ORIGINAL ARTICLE

Essential Role of Excessive Tryptophan and its Neurometabolites in Fatigue

Takanobu Yamamoto, Hirotsugu Azechi, Mary Board

ABSTRACT: ***Purpose:*** Serotonin, a neurotransmitter synthesized from tryptophan, has been proposed to play a key role in central fatigue. In this study, we examined whether tryptophan itself and/or its two metabolites, kynurenic acid (KYNA) and quinolinic acid (QUIN), are involved in central fatigue. ***Materials and Methods:*** Experiments were conducted using Sprague-Dawley rats (SDR) and Nagase analbuminemic rats (NAR). Central fatigue was assessed by treadmill running and a Morris water maze test. Microdialysis was used to collect samples for measurement of extracellular concentration of tryptophan, serotonin and 5-hydroxyindoleacetic acid (5-HIAA) and to infuse test agents. To examine the kinetics of release, synaptosomes in the striatum were prepared *in vitro* to measure intra- and extrasynaptosomal concentration of tryptophan, serotonin and 5-HIAA. ***Results:*** The concentration of tryptophan secreted into the extracellular space of the striatum was higher during fatigue only, and quickly returned to basal levels with recovery from fatigue. Running time to exhaustion was reduced by activation of tryptophan receptors. Time to exhaustion was shorter in NAR, which maintain a higher extracellular level of striatum tryptophan than SDR. Impaired memory performance in a water maze task after tryptophan treatment was attributable to high levels of KYNA and QUIN in the hippocampus acting synergistically on N-methyl-D-aspartic acid receptors. When branched-chain amino acids were administered, tryptophan transport to the extracellular space of the striatum was drastically inhibited. ***Conclusion:*** Our findings demonstrate that the increase in fatigue which occurs because of excessively elevated brain tryptophan can be further amplified by the use of synthetic KYNA and QUIN.

RÉSUMÉ: Rôle essentiel d'un excès de tryptophane et de ses neurométabolites dans la fatigue. ***Objectif :*** La sérotonine, un neurotransmetteur synthétisé à partir du tryptophane, jouerait peut-être un rôle clé dans la fatigue d'origine centrale. Dans cette étude, nous avons examiné si le tryptophane lui-même et/ou ses deux métabolites, l'acide kynurénique (KYNA) et l'acide quinolinique (QUIN), sont impliqués dans la fatigue d'origine centrale. ***Méthode :*** Nous avons effectué nos études sur des rats Sprague-Dawley (SDR) et des rats Nagase analbuminémiques (NAR). La fatigue d'origine centrale était évaluée par un test sur tapis roulant et par le test du labyrinthe aquatique de Morris. La cueillette des échantillons pour mesurer la concentration extracellulaire de tryptophane, de sérotonine et d'acide 5-hydroxyindoleacétique (5-HIAA) ainsi que l'injection des produits testés ont été effectuées par microdialyse. Nous avons préparé des synaptosomes du striatum in vitro pour mesurer la concentration de tryptophane, de sérotonine et de 5-HIAA intra et extrasymaptosomale dans le but d'examiner leur cinétique de libération. ***Résultats :*** La concentration de tryptophane secrétée dans l'espace extracellulaire du striatum était plus élevée seulement pendant la fatigue et revenait rapidement au niveau de base lorsque la fatigue était disparue. Le temps de course jusqu'à épuisement était diminué par l'activation des récepteurs du tryptophane. Le temps d'épuisement était plus court chez les RAN qui conservaient un niveau extracellulaire de tryptophane striatal plus élevé que les RSD. Une altération de la mémoire lors de l'exécution du test du labyrinthe aquatique après traitement par le tryptophane était attribuable à des niveaux élevés de KYNA et de QUIN agissant en synergie sur les récepteurs de l'acide N-méthyl-D-aspartique dans l'hippocampe. Quand des acides aminés à chaînes ramifiées étaient administrés, le transport du tryptophane vers l'espace extracellulaire du striatum était inhibé de façon drastique. ***Conclusion :*** Nos constatations démontrent que l'augmentation de la fatigue qui survient à cause d'un taux de tryptophane excessivement élevé dans le cerveau peut être amplifiée au moyen de KYNA et de QUIN synthétiques.

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Although quickly metabolized in the brain to kynurenine and serotonin, tryptophan produces intense psychological effects, including changes in mood[1], fatigue[2], and the perception of fatigue[3]. Moreover, quinolinic acid (QUIN), a tryptophan metabolite, was recently shown to be neurotoxic in the central nervous system[4], and an increase in QUIN levels has been identified in patients with myalgic encephalomyelitis, which is sometimes described as chronic fatigue[5]. Quinolinic acid is also an agonist of N-methyl-D-aspartic acid (NMDA) receptors and causes excitotoxic neural death[6].

On tryptophan loading, metabolism of kynurenine to kynurenic acid (KYNA) is greater in patients with chronic brain injury than in controls, and this might contribute to the continuation of cerebral dysfunction in these patients[7]. This finding suggests brain dysfunction, including chronic fatigue syndrome, is related to elevated tryptophan metabolism. Pharmacologically, KYNA is an antagonist of NMDA and α-7 nicotinic acetylcholine receptors[8], and its antagonistic effect on

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glutaminergic and cholinergic neurons during central fatigue may interfere with neurocognitive functions such as memory and exercise skills in brain regions, such as the hippocampus and striatum. However, it remains unclear whether the neuromechanism of central fatigue and its attendant behavioral inhibition result from a direct effect of tryptophan or its metabolites.

Various findings support the hypothesis that tryptophan plays a direct role in the production of central fatigue. Increased L-tryptophan induces central fatigue[9] and inhibits the firing of raphe neurons[10]. In the striatum, KYNA reduces extracellular dopamine levels[11]. These findings suggest that tryptophan and KYNA cause behavioral suppression and dysfunction in the brain.

In a human model of central fatigue (i.e., patients undergoing two major surgeries), significant increases were seen in post-operative plasma-free tryptophan level and the ratio of free tryptophan level to branched-chain amino acids (BCAA) level, while plasma albumin decreased[3,9], suggesting that elevated tryptophan levels are intimately associated with the development of fatigue in the brain. It has therefore been speculated that Nagase analbuminemic rats (NAR) could serve as an animal model of central fatigue, given their high plasma levels of free tryptophan as well as high free tryptophan/BCAA ratio in plasma.

These findings notwithstanding, however, the serotonin hypothesis of central fatigue is considered the most likely explanation of these phenomena[12], with tryptophan acting merely as the precursor for serotonin. Present knowledge concerning the role of tryptophan in fatigue thus remains largely incomplete. In the present study, we examined the relationship of tryptophan and its active neurometabolites, KYNA and QUIN, to fatigue in the brain using a variety of *in vivo* techniques (including microdialysis, and *in vivo* injection of the serotonin reuptake inhibitor [fluoxetine], serotonin and tryptophan receptor agonists [*m*-chlorophenylpiperazine {*m*-CPP} and D,L-β-(1-naphthyl) alanine, respectively], serotonin precursors [tryptophan and 5-hydroxytryptophan {5-HTP}], and tryptophan neurometabolites [QUIN, KYNA]) and *in vitro* techniques (including a kinetic assay to measure the amount of tryptophan release and uptake). We also investigated whether KYNA could induce central fatigue, given its role as an NMDA antagonist with the ability to induce behavioral and memory abnormalities[13].

Materials And Methods

Animal procedures

Adult female Sprague-Dawley rats (SDR) (n=45) and analbuminemic rats (NAR) (n=3), which genetically lack the ability to synthesize albumin (Japan SLC Inc., Hamamatsu, Japan)[14], were housed two or three per cage under a 12-hour (h) light-dark schedule (lights on at 07:00) in a humidity-controlled (55%) and temperature-controlled (22°C) colony room (CLEA Japan, Inc., Osaka, Japan). All experiments were conducted in the light phase of the cycle.

After microinjection of tryptophan, KYNA, and QUIN, central fatigue assessment was carried out via two performance tests, treadmill running and a Morris water maze test. Although the fatigue induced by treadmill running is muscle fatigue, it can be used as an indicator of central fatigue[15] because voluntary muscles are controlled by the brain and influenced by mood[16].

Samples (1 µl/minute (min)) were collected from the striatum by microdialysis during fatigue induced by treadmill running and were analyzed in real time for extracellular tryptophan, serotonin, and 5-hydroxyindoleacetic acid (5-HIAA) at fmol-pmol concentrations using high performance liquid chromatography (HPLC)[9]. The kinetics of striatal synaptosomal release and uptake of tryptophan added to the incubation medium were analyzed and compared to the kinetics of striatal synaptosomal release and uptake of serotonin. All animal procedures were conducted in accordance with guidelines of the Japanese Neuroscience Society. D, L-β-(1-naphthyl) alanine was from Bachem AG, Switzerland. Other compounds were from Sigma Chemical Co. (St. Louis, MO, USA).

In vivo microdialysis

Microdialysis was used to investigate extracellular tryptophan concentration in real time during fatigue in rats (SDR and NAR weighing 210–255 g) treated with either saline or BCAA (valine:leucine:isoleucine, 5:3:2, 250 mg/kg i.p.). Saline and BCAA were administered 30 min before the exhaustion test. A microdialysis guide cannula (CMA 12) was inserted into the left striatum (coordinates relative to the bregma: A 0.2 mm, L 3.0 mm, and V –5.0 mm) under anesthesia with sodium pentobarbital (25 mg/kg i.p.; Abbott Laboratories, Abbott Park, IL, USA). Five days later, a microdialysis probe (PC 12; probe shaft, 14 mm; membrane length, 3 mm) was inserted through the cannula. The probe was connected to a microdialysis pump (CMA 102 Microdialysis pump, CMA/Microdialysis, Solna, Sweden) for injection of modified Ringer's solution through the probe. Animals were allowed to move freely in a breeding cage (30 cm [W] × 36 cm [H] × 17 cm [D]) for 30 min before running to exhaustion, and extracellular fluid samples were collected for measurement of baseline values. Subsequently, samples (1 µl/min) were collected every 30 min during running to exhaustion and during the recovery period. Although each animal stopped running at a different exhaustion time, sampling was continued for up to 240 min in the saline group and 270 min in the BCAA group. Tryptophan, serotonin (5-HT), and 5-HIAA levels in these samples were measured by high performance liquid chromatography 9. 5-HIAA, the final metabolite of serotonin, was used as a measure of serotonin metabolism.

Using the same system, tryptophan, serotonin, D,L-β-(1-naphthyl) alanine, 5-HTP, m-CPP, fluoxetine, and KYNA (1 mM each) were infused via the microdialysis probe at a speed of 1 µl/min for 30 min. Fatigue was induced as described above.

In vitro synaptosomal preparation and release assay

The striatum from each SDR (200–250 g) was immersed in ice-cold 0.32 M sucrose (pH 7.4) (ten times the striatal weight) and then homogenized using a glass homogenizer equipped with a Teflon pestle. The supernatant obtained by centrifugation of the homogenate (1000 g for 10 min) was re-centrifuged (15,000 g for 30 min) to yield a crude synaptosomal pellet (P_2' fraction), which was resuspended in 0.32 M sucrose and re-centrifuged (15,000 g for 30 min) to yield the P_2 fraction. For the release assay, synaptosomes (200 µl) were resuspended in 250 µL of

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Krebs-Henseleit buffer which had been aerated with 95% O_2/5% CO_2 and then preincubated in a 95% O_2/5% CO_2 atmosphere at 37°C for 10 min. Tryptophan adjusted to a final concentration of 200 nM or 2 μM was added to the suspension of synaptosomes, which were then depolarized with 30 mM K^+ (30 min). Synaptosomal release was halted by the addition of ice-cold 10 mM Ethylenediaminetetraacetic acid (EDTA) (100 μl). The suspension was centrifuged (14,500 g for 15 min), and the upper layer was transferred using an aspirator to a new tube and deproteinated by the addition of 150 μl of 1 M perchloric acid with 0.1% ascorbic acid and 0.1% EDTA. The resulting mixture was vortexed vigorously and stored at –80°C until HPLC assay of tryptophan and serotonin concentrations. The lower layer (synaptosome layer) was also resuspended in 150 μl of Krebs-Henseleit buffer, deproteinated with 1 M perchloric acid, and assayed for tryptophan, serotonin, and 5-HIAA concentration.

Psychological and behavioral tests

Sprague-Dawley rats (180–200 g; n=8) were used in open-field and stereotyped behavior tests. The tests were performed in a circular apparatus (diameter, 90 cm; height, 30 cm, Shinano, Tokyo, Japan) with the numbers 1–19 marked on the floor. The frequency of rearing and duration of motor activity and immobility were observed for seven minutes, but only observations during the last five min after transfer to the open-field box were recorded using a video tracking system (Comp ACT VAS Ver. 3.0X, Muromachi Kikai, Tokyo, Japan). Motor activity was measured by counting the frequency of movements between numbers marked on the floor.

Spatial learning

Sprague-Dawley rats (180–200 g) were trained in a Morris water maze[17], a circular water tank 147 cm in diameter fitted with a refuge platform (height, 250 mm), for ten consecutive days of four daily sessions. The platform was hidden 1 cm below the water surface, and made opaque with black nontoxic paint. The starting point was changed every day. The time to reach the platform (latency), length of the swim path, and swim speed were recorded automatically by a video tracking system (Comp ACT VAS Ver. 3.0X, Muromachi Kikai). This method was used for the evaluation of spatial memory acquisition. To measure the recall of spatial memory, a 30-second probe test was performed after microinjection. The probe test involved removing the installed platform during training, and allowing the rat to search freely within the water maze. The time to search within a radius of 20 cm of the goal was measured. Similar to the training, the probe test was performed from four different starting points, with one trial for each. The test was performed weekly a total of three times under conditions in which cues could be given. In brief, the conditions employed during testing were the same as those employed during training and were defined as 100% if all cues were visible and as 50% if half the cues were hidden by a curtain. The rats were tested six to seven days after injection of 180 nmol QUIN or QUIN plus 20 nmol KYNA into the hippocampus CA3 region (AP=–5.3 mm, ML=±4.7 mm, DV=8.0 mm from the dura) through a guide cannula (CMA/12).

Probe test before drug injection

For the evaluation of recall, four different starting positions were used for training, and four trials were performed each day for ten days. The starting position for all trials was the edge of the pool directly opposite the platform used for training. For the probe test, the platform used at training was removed, and the rats were allowed to search freely within the maze. This test was performed weekly a total of three times. The 1st and 3rd trials were performed with all spatial cues visible and the 2nd trial was performed with only 50% of the spatial cues visible.

Results

Tryptophan release during fatigue

Intracerebral tryptophan levels were directly associated with the time to exhaustion and were shown to be easily reduced with BCAA treatment (Figure 1A). A decrease in tryptophan release was associated with an increase in time to exhaustion. Compared with the saline group (133 ± 6.3 min), time to exhaustion in the BCAA group was clearly prolonged via the dramatic reduction in extracellular tryptophan caused by BCAA treatment (187 ± 21.5 min) ($t=2.404$, $d.f.=7$, $p<0.05$). Moreover, return to basal level was associated with recovery from exhaustion, with real-time release of tryptophan in extracellular fluid showing that this response was sensitive and specific (Figure 1A, Figure 2A, C and E). In the *in vitro* model also, extrasynaptosomal release of tryptophan into the incubation medium over time was readily reproducible. Intrasynaptosomal tryptophan decreased significantly in the first 30 min ($p<0.01$, $t=11.26$), indicating that endogenous tryptophan and tryptophan taken up by synaptosomes was gradually released during this period into the extrasynaptosomal fluid (incubation medium) (Figure 1B). In the later stage of release, however, the concentration of extrasynaptosomal tryptophan derived from both the absorbed and endogenous tryptophan remained elevated. This state persisted as long as tryptophan was available in the incubation medium (Figure 1B). These findings show that the release of tryptophan from nerve endings can be induced by methods used to stimulate increased extracellular tryptophan.

In a similar *in vitro* experiment in which serotonin release was monitored instead of tryptophan release, serotonin was not continuously released (Figure 1C) even in the presence of 2 μM tryptophan. However, in the presence of tryptophan (200 nM) and clomipramine (10 μM), serotonin was transiently released (increased from 0.20 to 0.35 pmol/mg protein) at 30 min ($p<0.01$). *In vivo* microdialysis also showed the transient release of serotonin (at 30 min during fatigue) but this did not reflect the duration or state of fatigue (Figure 1D). The reason for this is shown in Figure 1E. The kinetics of the release of serotonin and its metabolite, 5-HIAA, from synaptosomes were inversely related. As shown in Figure 1E, the serotonin released in response to 30 mM K^+ was immediately taken up by nerve terminals (significant increase in concentration at 60 min, $p=0.015$, $t=8.0$) and then metabolized (concentration decreased 66% between 90 and 120 min: $t=4.1$, $p=0.056$; and $t=4.3$, $p=0.050$, respectively), confirming the transience of serotonin release. In contrast, the synaptosomal concentration of 5-HIAA decreased transiently as a consequence of serotonin release at 30 and 60 min: $p=0.006$, $t=11.9$; and $p=0.019$, $t=7.0$, respectively),

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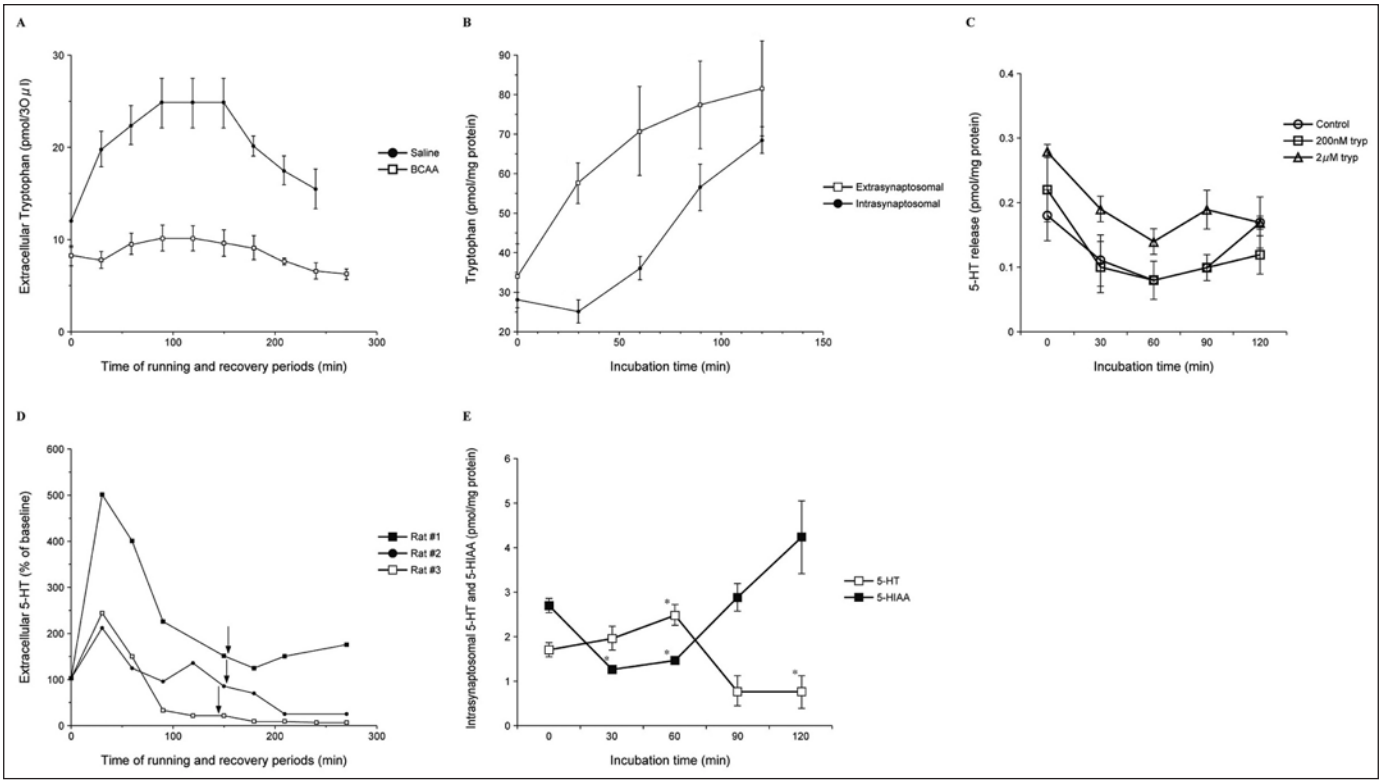

Figure 1: *Release and metabolism of tryptophan and serotonin in in vivo microdialysis (A and D) and in vitro experiments (B, C, and E). A: Effect of BCAA treatment on time to exhaustion and extracellular tryptophan concentration. The axis of the abscissas indicates the time course of sampling as well as that of exhaustion and subsequent recovery. Four animals injected with saline and five injected with BCAA ran for at least 150 min. Sampling of extracellular fluid was performed at intervals of 30 min for up to 240 min in the saline group and 270 min in the BCAA group. The range of time to exhaustion is shown because there were variations among animals. The time ranged between 120 and 149 min in the saline group and between 150 and 240 min in the BCAA group. The time to exhaustion of the BCAA group (187 ± 21.5 min) was longer than that of the saline group (133 ± 6.3 min). Results were compared between the saline control and BCAA using the unpaired Student's t test (t=2.404, d.f.=7, p<0.05). The difference in tryptophan concentration and running performance level between the two groups was significant (Mann-Whitney U-test: p<0.0001). Error bars, s.e.m. B: Release of tryptophan from synaptosomes (extrasynaptosomal level) and uptake of tryptophan into synaptosomes (intrasynaptosomal level) in the presence of L-tryptophan (200 nM). Results were compared to the control (0 min incubation time) using one-way ANOVA followed by multiple comparisons (Dunnett's test). n=3 per data point. Error bars, s.e.m. Intrasynaptosomes, F(4,10)=39.75, p<0.0001. Extrasynaptosomes, F(4,10)=14.58, p=0.0004. C: Effect of an increase in L-tryptophan (200 nM and 2 µM) availability on serotonin (5-HT) release. Two-way ANOVA followed by multiple comparisons (Scheffe's test) were conducted for effects of an increase in L-tryptophan (control, 200nM and 2 µM) availability on serotonin (5-HT) release by incubation time. Interaction of incubation time and L-tryptophan level was not significant (p=0.850). Error bars, s.e.m. D: Changes in extracellular serotonin in SDR (n=3) during running to fatigue and recovery periods. One-way ANOVA was followed by Fisher's PLSD test. F(7,22)=2.281, p<0.05 (before running vs 30 min during exercise). Baseline level was in the range of 50 fmol to 1.1 pmol per 30 µl. Arrows indicate the time to exhaustion. E: Time course of change in serotonin (5-HT) and 5-HIAA levels in synaptosomes (intrasynaptosomal level) during supplementation with 2 µM L-tryptophan. Following its reuptake, serotonin was rapidly metabolized to 5-HIAA, and hence serotonin concentration decreased rapidly. Results were compared to the control (0 min incubation time) by the paired Student's t-test. n=3 per data point. Error bars, s.e.m. * p≤0.05.*

but the gradually reabsorbed serotonin was rapidly metabolized to 5-HIAA, which returned the concentration to its original level at 90 min (p=0.438, t=1.0). These findings show that released serotonin is quickly re-uptaken and subsequently metabolized to 5-HIAA. Serotonin therefore has no effect on fatigue.

The treadmill run time to exhaustion was shorter in NAR than SDR (NAR, 135±14.4 min; SDR, 190±18.4 min; d.f.=16, t=2.38, p<0.05, unpaired Fisher's protected least significant difference [PLSD]). Among other differences, NARs had a higher extracellular tryptophan concentration when fatigued (Figure 2A, C and E) and lower extracellular 5-HIAA concentration (Figure 2B, D and F). These findings indicate that when fatigued, NAR have greater intracerebral uptake of tryptophan and less monoamine oxidase-catalysed conversion of serotonin to 5-HIAA.

Regulation of fatigue via tryptophan receptors

The time to exhaustion was significantly shorter in rats treated with L-tryptophan (125 mg/kg, i.p.) (103.3±20.3 min) than in control rats (129.8±19.3 min), demonstrating greater fatigability (F(1,9)=7.02, p<0.05). Moreover, the time to fatigue was also significantly shortened by direct intracerebral injection of L-tryptophan (Figure 3A). Exhaustion in tryptophan-injected rats was manifested by contact of the abdomen with the floor of the cage and immobility for more than 1 h (collapsed condition).

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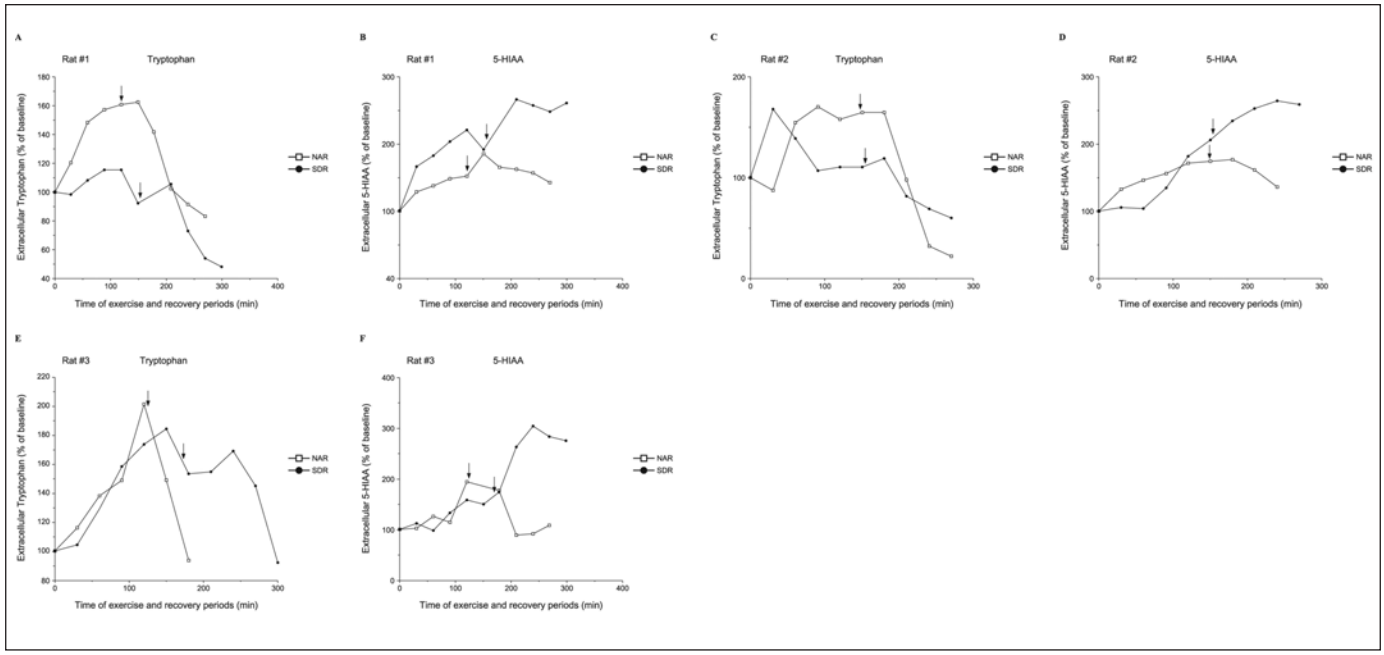

Figure 2: *Characteristic changes in extracellular tryptophan and 5-HIAA concentrations in the NAR and SDR groups measured by microdialysis. A clear fatigue-associated difference between the two groups was seen in the responses to tryptophan (A, C, E) and 5-HIAA (B, D, F). Exhaustion (indicated by arrows) appeared earlier in all NAR.*

On the other hand, exhaustion in the control group manifested as immobility but the body and limb position was maintained, showing that fatigability can be increased by the administration of tryptophan alone. To test whether these possible neuromodulatory functions of tryptophan were activated by a tryptophan receptor agonist[18], we repeated the experiment after intracerebral injection of the tryptophan receptor agonist D,L-β-(1-naphthyl)alanine. Time to exhaustion was reduced to about 63% of that in the control, again constituting a model of fatigability (Figure 3B). In contrast, fatigue mediated via serotonin receptors showed no synergistic effect on fatigue mediated via tryptophan receptors (Figure 3C). However, latency to stop running was unaffected by fluoxetine, a selective inhibitor of serotonin reuptake, (Figure 3A), the 5-HT2C agonist m-chlorophenylpiperazine (m-CPP) (Figure 3A), and 5-HTP itself (Figure 3C). Hence, serotonin induces fatigue at

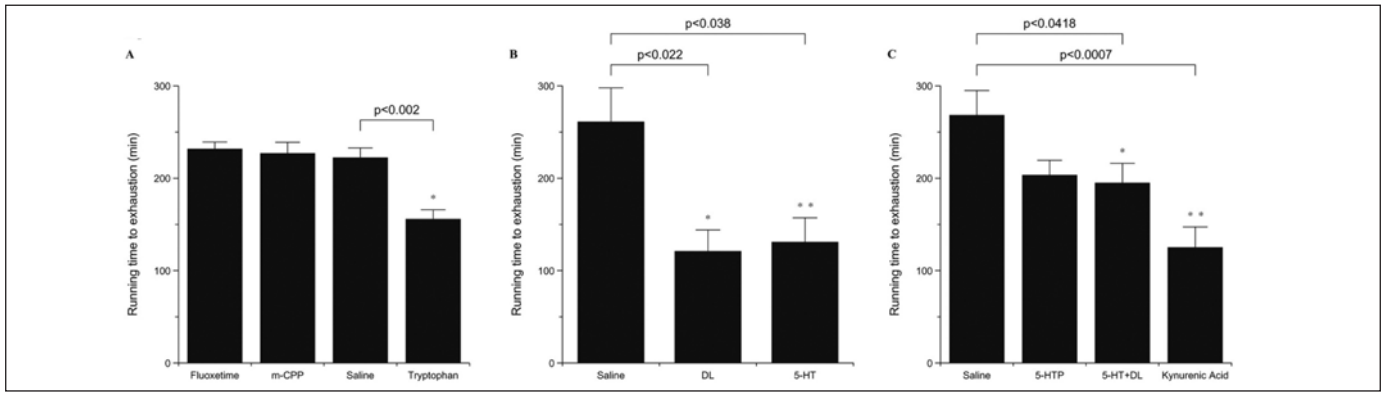

Figure 3: *Effect on running time to exhaustion induced by intracerebral (left striatum) injection of pharmacological agents including tryptophan and serotonin (5-HT). A: Time to exhaustion was measured after treatment with fluoxetine, m-CPP, and tryptophan and compared with time to exhaustion after treatment with saline. One-way ANOVA was followed by Fisher's PLSD test. F(3,15)=13.149, * p<0.002, Data are expressed as means ± s.e.m. B: Time to exhaustion was measured after treatment with D,L-β-(1-naphthyl)alanine (DL), a tryptophan analog, and serotonin (5-HT) and compared with time to exhaustion after treatment with saline. One-way ANOVA was followed by Fisher's PLSD test. F(2,10)=4.285, * p<0.0221, compared with saline. ** p<0.0389, compared with saline. Data are expressed as means± s.e.m. C: Time to exhaustion was measured after treatment with serotonin (5-HT), serotonin plus D,L-β-(1-naphthyl)alanine (DL), and kynurenic acid. One-way ANOVA was followed by Fisher's PLSD test. F(3,14)=6.345, * p<0.0418, compared with saline. ** p<0.0007, compared with saline. Data are expressed as means± s.e.m.*

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pharmacological doses only. It does not induce fatigue via 5-HT2C receptors, nor under physiological conditions which prompt the release of endogenously stored serotonin or serotonin after synthesis from 5-HTP.

Diminished performance in spatial learning

We next investigated the association of cognitive inhibition with fatigue induced by the tryptophan metabolite KYNA. Microinjection of 3 nmol of KYNA into the third cerebral ventricle led to a significant decrease in physical activity in open-field and rearing ($p<0.05$: $F(1,4)=7.48$ and $F(1,4)=6.36$, respectively). Injection of KYNA at 0.25 mM (d.f.=4, $p<0.005$), 0.4 mM (d.f.=6, $p<0.005$) and 1 mM (Figure 3C) caused a dose-dependent increase in fatigue induced by running compared to the injection of saline.

To investigate the effects of fatigue on learning and memory, we evaluated the relationship between the injection of tryptophan, QUIN, and KYNA and performance in the Morris water maze. For saline-treated rats, the mean time to reach the platform following spatial training of one trial per day for ten days (spatial training and fatiguing were done concurrently) was significantly longer in rats fatigued (Figure 4Aa) by continuous running at 30 m/min over 30 minutes for 12 days than in non-fatigued controls (Figure 4Ab). For tryptophan-treated rats, this mean time was significantly longer in the 1st and 2nd trials, demonstrating that the acquisition of memory was delayed by tryptophan in the initial learning stage (Figure 4B). In the re-learning phase with evaluation by the probe test (see spatial learning in Methods), tryptophan prolonged mean goal latency, indicating that it qualitatively decreased learning recall (Figure 4C). In addition, QUIN alone or co-administered with KYNA produced a decrease in recall retention. Thus, rather than antagonizing the effect of QUIN, KYNA elicited fatigue in the same way as QUIN (Figure 4D). These findings confirm that not only tryptophan but also its metabolites KYNA and QUIN decrease spatial memory performance (both acquisition and recall).

Discussion

This study demonstrates that increased flow of tryptophan from the peripheral circulation to the central nervous system is the first step in the mechanism of all central fatigue. Extracellular levels of tryptophan in the central nervous system increase during fatigue (Figure 1A), and readily return to basal levels during recovery. Tryptophan is released from synaptosomes (Figure 1B). We previously showed that the binding affinity of free tryptophan for albumin was decreased and that the plasma concentration ratio of free to total tryptophan was increased in a postoperative model of central fatigue in humans[9]. In contrast, tryptophan-deficient rats with tryptophan levels in the extracellular fluid of about half the normal level showed decreased fatigability[19]. Remarkably, we found that tryptophan concentration in the striatal extracellular fluid is a sensitive indicator of the persistence of fatigue, providing direct evidence for its fatigue-inducing role in the brain. The finding that the maximum concentration of tryptophan in extracellular fluid at fatigue was 1.2 μM (Figure1A), a level clearly below the Michaelis constant (Km value)[20,21] of the high-affinity tryptophan carrier in nerve terminals (high-affinity synaptosomal uptake system), indicates that tryptophan is

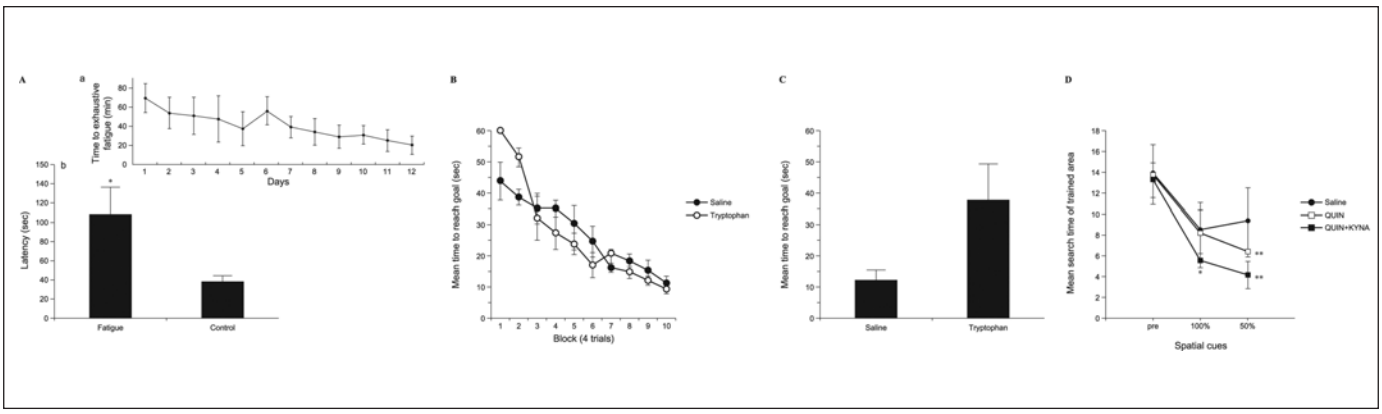

Figure 4: *Water maze learning in controls, under chronic fatigue conditions, and following administration of L-tryptophan, quinolinic acid and kynurenic acid. A: Water maze learning in control rats (non-fatigued) and (a) rats chronically fatigued by periodic treadmill running over 12 days. One-way ANOVA was followed by repeated measures, $F(2,18)=54.666$, $p<0.05$. (b) Latency was significantly different between the non-fatigued and chronic fatigued groups ($t=7.17$, d.f.=9, $p<0.001$). Results were compared to the saline control using the unpaired Student's t-test. Error bars, s.e.m. B: Learning curve for reaching the platform. $F(9,81)=24.826$, $p<0.001$, compared with saline control and L-tryptophan during Block 1; and $F(9,81)=2.332$, $p<0.05$, compared with saline control and L-tryptophan during Block 2. Saline control and L-tryptophan were given by intraperitoneal administration at 100 mg/kg, 30 minutes before the experiment. Data are expressed as means ±s.e.m. C: Effect of injected L-tryptophan on spatial learning and recall. Recall of spatial information was measured in terms of the time required to reach the platform and after the probe test. Latency was significantly different between the groups ($t=2.74$, d.f.=9, $p<0.05$). Results were compared to the saline control using the unpaired Student's t-test. Data are expressed as means ±s.e.m. D: Effect of microinjection (Micro Infusion Unit XF-320J, Nihon Kohden) of quinolinic acid or quinolinic acid plus kynurenic acid at 1.25 ml/min injected with a Hamilton 5 microliter syringe on the probe test. Pre: probe test with no spatial cues. Results were compared to the pre-microinjection values using the unpaired Student's t-test. * $p<0.05$, ** $p<0.01$. Error bars, s.e.m.*

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readily taken up and that the uptake is Maximum catalytic activity (Vmax)-dependent (Figure 1B).

As shown in Figure 1C, serotonin release from synaptosomes was not observed after the addition of tryptophan (200 nM and 2 μM) into the incubation medium. The very short duration of action of serotonin suggests that the serotonin hypothesis of central fatigue is flawed. Among other reasons, the extracellular concentration of serotonin is very low, as shown by our present results (Figure 1D), in which serotonin levels were from 50 fmol/30 μl to 1.1 pmol/30 μl. This concentration is approximately 1/200 to 1/10 that of tryptophan, which averages 10 pmol/30 μl. Fatigability is not affected by the increased extracellular concentration of serotonin induced by the microinjection of fluoxetine or by the administration of 5-HTP. The surprising and dramatic increase in synaptosomal tryptophan content was non-specific and occurred in all five regions of the central nervous system tested (striatum, motor cortex, hypothalamus, hippocampus, and thoracic spinal cord) in response to treadmill running to exhaustion, whereas the increase in serotonin occurred only in the corpus striatum[22]. In view of the postulated serotonin hypothesis, the intracerebral increase in serotonin is secondary (non-specific) rather than primary (specific). These results strongly suggest that tryptophan, which is released in a Ca^{2+}-dependent manner from nerve terminals, acts directly on pre- or post-synaptic terminals and is an inhibitory neuromodulator of neurotransmitter release. Confirming this, we previously observed increased fatigability after administration of the tryptophan receptor agonist D,L-β-(1-naphthyl)alanine[18].

Many lines of evidence help explain the above interpretation of tryptophan-related mechanisms of neuronal and behavioral suppression. Once transported into the brain, tryptophan is readily metabolized via kynurenine into KYNA and QUIN[7,23]. This is the major pathway of tryptophan metabolism in the mammalian brain[4,6], and is predominant over the serotonin synthetic pathway. These substances are not only antagonists and agonists of NMDA receptors, respectively, but KYNA has also been confirmed as an antagonist of the α-7 nicotine receptor[8, 24]. Furthermore, KYNA at nanomolar concentrations inhibits the extracellular release of striatal dopamine[11], while increased levels of QUIN have been reported in myalgic encephalomyelitis, a condition characterized by pathological fatigue[5]. In our experiments also, injection of KYNA impaired SDR performance in all three behavioral tests (running, open-field, and Morris water maze tests). These findings confirm that fatigue is caused by KYNA, and indicate that these tryptophan metabolites are neurometabolites. Behavior may be inhibited simply through physiologically-produced changes in mood such as increased lethargy. As shown in Figure 4D, a QUIN hypothesis is suggested for the effects on spatial learning performance. In fact, central fatigue induced by tryptophan and its neurometabolites decreases the function of higher brain structures that play a role in memory and cognitive ability, such as the hippocampus. As evidence, the present study showed that fatigue decreased water maze learning ability (Figure 4A). Administration of tryptophan not only caused fatigue but also decreased water maze performance during both memory acquisition (Figure 4B) and recall (Figure 4C). Furthermore, KYNA increased fatigability (i.e., decreased motor performance; Figure 3C), and interestingly the co-administration of KYNA plus QUIN into the hippocampus synergistically decreased neurocognition (i.e., decreased spatial learning performance) (Figure 4D). This finding supports our new KYNA-QUIN hypothesis that central fatigue arises due to a rapid increase in concentrations of active neurometabolites.

As shown in Figure 1A, pre-treatment with BCAA decreased the concentration of tryptophan in striatal extracellular fluid by 60%, providing considerable alleviation of fatigue. The increase in running time after BCAA treatment means that the rat retains its physical capability because it retains its motivation to engage in physical activity, which is controlled by the central nervous system. Thus, because the brain controls voluntary muscle movement, peripheral fatigue can serve as an indicator of central fatigue. To our knowledge, this finding, obtained under non-restrained conditions, is the first direct evidence that BCAA inhibit intracerebral tryptophan release and uptake from the circulation, and contribute to the lessening of fatigue, albeit that our previous data suggested this indirectly[15]. This mechanism may explain why resistance to central fatigue via inhibition of the L-system transporter for the uptake of tryptophan also improves exercise performance, i.e., increases treadmill running time to exhaustion. Branched-chain amino acids are expected to exert their effect against fatigue by inhibiting metabolic activities in both the tryptophan and kynurenine pathways simultaneously, thereby blocking the root cause of fatigue. Both humans and rats are affected by this enhanced fatigue phenomenon and therefore should benefit from this treatment. Our findings demonstrate that fatigue in the brain can be controlled by changing the intracerebral content of tryptophan, and that excessive levels of tryptophan can promote the release of tryptophan from nerve terminals, leading to central fatigue mediated via tryptophan receptors and enhanced kynurenine metabolism, but not to serotonin metabolism.

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