peripheral kynurenine pathway (KP) activity was estimated by measuring the kynurenine to tryptophan (KYN/TRP) ratio in the lungs of both *Unpredictable Chronic Mild Stress* (UCMS) and non-stressed mice. Anxiety and depression-like behaviors were respectively assessed in the elevated plus maze (EPM) test and the forced swim test (FST). (A) Spearman's coefficient and associated p value are reported for each correlation. Bold values indicate a significant correlation ($p<0.05$ Bonferroni-corrected). (B) Significant correlated variables are depicted as dot plots.

3.2. Link between peripheral (lung) KP and brain chemistry

3.2.1. 5-HT metabolism (Fig. 4)

3.2.1.1. In UCMS mice. A positive correlation was found between the peripheral (lung) KYN/TRP ratio and the concentration of 5-HT in CC ($r=0.56$; $p=0.04$). While a similar positive correlation was observed between KYN/TRP and 5-HIAA in CC ($r=0.64$; $p=0.02$), no such associations were seen with 5-HT ($r=0.19$; $p=0.65$) and 5-HIAA ($r=0.33$; $p=0.27$) in AMY.

3.2.1.2. In non-stressed mice. CC 5-HT ($r=0.07$; $p=0.80$), AMY 5-HT ($r=-0.22$; $p=0.44$), CC 5-HIAA ($r=-0.09$;$p=0.76$) and AMY 5-HIAA ($r=-0.08$;$p=0.78$) were not found to be correlated with peripheral KYN/TRP.

3.2.2. KYN metabolism (Fig. 5)

3.2.2.1. In UCMS mice. Like KYNA levels in CC, the concentrations of QUIN in CC and AMY were not found to be correlated with peripheral (lung) KYN/TRP ratio ($r=-0.44$; $p=0.15$–$r=0.04$; $p=0.88$–$r=-0.02$; $p=0.96$ respectively). However, the content of KYNA in AMY was negatively associated with peripheral KYN/TRP ($r=-0.62$; $p=0.03$).

3.2.2.2. In non-stressed mice. Similarly, neither QUIN nor KYNA were linked to peripheral KYN/TRP in any of the two structures investigated: CC QUIN ($r=-0.08$; $p=0.77$), AMY QUIN ($r=0.08$; $p=0.77$), CC KYNA ($r=0.07$; $p=0.80$) and AMY KYNA ($r=0.20$; $p=0.50$).

4. Discussion

Given that clinical evidence suggest a close association between anxiety/depression symptoms and elevated peripheral KP activity, we investigated the extent to which peripheral (lung) KP could be related to both behavioral and biochemical parameters in both UCMS and non-stressed mice. We showed that mice with a high peripheral KP activity are also those expressing more anxiety-like and depressive-like behaviors, but this association is only seen in UCMS mice. Moreover, we found that these mice also have higher levels of 5-HT and 5-HIAA in CC and lower levels of KYNA in AMY, which could likely underlie the expression of anxiety and depressive-like behavioral patterns. Even if it was not the purpose of the study to compare control *vs.* UCMS mice, the effect of the UCMS on all the biochemical measures can be appreciated in Laugeray et al., 2010 and the behavioral effect is described on Table S1 as supplementary data in this report.

4.1. Role of the peripheral (lung) KP in the modulation of anxio-depressive states

Peripheral KP activity, mediated by TDO/IDO activation, seems to play a key role in the modulation of affective behaviors both in humans and rodents (Moreau et al., 2008; O'Connor et al., 2009b, 2008). Several clinical works reported an increased activity of the KP in the plasma of patients experiencing anxiety/depression (Maes et al., 2002; Myint et al., 2006). Similarly, a large preclinical evidence suggests that activation of the peripheral KP is related to the expression of a depressive-like syndrome (O'Connor et al., 2008). Despite these data, the question of the respective roles of peripheral and brain KP in these effects was left unresolved. With regard to this question, important clues can be provided by our results. Indeed, we previously showed that peripheral KP is activated after a 6-week period of UCMS (Laugeray et al., 2010). In the present study, we observe that UCMS-mice having higher peripheral KP activity express more risk assessment behaviors in the EPM and are more resigned in the FST. Interestingly this result is not observed in non-stressed animals, indicating that activity of the peripheral KP is obviously only involved in the modulation of UCMS-induced behavioral responses. These findings are not in agreement with those of O'Connor et al. who

(A)

	UCMS				NON STRESSED			
	5-HT (pmol/mg)		5-HIAA (pmol/mg)		5-HT (pmol/mg)		5-HIAA (pmol/mg)	
	CC	AMY	CC	AMY	CC	AMY	CC	AMY
Peripheral KP activity (KYN/TRP ratio)	**r=0.56 p=0.04**	r=0.19 p=0.65	**r=0.64 p=0.02**	r=0.33 p=0.27	r=0.07 p=0.80	r=-0.22 p=0.44	r=-0.09 p=0.76	r=-0.08 p=0.78

(B)

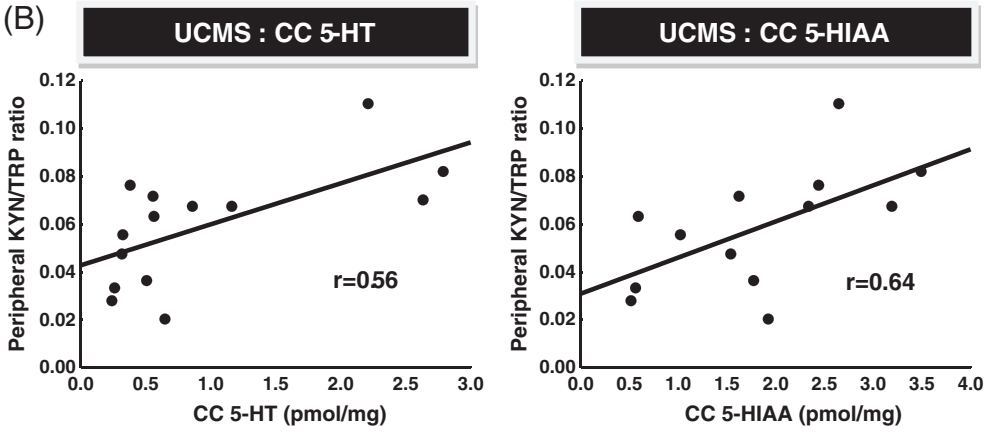


Fig. 4. Correlations between peripheral (lung) kynurenine pathway and cerebral 5-HT metabolism. Peripheral kynurenine pathway (KP) activity was estimated by measuring the kynurenine to tryptophan (KYN/TRP) ratio in the lungs of both *Unpredictable Chronic Mild Stress* (UCMS) and non-stressed mice. 5-HIAA and 5-HT levels were determined in amygdala (AMY) and cingulate cortex (CC) of all mice by HPLC. (A) Spearman's coefficient and associated p value are reported for each correlation. Bold values indicate a significant correlation ($p<0.05$ Bonferroni-corrected). (B) Significant correlated variables are depicted as dot plots.

(A)

	UCMS				NON STRESSED			
	QUIN (nmol/mg)		KYNA (pmol/mg)		QUIN (nmol/mg)		KYNA (pmol/mg)	
	CC	AMY	CC	AMY	CC	AMY	CC	AMY
Peripheral KP activity (KYN/TRP ratio)	r=0.04 p=0.88	r=-0.02 p=0.96	r=-0.44 p=0.15	**r=-0.62 p=0.03**	r=-0.08 p=0.77	r=0.08 p=0.77	r=0.07 p=0.80	r=0.20 p=0.50

(B)

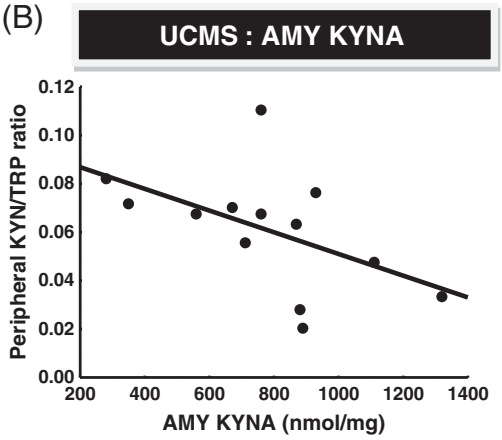


Fig. 5. Correlations between peripheral (lung) kynurenine pathway and cerebral kynurenine pathway metabolites. Peripheral kynurenine pathway (KP) activity was estimated by measuring the kynurenine to tryptophan (KYN/TRP) ratio in the lungs of both *Unpredictable Chronic Mild Stress* (UCMS) and non-stressed mice. Quinolinic acid (QUIN) and kynurenic acid (KYNA) levels were determined in amygdala (AMY) and cingulate cortex (CC) as described in "Materials and methods" section. (A) Spearman's coefficient and associated p value are reported for each correlation. Bold values indicate a significant correlation ($p<0.05$ Bonferroni-corrected). (B) Significant correlated variables are depicted as dot plots.

found that non-stressed mice receiving a systemic administration of KYN expressed more pro-depressant behaviors in the FST (O'Connor et al., 2008). In addition, our results are not in accordance with those of Lapin *et al.* who reported a raised expression of anxiety-like behaviors in non stressed mice (Lapin, 2003). These discrepancies could be due to the fact that pharmacological doses of KYN were used in these studies. Such conditions are likely to mimic an important activation of the KP, maybe more than it is during UCMS, and consequently, may alter behavior through high levels of KYN metabolites in the brain. Given that the lung KYN/TRP ratio is classically used as an index of IDO activity, it would be easy to conclude that the expression of stress-related behaviors is associated with the activity of IDO. However, as the animals were not blood-perfused, blood-derived contents of KYN and TRP (largely affected by the activity of TDO in the liver) might have significantly contributed to our dosage. That is why it is not possible to conclude on what enzyme is involved in associations reported in this study.

4.2. Peripheral (lung) KP activity is related to brain 5-HT neurochemistry

These findings raise the question of the mechanism by which a high peripheral KP activity could promote stress-related behaviors in UCMS mice. Several hypotheses have been formulated to answer this question. Some authors proposed that activated KP could deplete circulating TRP and then reduce brain 5-HT synthesis inducing serotonin-depletion-related behavioral abnormalities (Konsman et al., 2002; Myint and Kim, 2003). But we show here that it is obviously not the case. Indeed, if this assumption was true we should have obtained a negative correlation between cerebral 5-HT levels and peripheral KYN/TRP ratio which is not the case. On the contrary we found a positive correlation between CC 5-HT and the KYN/TRP ratio. This is in agreement with our previous study in which we did not observe any effect of UCMS-related KP activation on brain 5-HT metabolism (Laugeray et al., 2010). This demonstrates that behavioural differences observed in these mice are not due to depleted levels of 5-HT. Interestingly, we report a more elevated level of 5-HT and 5-HIAA in the CC of UCMS mice presenting a high peripheral KP activity.

CC is a part of the medial prefrontal cortex (mPFC) and it is well established that mPFC is responsive to stressors (Bland et al., 2003). At a behavioral level, 5-HT in the mPFC has been implicated in stress-related anxiety and fear. 5-HT efflux has been shown to be increased in this structure as a result of the psychological stress induced by conditioned fear (Hashimoto et al., 1999) or by the confrontation to aversive situations such as an elevated platform (Rex et al., 1993). Moreover, it has been argued that uncontrollable stress produced anxiety associated with increases in extracellular 5-HT (Bland et al., 2003). According to these data, one can suppose that a high peripheral KP could be related to negative affective state induced by UCMS as we found high levels of 5-HT and 5-HIAA in the CC of UCMS having the highest peripheral KYN/TRP ratio. This is in agreement with present findings showing that UCMS mice having a high peripheral KP activity express more anxiety- and depression-like behaviors. Taken together, these data indicate that, under pathological conditions such as chronic stress, activated peripheral KP might be associated with both behavioral and cerebral serotoninergic changes.

4.3. Cerebral KP and modulation of stress-related behaviors

To explain the link between a high peripheral (lung) KP activity and the rise in anxio-depressive behaviors, it was also suggested that neuroactive compounds further produced along the KP could be of importance as some of them are known to modulate glutamatergic neurotransmission. This is the case for QUIN and KYNA that act respectively as pro- and anti-glutamatergic compounds (Connick and Stone, 1988; Hilmas et al., 2001).

A new point that is put forward in this study is the involvement of cerebral KP in the modulation of emotional reactivity. Indeed, we show that, in addition to be related to both behavioral and cerebral serotoninergic changes in UCMS mice, peripheral (lung) KP is also

associated with cerebral KP changes. Indeed, the level of KYNA in AMY was found to be negatively correlated with the peripheral KYN/TRP ratio, suggesting that UCMS mice having an elevated peripheral KP activity are also those that have the lowest levels of KYNA in AMY. This is of particular importance considering the role of KYNA. Initially described as a broad spectrum antagonist of ionotropic glutamate receptors (Perkins and Stone, 1982), KYNA was later shown to block the glycine site of the NMDA receptor with much higher potency (Kessler et al., 1989). However, KYNA is unlikely to inhibit this site under physiological conditions (Hilmas et al., 2001). Rather, endogenous KYNA (at the nanomolar range) can exert its anti-glutamatergic action by antagonizing α7 nicotinic receptors (Hilmas et al., 2001). Indeed, microdialysis studies show that nanomolar concentrations of KYNA are able to reduce the extracellular content of glutamate in prefrontal cortex (Wu et al., 2009) and striatum (Carpenedo et al., 2001), resulting in a decrease of glutamate-related excitatory effect. What is interesting regarding our study is that the mean KYNA concentration is about 30 nM regardless of the structure, which is in the same range that those reported in the work of Wu et al. Therefore, one can suppose that the lowered level of KYNA observed in the amygdala of UCMS mice with a high peripheral KP activity can locally promote a neuronal excitation mediated by an increased glutamate release. A rise in excitatory activity within the AMY is known to strongly activate the stress response system (Herman et al., 2005) and then to be responsible for the expression of stress-related behaviors; that is in agreement with our finding that these mice express stress-related behaviors more importantly. Moreover, such a hypothesis is in accordance with numerous works highlighting the crucial role of glutamatergic neurotransmission in the etiology and the pathophysiology of anxiety (Barkus et al., 2010; Mathew et al., 2008; Simon and Gorman, 2006) and depressive symptoms (Hashimoto, 2009; Sanacora et al., 2008; Skolnick et al., 2009).

5. Conclusion

We previously reported that confronting mice to recurrent psychogenic stressors resulted in the increase of the peripheral (lung) KYN/TRP ratio traducing an activation of the peripheral KP. With the present study, we are able to go into detail by showing that UCMS mice having higher peripheral KP activity are also those that have 1) higher expression of stress-related behaviors in the EPM and the FST, 2) increased 5-HT metabolism in CC and 3) reduced level of KYNA in AMY. However, the correlative approach precludes any conclusion as to whether the variations revealed in this study are causally linked to the observed behavioral variations (or *vice versa*).

These results might be of importance regarding the comprehension of the complex role played by peripheral alterations in the occurrence of emotional disturbances induced by chronic stress. Beyond the functional implication of the peripheral KP in behavior, we also modestly contributed to better understand the role of the cerebral KP in behavioral changes observed in response to a chronic stress exposure by showing that corticolimbic modifications of KP metabolites may be related to stress-related disorders in chronically stressed animals. Even if several lines of evidence point out the glutamatergic system as a key factor between KP alterations and behavioral changes, further experiments are needed to fully validate this assumption.

The fact that the KP is under the control of both the neuroendocrine and immune systems, two of the most important physiological systems involved in the response to stress, and that it is able to modulate corticolimbic systems of neurotransmission, makes this metabolic pathway a relevant candidate for explaining alterations encountered during chronic stress.

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Conflicts of interest

A. Laugeray, JM. Launay, J Callebert, A. Surget and P. Barone report no biomedical financial interests or potential conflicts of interest.

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