Central nervous system-specific alterations in the tryptophan metabolism in the 3-nitropropionic acid model of Huntington's disease

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Abstract

Experiments on human samples and on genetic animal models of Huntington's disease (HD) suggest that a number of neuroactive metabolites in the kynurenine (KYN) pathway (KP) of the tryptophan (TRP) catabolism may play a role in the development of HD. Our goal in this study was to assess the concentrations of TRP, KYN, kynurenic acid and 3-hydroxykynurenine (3-OHK) in the serum and brain of 5-month-old C57Bl/6 mice in the widely used 3-nitropropionic acid (3-NP) toxin model of HD. We additionally investigated the behavioral changes through open-field, rotarod and Y-maze tests. Our findings revealed an increased TRP catabolism via the KP as reflected by elevated KYN/TRP ratios in the striatum, hippocampus, cerebellum and brainstem. As regards to the other examined metabolites of KP, we found only a significant decrease in the 3-OHK level in the cerebellum of the 3-NP-treated mice. The open-field and rotarod tests demonstrated that treatment with 3-NP resulted in a reduced motor ability, though this had almost totally disappeared a week after the last injection, similarly as observed previously in most murine 3-NP studies. The relevance of the alterations observed in our biochemical and behavioral analyses is discussed. We propose that the identified biochemical alterations could serve as applicable therapeutic endpoints in studies of drug effects on delayed-type neurodegeneration in a relatively fast and cost-effective toxin model of HD.

Keywords: Huntington's disease, 3-nitropropionic acid, tryptophan metabolism, HPLC, behavioral alterations

1. Introduction

Huntington's disease (HD) is an autosomal dominantly inherited progressive neurodegenerative disorder which results in cognitive, psychiatric and motor disturbances. HD is caused by an expansion of the cytosine-adenine-guanine (CAG) repeat in the gene coding for the *N*-terminal region of the huntingtin protein (Htt), which leads to the formation of a polyglutamine stretch. Above 39 CAGs, there is obligatory disease development (The Huntington's Disease Collaborative Research Group 1993). Although the exact mechanisms through which mutant Htt (mHtt) leads to the characteristic neuropathology are not fully understood, the potential roles of excitotoxicity and a neuronal mitochondrial dysfunction are among the best-established concepts (Szalardy et al. 2012; Zádori et al. 2012).

Various evidence suggests that the involvement of striatal glutamatergic excitotoxicity in the development of HD is mediated predominantly by the overactivation of *N*-methyl-D-aspartate receptors (NMDARs), and most specifically through NR2B subunit-containing NMDARs at the extrasynaptic sites (Milnerwood et al. 2010). In line with this, the expression of mHtt has been shown to sensitize the NR2B subunit-containing NMDARs (Chen et al. 1999). There is evidence indicating that such excitotoxic injury is mediated at least in part by endogenous substances, including certain metabolites of the kynurenine pathway (KP) of the tryptophan (TRP) metabolism (Fig. 1; Zádori et al. 2011).

This pathway involves of a number of neuroactive compounds. Among them, quinolinic acid (QUIN) is a weak NMDAR agonist (Stone and Darlington 2002) with demonstrated ability to induce excitotoxic injury, which led to QUIN toxicity being utilized as an early toxin model of HD (Beal et al. 1986). This toxic effect of QUIN was revealed to be augmented by another deleterious KP metabolite, 3-hydroxykynurenine (3-OHK), which can generate oxidative stress via the production of reactive oxygen species (ROS) (Guidetti et al. 2000). On the other hand, kynurenic

acid (KYNA) is an NMDAR antagonist at the strychnine-insensitive glycine coagonist site (Perkins and Stone 1982) and a weak antagonist on kainate- and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-sensitive ionotropic glutamate receptors (Kessler et al. 1989). Furthermore, its inhibitory potential on presynaptic α7-nicotinic acetylcholine receptors is proposed to be of high relevance at physiological KYNA levels, due to the suppression of presynaptic glutamate release (Hilmas et al. 2001). On this basis, KYNA was considered to be neuroprotective against NMDAR-modulated glutamatergic excitotoxicity, which has since been demonstrated by a variety of experimental evidence (Miranda et al. 1997; Sinor et al. 2000).

Besides the theoretical considerations, evidence is emerging of the presence of alterations in the KP of the TRP metabolism in tissues from HD patients and transgenic animals and in human post-mortem HD brains (Schwarcz et al. 1988; Beal et al. 1990, 1992; Heyes et al. 1992; Pearson and Reynolds 1992; Jauch et al. 1995; Guidetti et al. 2004, 2006). Briefly, these results indicate a relative decrease in KYNA concentrations relative to the levels of toxic neuroactive kynurenines (Zádori et al. 2011).

A decreased activity of the succinate dehydrogenase (SDH), complex II of the electron transport chain in post-mortem HD brains was one of the early findings suggestive of the role of a mitochondrial dysfunction in the development of HD (Stahl and Swanson 1974). Furthermore, mHtt has been shown to be able to bind directly to the mitochondria, altering their normal function (Choo et al. 2004). In line with the decreased SDH activity, mitochondrial II complex inhibitors such as 3-nitropropionic acid (3-NP) or malonate have been found to be useful in the investigation of HD through their utilization in animal toxin models (Túnez et al. 2010). The 3-NP model is frequently applied as an easy and rapid way to study certain aspects of neurodegenerative processes in HD (Brouillet 2014). Treatment with 3-NP evokes increases in the production of ROS and ·NO (La Fontaine et al. 2000) and the activation of apoptosis-related factors including caspase-3 and calpain (Duan et al. 2000; Bizat et al. 2003a), mechanisms through which 3-NP evokes striatal lesions in

both rodents and primates in a rather selective manner. Several dosing regimens are applied to evoke characteristic neuropathological and behavioral alterations in rodents (Brouillet et al. 2005). Briefly, striatal neurodegeneration in rats occurs when the steady inhibition of SDH attains 50–60% (Alexi et al. 1998; Brouillet et al. 1998; Blum et al. 2002; Bizat et al. 2003b). In mice, there have been only a few studies of the dosage-related reduction in SDH activity. Lower cumulative doses (320 mg/kg) have been shown to induce an approximately 20% reduction of enzyme activity, which is not associated with marked striatal lesions (Fernagut et al. 2002a). However, higher cumulative doses (400–450 mg/kg) were widely used to induce measurable striatal lesions (Klivenyi et al. 2000, 2006). Accordingly, in a previous study we demonstrated a significant reduction in striatal neuronal density via the application of unbiased semiquantitative densitometric analysis by using a subacute dosing regimen with a cumulative dose of 500 mg/kg (Török et al. 2014).

A number of publications have reported 3-NP-evoked alterations in motor and cognitive performance in rodents (Borlongan et al. 1995b; El Massioui et al. 2001; Brouillet et al. 2005; Li et al. 2009). The described alterations in motor functioning are highly reminiscent of those characteristic of human juvenile HD, such as rigidity, bradykinesia, dystonia and gait disturbances (Nance and Myers 2001). Although it seems clear that higher cumulative doses of 3-NP cause motor and cognitive impairments, the motor dysfunctions observed undergo a considerable amelioration after the cessation of toxin administration (El Massioui et al. 2001; Fernagut et al. 2002b; Li et al. 2009). Early studies raised the possibility that the KP is altered in 3-NP models (Csillik et al. 2002; Luchowski et al. 2002), but this has not yet been further investigated.

The aims of the present study were a delayed assessment of the TRP metabolism with regard to certain metabolites of the KP following subacute 3-NP treatment, and a comparison of the time-course of the toxin-induced behavioral alterations with the data available from the literature.

2. Materials and methods

2.1. Animals and 3-NP treatment

We used 30 5-month-old male C57Bl/6 mice in this study. The animals were housed in cages under standard conditions with a 12-12-h light-dark cycle and free access to food and water. The experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local animal care committee. The animals were randomly divided into two groups (n = 16 and n = 14, respectively). In the first group, 3-NP (Sigma-Aldrich, Saint Louis, MO, USA; dissolved in phosphate-buffered saline (PBS), pH adjusted to 7.4) was administered in a subacute dosing regimen as reported previously (Török et al. 2014), with twice daily intraperitoneal (i.p.) injections (50 mg/kg each) for 5 consecutive days. The second group served as the vehicle-injected control.

2.2. Behavioral assessment

2.2.1. Open-field test

Spontaneous locomotor and exploratory activities of the animals were assessed with the open-field paradigm. The first analysis was performed 3 days before the beginning of 3-NP administration to exclude any spontaneous difference between the randomly distributed animals. The second and third analyses were performed on the 3rd and the 11th day subsequent to the last day of the toxin regimen. The tests were performed at the same time of day to avoid possible changes due to the diurnal rhythm. Each mouse was placed in the center of an open-field motimeter box measuring 48*48*40 cm for 15-min tracking periods (analyzed in three 5-min periods). The movement patterns of the animals were tracked and recorded with the Conducta 1.0 system, with the use of infrared beams (Experimetria Ltd., Budapest, Hungary). The ambulation distance, the time spent in

immobility and the total time spent with consecutive rearings were the parameters used to assess the motor and exploratory characteristics of the mice. The grid and the walls of the setup were thoroughly wiped with a cloth containing ethanol solution 70 v/v% after each session to prevent any bias from olfactory cues.

2.2.2. Rotarod test

The rotarod test was applied to characterize the effects of 3-NP on motor coordination. The animals were trained on the rotarod for a 3-session period for 5 min on 2 consecutive days prior to the first test day. On the first and second days of the training sessions, a constant speed of 5 and 10 rpm, respectively, was used. Following the training sessions, the first test was performed prior to the first 3-NP injection in order to exclude any inborn difference between the randomly distributed 2 groups. During the tracking sessions on the test days, an accelerating speed profile from 1 rpm to 30 rpm was set for a 5-min tracking period. The performance of each mouse was measured three times; resting periods of 30 min were allowed between consecutive tracking sessions. The latency to fall values were recorded with the TSE Rotarod Advanced system (TSE Systems International Group, Frankfurt am Main, Germany). The average latency to fall values were used for the comparative analyses. The second, third and fourth tests were performed 2 h following the second 3-NP injection and on the 1^{st} and 10^{th} days following the last 3-NP administration, respectively. On the day before the respective test days, a 3-session retraining at 10 rpm for 5-min with 30-min resting periods was carried out to enable the animals to recall the rotarod experience. The rods and the separating walls were thoroughly wiped with a cloth containing ethanol solution 70 v/v% after each session to prevent any bias from olfactory cues.

2.2.3. Y-maze test

An asymmetric Y-maze paradigm was applied to assess the short-term spatial memory of the mice.

The Y-shaped translucent maze was placed on a black board enclosed by black walls, which prevented any view of the external environment. Unique visual cues were placed around the three arms of the maze. The test was performed on the 9th day after the end of the toxin regimen. The animals were first placed into the labyrinth and allowed to explore the common arm and only 1 of the 2 shorter arms of the maze until each mouse had spent a total of 2 min in the open short arm. After the session of acquisition, the mice were returned to their home cages for 30 min. In the trial of recall, the animals were free to enter all arms, and the tracking was stopped after a total of 2 min had been spent inside the 2 short arms. The exploratory activity of the mice was tracked and recorded with the SMART v2.5 system (Panlab, Barcelona, Spain). For evaluation, the recognition indices of the time spent and the distances travelled in the novel arm of interest were used. The recognition index was calculated via the following formula, where P is the examined parameter:

$$\text{recognition index} = P_{\text{novel arm}} / (P_{\text{novel arm}} + P_{\text{known arm}})$$

The side of the novel arm was randomly distributed in each group, and the board and walls of the setup were thoroughly wiped with a cloth containing ethanol solution 70 v/v% after each session to prevent any bias from olfactory cues.

2.3. High-performance liquid chromatography (HPLC)

2.3.1. Sample preparation

The investigated reference compounds (L-TRP, L-KYN-sulfate, KYNA and 3-OHK), the substances used as internal standards (3-nitro-L-tyrosine (3-NLT) and isoproterenol (IPR)), zinc acetate dihydrate and sodium octylsulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and perchloric acid (PCA) were purchased from Scharlau (Barcelona, Spain), and acetic acid, phosphoric acid, sodium dihydrogenophosphate and disodium ethylenediaminetetraacetate were purchased from VWR International (Radnar, PA, USA).

On the 18th day of the experiment (12 days after the last 3-NP injection), the mice were deeply anesthetized with isoflurane (Forane®; Abott Laboratories Hungary Ltd., Budapest, Hungary). After thoracotomy, 0.3–0.7 ml venous blood was obtained from the right ventricle by intracardial puncture, followed by perfusion with artificial cerebrospinal fluid (composition in mM: 122 NaCl, 3 KCl, 1 Na_2SO_4, 1.25 KH_2PO_4, 10 D-glucose*H_2O, 1 $MgCl_2$*$6H_2O$, 2 $CaCl_2$*$2H_2O$, 6 $NaHCO_3$) for 2 min by an automatic peristaltic pump. The blood samples were left to coagulate for 30 min, and were then centrifuged for 10 min at 12,000 rpm. The supernatant sera were pipetted into polypropylene Eppendorf tubes and stored at -80 °C until further sample handling. After perfusion, the brains were rapidly removed on ice and stored at -80 °C until analysis. Before analysis, the brain samples were weighed and then sonicated in an ice-cooled solution (250 µl) containing 2.5 w/w% PCA with 2 µM 3-NLT and 600 nM IPR as internal standards. The samples were next centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were collected.

Before analysis, the serum samples were thawed and, after a brief vortex 200 µl of serum sample was 'shot' onto 200 µl of precipitation solvent (containing 5 w/w% PCA with 4 µM 3-NLT and 2 µM IPR as internal standards). The samples were subsequently centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were collected.

2.3.2. Measurement of TRP, KYN and KYNA

The TRP, KYN and KYNA concentrations of the samples were quantified by a slight modification of the method of Hervé et al. (Hervé et al. 1996), with the use of an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a fluorescent and a UV detector, the fluorescent detector was set at excitation and emission wavelengths of 344 nm and 398 nm for the determination of KYNA and TRP, and the UV detector was set at 365 nm for the determination of KYN and the internal standard 3-NLT. Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm x 4.6 mm I.D. (Phenomenex Inc.,

Torrance, CA, USA) after passage through a Hypersil ODS pre-column, 20×2.1 mm I.D., 5 μm particle size (Agilent Technologies, Santa Clara, CA, USA) with a mobile phase composition of 0.2 M zinc acetate/ACN = 95/5 (v/v%) with the pH adjusted to 6.2 with glacial acetic acid, applying isocratic elution. The flow rate and the injection volume were 1.5 ml/min and 50 μl, respectively. The peaks of the different compounds obtained during chromatographic analysis are presented in Fig. 2.

2.3.3. Measurement of 3-OHK

For determination of the concentrations of 3-OHK and its internal standard (IPR), we applied the Agilent 1100 HPLC system equipped with Model 105 electrochemical detector (Precision Instruments, Marseille, France). In brief, the working potential of the detector was set at +650 mV, using a glassy carbon electrode and an Ag/AgCl reference electrode. The mobile phase containing sodium octylsulfate (2.8 mM), sodium dihydrogenophosphate (75 mM) and disodium ethylenediaminetetraacetate (100 μM) was supplemented with acetonitrile (5 v/v%) and the pH was adjusted to 3.0 with phosphoric acid (85 w/w%). The mobile phase was delivered at a rate of 1 ml/min at 40 °C onto the reversed-phase column (HR-80 C18, 80×4.6mm, 3-μm particle size; ESA Biosciences, Chelmsford, MA, USA) after passage through a pre-column (Hypersil ODS, 20×2.1 mm, 5-μm particle size; Agilent Technologies, Santa Clara, CA, USA). Ten-microliter aliquots were injected by the auto-sampler with the cooling module set at 4 °C. The peaks of the different compounds obtained during chromatographic analysis are presented in Fig. 2.

2.4. HPLC method validation

2.4.1. Calibration curve and linearity

Calibrants were prepared at 6 different concentration levels, from 0.1 to 50 μM, 0.05 to 5 μM, 1 to

100 nM, 10 to 200 nM, 0.5 to 7.5 μM and 25 to 600 nM for TRP, KYN, KYNA, 3-OHK and the internal standards, 3-NLT and IPR, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R software (R Development Core Team 2002). Very good linearity was observed throughout the investigated concentration ranges for TRP, KYNA, KYN, 3-OHK and the internal standards when either fluorescence, UV or electrochemical detection was applied.

2.4.2. Selectivity

The selectivity of the method was checked by comparing the chromatograms of TRP, KYN, KYNA, 3-OHK and the internal standards for blank serum and homogenized brain samples and those for spiked serum and homogenized brain samples. All compounds could be detected in their own selected chromatograms without any significant interference.

2.4.3. LOD and LLOQ

The limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined via the signal-to-noise ratio with a threshold of 3 and 10, respectively, according to the ICH guidelines (ICH 1995). The LOD for brain samples was 10, 40, 0.4 and 10 nM, while LLOQ was 20, 130, 1 and 30 nM for TRP, KYN, KYNA, and 3-OHK, respectively. The LOD for the serum samples was 15, 100, 1 and 10 nM, while LLOQ was 35, 275, 3.75 and 30 nM for TRP, KYN, KYNA and 3-OHK, respectively.

2.4.4. Precision

Replicate HPLC analysis showed that the relative standard deviation was ≤ 2.2% for the peak area

response and ≤ 0.1% for the retention time for TRP, KYN and KYNA, whereas in the case of 3-OHK the relative standard deviation was ≤ 8.6% for the peak area response and ≤ 0.3% for the retention time.

2.4.5. Recovery

The relative recoveries were estimated by measuring spiked samples of TRP, KYN, KYNA and 3-OHK at 2 concentrations with 3 replicates of each. No significant differences were observed for the lower and higher concentrations. The recoveries for the brain samples were 86 to 91%, 98 to 100%, 82 to 92% and 69 to 74% for TRP, KYN, KYNA and 3-OHK, respectively. The recoveries for the serum samples ranged from 77 to 90%, 77 to 82%, 103 to 108% and 28 to 34% for TRP, KYN, KYNA and 3-OHK, respectively.

2.5. Statistics

All statistical analyses were performed with the use of the R software (R Development Core Team 2002). We first checked the distribution of data populations with the Shapiro-Wilk test, and we also performed the Levene test for analysis of the homogeneity of variances. In the behavioral analyses, all the data exhibited normal distribution and equal variances were assumed, and we therefore used the independent t-test to compare the 2 groups. As a consequence of the multiple comparisons of the same 2 groups, the necessary corrections were carried out in p values according to the Bonferroni method. In the case of the HPLC analyses, due to the necessity of a large number of comparisons of data from the 2 groups obtained via a single measurement, two-sample t-tests via Monte-Carlo permutation (with 10,000 random permutations) were applied. We rejected the null hypothesis when the corrected p values were < 0.05, and in such cases the differences were considered significant. Data with Gaussian or non-Gaussian distributions were plotted as means (± S.E.M.) or medians

(and interquartile range), respectively.

3. Results

3.1 Behavioral assessment

Our results demonstrated a significant decrease in the latency to fall period in the 3-NP-treated animals (35.33 ± 6.97 s; $p < 0.001$, independent t-test) in the rotarod test 1 day after the last toxin injection in the subacute treatment regime as compared with the control group (126.34 ± 8.08 s). 10 days after the last injection, this difference had disappeared ($p = 0.16$, independent t-test; Fig. 3A). In the Y-maze test, there was no difference between the groups 9 days after the last injection ($p = 0.37$, independent t-test; Fig. 3B). In the open-field test performed 3 days after the last injection, a significant decrease was observed in the ambulation distance during the total 15-min tracking period (3-NP: 3203.2 ± 249.9 cm; control: 4874.9 ± 409.6 cm; $p = 0.003$, independent t-test; Fig. 3C) including that in the last 5-min period (3-NP: 950.4 ± 108.6 cm; control: 1359.5 ± 116 cm; $p = 0.028$, independent t-test; Fig. 3E). However, 11 days after the last 3-NP injection, the difference was significant only as regards the last 5-min period (3-NP: 985.2 ± 69.9 cm; control: 1417.1 ± 106 cm; $p = 0.001$, independent t-test; Fig. 3C). No difference in rearing time was detected between the groups either in the total 15-min (Fig. 3D) or in the last 5-min (Fig. 3F) tracking period.

3.2. HPLC analysis

As shown in Table 1, no difference was found in the KYNA concentration in the serum or in the investigated brain regions of the toxin-treated and control mice 12 days after the last injection, and there was no significant difference between the TRP levels of the serum samples of the toxin-treated

and control mice, whereas in the case of KYN, a significant difference was detected in the cortex (2,679 ± 110 pmol/g wet weight (ww); p = 0.017, permutation test) relative to the control value (3,110 ± 112 pmol/g ww). However, significantly decreased TRP levels were found in the striatum (19,530 (17,700–20,440) pmol/g ww; p = 0.0002, permutation test), cortex (14,360 (13,540–16,300) pmol/g ww; p = 0.003, permutation test), hippocampus (16,550 (14,790–17,580) pmol/g ww; p = 0.006, permutation test), cerebellum (14,930 (14,210–16,260) pmol/g ww; p = 0.0002, permutation test) and brainstem (12,740 (12,280–14,100) pmol/g ww; p = 0.0004, permutation test) of 3-NP-treated mice as compared with the controls (27,950 (23,820–35,700); 22,860 (17,260–28,430); 26,590 (18,020–30,730); 24,810 (17,630–28,510); 20,960 (15,920–29,890) pmol/g ww, respectively). The observed decreases in TRP level were generally associated with an increased KYN/TRP ratio, an index widely used to assess the metabolic activity of KP. Indeed, a significant increase in KYN/TRP ratio were observed in the striatum (0.156 ± 0.014; p = 0.008, permutation test), hippocampus (0.158 ± 0.008; p = 0.023, permutation test), cerebellum (0.166 ± 0.01; p = 0.008, permutation test) and brainstem (0.166 ± 0.008; p = 0.009, permutation test) as compared with the control values (0.102 ± 0.011; 0.122 ± 0.011; 0.129 ± 0.007; 0.117 ± 0.013, respectively; Fig. 4); however, the difference in the cortex did not reach statistical significance. No difference in the KYN/TRP ratio was observed in the serum. Additionally, a significant decrease in 3-OHK level was observed in the cerebellum (to below the LOD; p = 0.005, permutation test) in the 3-NP-treated mice in comparison with the control value (66.84 (28.99–97.64) pmol/g ww), but not in the other brain regions.

4. Discussion

Although HD is a rare condition, it serves as a prototype of monogenically determined, but multifactorially affected neurodegenerative disorders (Kim and Fung 2014). Study of the

pathogenetic process in animal models of HD could therefore provide a valuable tool for insight into the general neurodegenerative process, yielding a possibility of finding promising targets for drug development.

From this respect, genetic rodent models are optimal tools for the study of HD in view of the possible molecular and genetic translatability to humans (Ramaswamy et al. 2007). However, animal genetic models also fail to demonstrate the pathological alterations characteristic of human HD completely. Furthermore, the relatively long duration of disease development may be a disadvantage in studies in which the therapeutic effects of potential neuroprotective agents are assessed.

Before genetic models were established, a number of toxin-based models were utilized to study the neurodegenerative processes in HD, which may still be a rapid and cost-effective way of screening the therapeutic effects of potential drug candidates prior to the costly and demanding application of genetic models. These inhibitors of the mitochondrial functions are widely used to model the mitochondrial dysfunction in HD (Browne 2008).

SDH, the enzyme of complex II in the mitochondrial electron transport chain (Alston et al. 1977), is reversibly and irreversibly inhibited by malonate and 3-NP, respectively, toxins which have proved to furnish applicable phenotypic models of HD (Beal and Brouillet 1993; Beal et al. 1993). 3-NP is currently the toxin most widely used to study certain neuropathological aspects of the disease.

The consecutive or continual administration of 3-NP to rodents (i.p. or subcutaneously, respectively) leads to bilateral dystonia, rigidity, gait disturbances and uncoordinated movements. At lower cumulative doses of 3-NP (<60 mg/kg), motor symptoms have been described to be manifested in mild movement disturbances, such as slowness and a subtle wobbling gait. However, some authors have also reported early-stage hyperactivity at lower cumulative doses (<40 mg/kg) (Borlongan et al. 1995a). When the cumulative dose is elevated up to 80 mg/kg, the general weakness becomes more marked and dystonia develops, the latter being particularly pronounced in

the hind-limbs at this dose, and appearing soon after toxin administration (Brouillet 2014). A further elevation of the cumulative dose (>100 mg/kg) results in more expressed weakness and rigidity (Brouillet 2014). However, subsequent to the termination of the subacute toxin regimen in mice in the widely-applied experimental setup (as used in the current study), the detected alterations in motor functioning tend to abate and then cease (Fernagut et al. 2002a, 2002b; Li et al. 2009). It should be noted, however, that the extended application of 3-NP in a considerably higher cumulative dose resulted in persistent locomotor deficits in one study on mice (Stefanova et al. 2003). Studies involving 3-NP intoxication in rats yielded rather inconclusive results in terms of a persistent motor dysfunction (Teunissen et al. 2001; Tasset et al. 2012).

As regards a cognitive impairment, rats treated with a cumulative dose of 190 mg/kg demonstrated a severely decreased memory function in a fear-associated memory test (a passive avoidance task) and a perceptual-learning task (an attentional set-shifting task) (El Massioui et al. 2001). However, in a simple 2-trial Y-maze paradigm, performed on the 15th day after termination of the administration of 3-NP to mice (in a cumulative dose of 320 mg/kg), the intoxicated animals did not display any 3-NP-induced memory impairment (Li et al. 2009).

The above observations in mice were confirmed by our findings, i.e. most of the behavioral changes disappeared (i.e. the latency to fall, tested by rotarod; and the ambulation distance, tested in an open-field) or could not be detected (the recognition index, tested with the Y-maze test) 11 days after the last injection. The lack of difference in cognitive performance was not influenced by any incidental difference in locomotor acitivity, because we did not observe any significant difference in the total ambulation distance within the Y-maze (not shown). It is noteworthy, however, that we observed a significant reduction in the ambulation distance parameter in the last 5 min of the open-field test in the toxin-treated animals as compared with the controls, which may reflect a slight persistent behavioral dysfunction.

As concerns to the previously observed alterations in the TRP metabolism in transgenic HD

animals, the 3-OHK and KYNA levels were reported to be elevated in the striatum and cortex of a heterozygous transgenic mouse (FVB/N background, 89 CAG repeats, 12-15 months of age) model of HD (Guidetti et al. 2000). Another study involving the R6/2 mouse model of HD revealed increased kynurenine 3-monooxygenase activity in the striatum, cortex and cerebellum of 6-12-week-old animals, while the 3-hydroxyanthranilic acid oxygenase, kynurenine aminotransferase (KAT)-I and KAT-II activities (catalyzing the conversion of KYN to KYNA) in the cortex and cerebellum were unchanged. An elevated synthesis of 3-OHK was found in the striatum and a decreased kynureninase activity in the striatum and cortex of 4-12-week-old animals, but not in the cerebellum. Other investigated compounds (KYN, KYNA and QUIN) did not display any difference (Sathyasaikumar et al. 2010). In the R6/2 model, enhanced 3-OHK concentrations were observed in the striatum, cortex and cerebellum of 4-12-week-old animals, but no difference was found in younger mice (Guidetti et al. 2006). In the Hdh^{Q92} and Hdh^{Q111} models, 5-17-month-old animals demonstrated increased levels of 3-OHK in the striatum, cortex and cerebellum, and of QUIN in the striatum and cortex, whereas there were no differences in younger animals (Guidetti et al. 2006). In the YAC128 model, the 3-OHK concentrations were increased in the striatum, cortex and cerebellum of 5-12-month-old mice, while the QUIN levels were elevated only in the striatum and cortex of 8-12-month-old animals (Guidetti et al. 2006). In the same YAC128 mouse model, the striatal expression of indoleamine 2,3-dioxygenase (IDO)-1 (probably the most important enzyme participating in the TRP catabolism in the brain), was found to be up-regulated, whereas IDO-2 and tryptophan 2,3-dioxygenase did not undergo any significant change. As concerns metabolite concentrations, the levels of TRP and 3-OHK proved to be decreased in the striatum of 3-month-old animals. However, no difference in TRP level was found in 12-month-old mice, while 3-OHK and KYN were significantly elevated in the striatum. The KYNA and QUIN concentrations did not change in either group (Mazarei et al. 2013). Studies on the hippocampus did not indicate significant difference in any examined model or age group (Guidetti et al. 2006).

In contrast with genetic models, there are no available published data on the effects of 3-NP toxicity on the concentrations of TRP and KP metabolites under *in vivo* conditions in mice. One of the two studies of the effects of the chronic treatment of rats with 3-NP demonstrated a significant decrease in the amount of KAT-I-immunoreactive cells, predominantly in the striatum (Csillik et al. 2002). The other study on *ex vivo* rat cortical slices, showed that 3-NP dose-dependently inhibited the production of KYNA and led to decreased KAT-I and KAT-II activities in the cortical tissue homogenates (Luchowski et al. 2002). **No murine studies have been conducted previously to assess the effects of *in vivo* applied 3-NP on the KP metabolism at the metabolite level *per se*, and there have been no studies of KP alterations due to 3-NP intoxication in the serum, which would allow conclusions as to the possible effects of systemic KP alterations within the CNS. We have now quantitatively assessed the levels of 4 KP metabolites in the striatum, cortex, hippocampus, cerebellum and brainstem of mice, with a relatively high number of animals per group (n = 14-16).**

Our findings indicated a decreased TRP level in association with an increased KYN/TRP ratio in most of the examined brain regions of C57Bl/6 mice treated with 3-NP, and also a reduced concentration of 3-OHK in the cerebellum. It is noteworthy that we did not detect any significant difference in serum samples, which suggests that the observed alterations are specific for the examined brain regions and are not affected by a systemic change and/or an altered permeability of the blood-brain barrier. Our results on 5-month-old 3-NP-treated animals resemble those from studies of 3-month-old transgenic YAC128 animals (Mazarei et al. 2013), which is known to be one of the best animal strains for the modeling of the alterations in human HD (Slow 2003; Pouladi et al. 2013). The increase observed in the KYN/TRP ratio is comparable with the previous finding of increased IDO-1 activity in the brain (but not the serum) of YAC128 animals, an alteration reflecting that observed in several neurodegenerative diseases and their animal models (Vécsei et al. 2013), and suggested to contribute to the neurodegenerative process. The similarity of the findings

of the KP alterations in this 3-NP model with those observed previously in genetic models (and especially the YAC128 model) lead us to presume that the KP alterations observed in transgenic animals might be secondary to a mitochondrial dysfunction as present in the pathogenesis of HD, and that 3-NP toxicity may comprise a useful and cheap tool with which to screen the efficacy of potential drug candidates before the application of more demanding genetic models.

The rationale of our study was to find a valuable biomarker for therapeutic studies in the widely applied subacute 3-NP model of HD with previously demonstrated neurodegenerative characteristics, at a time point when merely minor behavioral alterations persist. Accordingly, we were confirmed that the behavioral alterations evoked by 3-NP toxicity are mostly intermittent, similarly as observed previously by others, which suggests that these alterations might be only functional and do not reflect the long-term effects of delayed-type neurodegeneration. On the other hand, at this delayed time point, we detected a marked persistent increases in the KYN/TRP ratio in the striatum, hippocampus, cerebellum and brainstem and a slight reduction in the level of 3-OHK in the cerebellum. Such alterations have not been reported earlier in 3-NP studies and are similar to those seen in young genetic animal models (especially in 3-month-old YAC128 transgenic mice). In the era of advanced genetic models of HD, our findings demonstrate the continued relevance and applicability of toxin models, which may provide cost-effective and rapid ways of screening potential drug candidates to treat this currently intractable disease.

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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8. Figure legends

Fig. 1 Schematic depiction of the kynurenine pathway of the tryptophan metabolism.

Fig. 2 Chromatograms of the analyzed compounds of the kynurenine pathway of the tryoptophan metabolism, from C57Bl/6 murine brain samples. *KYNA* kynurenic acid, *TRP* tryptophan, *KYN* kynurenine, *3-NLT* 3-nitro-L-tyrosine, *3-OHK* 3-hydroxykynurenine, *IPR* isoproterenol, *FLD* fluorescent detector, *ECD* electrochemical detector

Fig. 3 The effects of 3-NP treatment on different behavioral parameters in C57Bl/6 mice. Shortly after the last 3-NP injection, the latency to fall was significantly decreased in the rotarod test (**A**), but this difference had disappeared 9 days later. The Y-maze test, performed 9 days after the last 3-NP injection, did not reveal any difference between the groups (**B**). In the open-field paradigm 3 days after the last 3-NP injection, the ambulation distance was significantly decreased in the total 15-min examination period (**C**) and in the last 5-min period (**E**). However, 11 days after the last 3-NP injection, the difference was significant only in the last 5 min. No difference in rearing time was detected (**D**, **F**). The gray area indicates the 3-NP treatment period.
3-NP mice: n = 16, control mice: n = 14; data are means ± S.E.M; *3-NP* 3-nitropropionic acid, *LTF* latency to fall, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 4 The effects of 3-NP treatment on the KYN/TRP ratio in the serum and different brain regions of C57Bl/6 mice. The KYN/TRP ratio was significantly elevated in the striatum, hippocampus, cerebellum and brainstem of the 3-NP-treated mice. The increase in the cortex did not reach the level of statistical significance. No alteration was observed in the serum. Data are means ± S.E.M; *3-NP* 3-nitropropionic acid, *KYN* kynurenine, *TRP* tryptophan, * $p < 0.05$, ** $p < 0.01$