ORIGINAL ARTICLE

Age- and disease-specific changes of the kynurenine pathway in Parkinson's and Alzheimer's disease

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Abstract

The kynurenine (Kyn) pathway, which regulates neuroinflammation and N-methyl-D-aspartate receptor activation, is implicated in Parkinson's disease (PD) and Alzheimer's disease (AD). Age-related changes in Kyn metabolism and altered cerebral Kyn uptake along large neutral amino acid transporters, could contribute to these diseases. To gain further insight into the role and prognostic potential of the Kyn pathway in PD and AD, we investigated systemic and cerebral Kyn metabolite production and estimations of their transporter-mediated uptake in the brain. Kyn metabolites and large neutral amino acids were retrospectively measured in serum and cerebrospinal fluid (CSF) of clinically well-characterized PD patients ($n = 33$), AD patients ($n = 33$), and age-matched controls ($n = 39$) using solid-phase extraction-liquid chromatographic-tandem mass spectrometry. Aging was disease independently associated with increased Kyn, kynurenic acid and quinolinic acid in serum and CSF. Concentrations of kynurenic acid were reduced in CSF of PD and AD patients ($p = 0.001$; $p = 0.002$) but estimations of Kyn brain uptake did not differ between diseased and controls. Furthermore, serum Kyn and quinolinic acid levels strongly correlated with their respective content in CSF and Kyn in serum negatively correlated with AD disease severity ($p = 0.002$). Kyn metabolites accumulated with aging in serum and CSF similarly in PD patients, AD patients, and control subjects. In contrast, kynurenic acid was strongly reduced in CSF of PD and AD patients. Differential transporter-mediated Kyn uptake is unlikely to majorly contribute to these cerebral Kyn pathway disturbances. We hypothesize that the combination of age- and disease-specific changes in cerebral Kyn pathway activity could contribute to reduced neurogenesis and increased excitotoxicity in neurodegenerative disease.

Keywords: ageing, Alzheimer's disease, kynurenine, kynurenic acid, neurodegeneration, Parkinson's disease.
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As global life-expectancy rises, age-related neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's disease (AD) become a substantial burden to our healthcare systems (Feigin *et al.* 2017). Although they have a distinct neuropathological outcome and clinical profile, PD and AD share common pathophysiological features including neuro-inflammation, chronic *N*-methyl-D-aspartate (NMDA) receptor activation and metabolic dysfunction (Glass *et al.* 2010; Olivares *et al.* 2012; Jha *et al.* 2017). Kynurenine (Kyn) pathway metabolites are emerging as cross-organ signaling molecules that regulate these pathophysiological events and age-related changes in Kyn pathway activity could contribute to the onset and progression of PD and AD (Cervenka *et al.* 2017; Lim *et al.* 2017; Schwarcz and Stone 2017).

The Kyn pathway uses the amino acid tryptophan (Trp) as a substrate and produces several metabolites including kynurenic acid (KA), 3-hydroxykynurenine (3-Hk), xanthurenic acid (XA) and quinolinic acid (QA) (Fig. 1a). These metabolites have a wide range of physiological effects both in peripheral tissue and in the brain. For example, Kyn modulates the immune cell responses and dampens inflammation (Munn and Mellor 2013) and KA controls energy homeostasis and inflammation in adipose tissue (Agudelo *et al.* 2018). In the brain, KA acts as an endogenous NMDA receptor antagonist while QA has neurotoxic properties by agonizing the NMDA receptor (Stone *et al.* 2013) (Fig. 1c). In addition, KA, 3-Hk, and QA exert anti- and pro-oxidant effects (González Esquivel *et al.* 2017).

The activity of the Kyn pathway is controlled by the enzymes tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase that catalyze the conversion of Trp into Kyn. The expression of tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase in the brain is normally low and confined to specific brain regions. Cerebral Kyn pathway activity is therefore largely driven by Kyn and 3-Hk that are transported from the blood across the blood–brain barrier (BBB) by large-neutral amino acid transporters (Schwarcz *et al.* 2012) (Fig. 1b). The rate at which Kyn and 3-Hk are transported across the BBB depends on their concentration in the blood relative to that of large neutral amino acids (LNAA) [including Trp, leucine (Leu), isoleucine (Ile), valine (Val), phenylalanine (Phe), and tyrosine (Tyr)] (Fernstrom and Wurtman 1971; Fukui *et al.* 1991). This rate can be estimated by constructing ratios between Kyn or 3-Hk and LNAAs (Van

Gool *et al.* 2008; Fernstrom 2013). Dysregulation of the Kyn pathway or altered metabolism of LNAAs in peripheral organs could thus influence cerebral Kyn pathway activity.

Disturbances of Kyn pathway metabolites in the blood of PD and AD patients are common (Widner *et al.* 2000; Hartai *et al.* 2007; Gulaj *et al.* 2010; Schwarz *et al.* 2013; Giil *et al.* 2017; Havelund *et al.* 2017; Chang *et al.* 2018) but Kyn metabolites concentrations in cerebrospinal fluid (CSF), as a measure of their cerebral production, have been less well documented (Heyes *et al.* 1992; Wennstrom *et al.* 2014; Havelund *et al.* 2017). In addition, with the exception of two (Giil *et al.* 2017; Chang *et al.* 2018), most of the above-mentioned studies used a relatively small sample size and only one study analyzed Kyn metabolites in time-linked serum and CSF samples (Havelund *et al.* 2017). To our knowledge, no study estimated the rate of transporter-mediated cerebral uptake of Kyn in PD or AD.

To further elucidate the relevance of the Kyn pathway in age-related neurodegenerative diseases we assessed an extensive panel of Kyn metabolites in time-linked serum and CSF within a large cohort of PD patients, AD patients and age-matched cognitively healthy controls. We investigated the effect of age on Kyn metabolites and included a thorough analysis of the influence of medication use. Moreover, we measured LNAA to estimate transporter-mediated cerebral uptake of Kyn and 3-Hk and determined whether Kyn pathway metabolite concentrations correlated with measures of disease severity in AD patients.

Methods

Study population and sampling procedure

The study population comprised 105 subjects among whom AD patients ($n = 33$), PD patients ($n = 33$), and control individuals in good cognitive health ($n = 39$) (Table 1). Time-linked serum and CSF samples were retrospectively selected from the biobank of the Institute Born-Bunge (Wilrijk, Belgium) by an investigator other than the investigator performing biochemical and statistical analyses. Patients and controls were age- and sex-matched. Individuals with active oncological disease or with pre-existing kidney disease were not selected (based on clinical records) as these diseases can affect Trp metabolism (Platten *et al.* 2012; Theofylaktopoulou *et al.* 2013). All participants were recruited between 1991 and 2014 at the Memory Clinic of the Hospital Network Antwerp Middelheim (ZNA) and Hoge Beuken (Antwerp, Belgium) according to a

Fig. 1 Aging affects peripheral and central Kyn pathway metabolites but not LNAAs. (a) Trp is taken up from the diet and processed intra- and extrahepatically. Kyn metabolites are mainly produced in extra-hepatic tissue. (b) Trp, Kyn, and 3-HK compete with LNAA for transport across the BBB. (c) In the brain, the Kyn pathway is segregated based on cell-type; astrocytes mainly produce KA while microglia produce quinolinic acid. These metabolites can be released in the extracellular space and CSF. (d) Scatterplot showing the relationship between age and standardized scores for Trp and Kyn metabolites or LNAAs (e) in serum and CSF of controls, PD and AD patients. The best-fit line from linear regression (including 95% confidence interval) as well as the F-value is provided for model comparison. *$p < 0.01$ for F-tests. 3-Hk, 3-hydroxykynurenine; AD, Alzheimer's disease; BBB, blood–brain barrier; CSF, cerebrospinal fluid; Ile, isoleucine; KA, kynurenic acid; Kyn, kynurenine; Leu, leucine; LNAA, large neutral amino acids; PD, Parkinson's disease; Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; Val, valine; XA, xanthurenic acid. *$p < 0.01$.
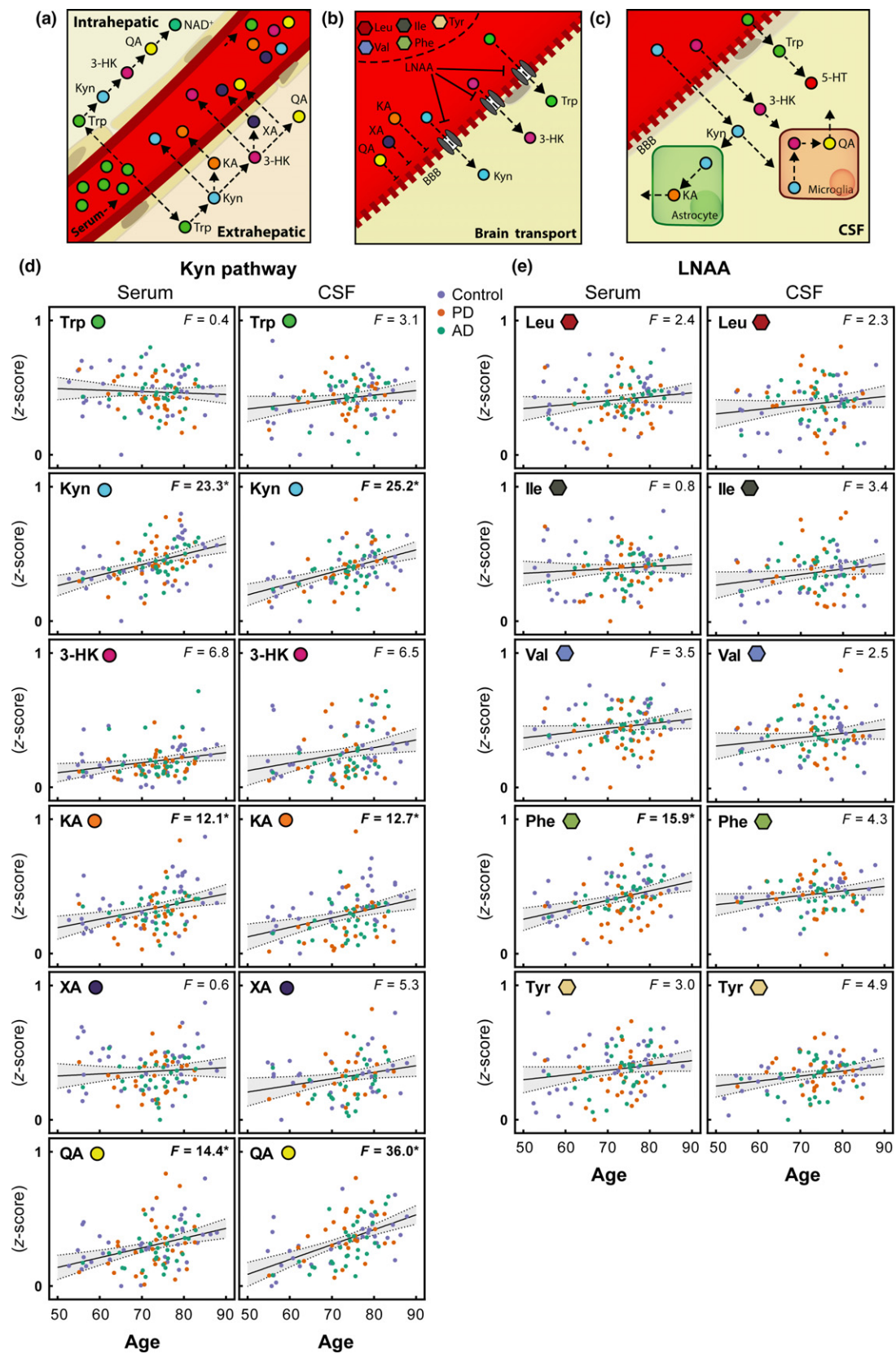
Table 1 Demographic and clinical characteristics

	Control $n = 39$	PD $n = 33$	AD $n = 33$
Sex			
Female, %	53.8	39.4	54.5
Age, years	71.3 (10.7)	73.4 (6.5)	73.7 (6.0)
Medication, number available	37/39	20/33	33/33
Antidepressant	5	8	13
Antipsychotic	1	5	17
Anticholinesterase	–	–	17
Levodopa use	–	13	–
Dopamine agonist	–	6	–
Duration of disease, number available	–	21/33	27/33
Years	–	3.0 (1.0–8.0)	3.0 (2.0–5.0)
Alzheimer's diagnosis			
Neuropathologically confirmed, %	–	–	39.4
Parkinson's diagnosis			
MCI, %	–	39.4%	–
MMSE, number available	–	17/33	28/33
Mean score	–	20.8 (6.4)	16.2 (6.5)

Table showing mean and SD or, when indicated, percentage for demographics, physical parameters and disease parameters for control subjects, PD patients and AD patients.

AD, Alzheimer's disease; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; PD, Parkinson's disease.

previously described protocol (Vermeiren *et al.* 2013; Van Der Zee *et al.* 2018). More specifically, patients were recruited for inclusion in a longitudinal, prospective study on neuropsychiatric symptoms in dementia (Engelborghs *et al.* 2005), according to which patients underwent a diagnostic work-up consisting of a physical, neurological and neuropsychological examination, routine blood screening, a lumbar puncture, and structural neuroimaging.

The diagnostic criteria for probable AD were applied according to the NINCDS-ADRDA criteria (McKhann *et al.* 1984) and were in agreement with the DSM–IV–TR criteria for dementia (American Psychiatric Association 2000). In case patients who consented deceased, brain autopsy was performed within 6–8 h following death according to a standard procedure in which the right hemisphere was placed in 12% formaline for fixation and consequent neuropathological assessment (Vermeiren *et al.* 2014). Diagnostic criteria for PD were the presence of at least two of four characteristic symptoms (resting tremor, bradykinesia, muscular rigidity, and impaired postural reflexes) combined with an insidious onset (Hoehn and Yahr 1967; Engelborghs *et al.* 2003). The control population consisted of individuals with disorders of the peripheral nervous system (e.g. peripheral facial nerve palsy) and complaints of lower back pain requiring a selective lumbar radiculography (Engelborghs *et al.* 2008). Sample size calculation was not performed given the exploratory nature of the study.

Data regarding disease duration, medication use, medication side-effects, comorbidities, and results from the Mini-Mental State

Examination (MMSE) as a measure of AD severity/stage at the time of blood and CSF sampling were obtained through thorough retrospective examination of medical records.

Written informed consent was provided by all patients. The study was conducted in compliance with the Helsinki Declaration. Ethics approval for human sample collection of CSF and serum was granted by the Medical Ethical Committee of the Middelheim General Hospital (Antwerp, Belgium; approval numbers 2805 and 2806). The study was not pre-registered.

Biochemical analysis of Trp, Kyn metabolites, and large neutral amino acids

Sampling of paired serum and CSF was performed according to a standard procedure between 08.00 and 10.00 a.m. (Van Der Zee *et al.* 2018). Samples were immediately snap frozen in liquid nitrogen and stored at −80°C until biochemical analyses. Samples were blinded prior to biochemical analyses.

Concentrations of Trp, Kyn, 3-Hk, KA, XA, and QA in blood and CSF were measured at the department of Laboratory Medicine of the University Medical Center Groningen using an automated online solid-phase extraction-liquid chromatographic-tandem mass spectrometric method with deuterated internal standards. Trp, Kyn, and 3-Hk were analyzed according to a previously described method (de Jong *et al.* 2009). KA, XA, and QA were analyzed essentially as previously described (Meinitzer *et al.* 2014). In short, 50 μL serum or 100 μL CSF was mixed with deuterated analogues [$(^2H_5)$ KA, $(^2H_4)$ XA, and $(^2H_3)$ QA], subsequently samples were extracted using Strata X-A 96-well plates (Phenomenex, Utrecht, The Netherlands). After sample clean-up 1 μL was injected into an Acquity UPLC (Waters, Milfords, MA, USA) coupled to a XEVO TQ-S MS/MS (Waters). Interassay coefficient of variations for KA, XA, and QA were below 4.2%, < 5.7 and 4%, respectively.

For the analysis of LNAAs (Leu, Ile, Val, Phe, and Tyr), 25 μL of plasma or CSF was mixed with a mix of stable isotope analogs ($^{13}C_6$-Leu, $^{13}C_6$-Ile, $^{13}C_5$-^{15}N-Val, $^{13}C_9$-^{15}N-Phe, $^{13}C_9$-^{15}N-Tyr). Subsequently, samples were passed through an Ostro Protein and Phospholipid removal plate (Waters). Eluate was injected (0.15 μL) into an Acquity UPLC (Waters) coupled to a XEVO TQ-S MS/MS (Waters). Mass spectrometer was run in positive electrospray ionization and selective reaction monitoring mode. Chromatography was performed on a Xbridge BEH Amide, 2.1 × 100 mm, 2.5 μm column (Waters). Interassay coefficient of variations was below 5.0% for all components.

The ratios Trp/LNAA, Kyn/LNAA, and 3-Hk/LNAA (with the LNAA being the sum of all LNAA including Trp) were calculated to provide a relative estimation of the transport of Trp, Kyn, and 3-Hk across the BBB. In addition, the following ratios were calculated: The Kyn/Trp ratio (multiplied by 100) in serum and CSF as an indicator of increased IFN-y-mediated Trp metabolism (Oxenkrug 2010), the KA/QA ratio (multiplied by 1000) in serum and CSF as a readout for relative neuroprotection (Vancassel *et al.* 2018) and the XA/3-HK ratio (multiplied by 100) in serum and CSF as a marker for vitamin B status (Ulvik *et al.* 2013).

Statistics

Statistical analyses were performed using SPSS 24 (IBM corp, Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Baseline characteristics are

reported as percentage, mean and standard deviation of the mean or median and interquartile range. Normality of the data was assessed using QQ-plots and the Shapiro–Wilk test. Group characteristics were compared using chi-square tests, ANOVA or Kruskall–Wallis tests.

Linear regression analyses were conducted to investigate the association between age and metabolite concentrations and the associations between serum and corresponding CSF metabolite concentrations. For these analyses Z-scores were calculated {[(metabolite) − mean]/standard deviation} and the lowest concentrations was set to 1. These were then log-transformed and used as dependent variables in regression analyses with age or the corresponding metabolite in serum as independent variable. Age-by-disease interaction analyses were conducted to investigate whether these associations differed between PD, AD, and control. The best-fit line (including 95% confidence interval) as well as the F-value is provided for model comparison.

Metabolite concentrations were first compared between control and PD and between control and AD using Mann–Whitney U tests. Next, in order to take into account the intercorrelation between the metabolites in each pathway while adjusting for covariates, we performed four MANCOVA using groups of correlated-dependent variables: (i) serum concentrations of Kyn pathway metabolites (Trp, Kyn, 3-HK, KA, XA, QA), (ii) serum concentrations of LNAAs (Trp, Leu, Ile, Val, Phe, Tyr), (iii) CSF concentrations of Trp and Kyn pathway metabolites or (iv) CSF concentrations of LNAAs. These models included age and sex as covariates. Prior to multivariate analyses, univariate normality of the residuals and homogeneity of variance and covariance was achieved by transforming metabolite concentrations (natural log) (Tabachnik and Fidell 2013). Multivariate outliers ($n = 4$ for serum and $n = 1$ for CSF) were detected and removed using Mahalanobis distance at $p < 0.001$ based on the χ^2 distribution (Tabachnik and Fidell 2013). Because of high multicollinearity among Val, Leu, and Ile (the branched-chain amino acids), these were imputed as the sum of their concentrations. Univariate ANCOVA were performed to compare the age- and sex-adjusted group means and to establish the association between age, sex and the individual metabolites. Contrasts were set to compare control to PD and control to AD.

Finally, the correlations between Kyn metabolites and disease severity of AD was analyzed using Spearman's rank correlation analyses.

The criterion α was set to 0.010 for all tests of significance to adjust for the inflated Type I error rate because of multiple testing (Tabachnik and Fidell 2013).

Results

Study population

Table 1 shows the characteristics for the study population. There were no significant differences among control, PD, and AD patients with respect to age and sex.

Time-linked serum and CSF samples were available for 90 out of 105 patients (serum only for 12 and CSF only for 3 patients). The storage time was different between the control, PD patient and AD patient group [median number of months (interquartile range) of 151 (134–164), 179 (148–235), and 125 (83–163), respectively], but correlated with none of the measured Kyn metabolites or LNAA in CSF or serum. Univariate and multivariate models (additionally adjusted for age and sex) were used to further investigate the potential confounding effects of storage time on metabolite concentrations. These models indicated that storage time was not associated with concentrations of Kyn metabolites and LNAAs in serum and CSF. Moreover, interaction analyses indicated that storage time did not interact with disease group to affect Kyn metabolite or LNAA concentrations.

Aging is associated with altered peripheral and central Kyn pathway activity

Linear regression analyses were used to investigate the associations between age and concentrations of Kyn metabolites or LNAAs in serum and CSF. The results indicated that aging was associated with increased serum levels of Kyn, KA, and QA [$F(1, 100) = 23.3$, $p < 0.001$; $F = 12.1$, $p < 0.001$ and $F = 14.4$, $p < 0.001$, respectively] (Fig. 1d). Similarly, aging was associated with increased CSF concentrations of Kyn, KA, and QA [$F(1, 91) = 25.2$, $p < 0.001$; $F = 12.7$, $p < 0.001$ and $F = 36.0$, $p < 0.001$]. Age-by-disease interaction analyses indicated that the associations between age and Kyn metabolites were not different among control, PD, and AD.

Regarding the LNAAs, aging was associated with increased Phe concentrations in serum [$F(1, 99) = 15.9$, $p < 0.001$]. The analyses showed no association between age and concentrations of LNAAs in CSF (Fig. 1e). Interaction analyses indicated neither an effect of disease on the associations between age and concentrations of LNAAs in serum nor in CSF.

KA is reduced in CSF of PD and AD patients

We first conducted multivariate analyses to investigate whether PD and AD were associated with changes in the combined concentrations of metabolites, considered as groups of correlated metabolites. These models indicated that the combined abundance of Kyn metabolites in serum – when adjusting for the intercorrelation between these metabolites and correcting for age and sex – differed between patients and controls [Wilks' lambda $F(12, 172) = 2.72$, $p = 0.002$]. The same was true for Kyn metabolites in CSF [Wilks' lambda $F(12, 158) = 2.97$, $p = 0.001$]. Disease state was also associated with changes in LNAAs in serum [Wilks' lambda $F(8, 176) = 2.65$, $p = 0.009$] but not in CSF [Wilks' lambda $F(8, 160) = 1.00$, $p = 0.438$] (Table S1). Sex was not associated with changes in Kyn metabolites or LNAAs when considered as groups of metabolites.

To further investigate these associations, we then conducted non-parametric tests and univariate tests controlling

for age and sex. For PD patients, non-parametric analyses showed trends towards reduced concentrations of Trp, KA, and XA in serum compared to serum of control subjects ($Z = -2.11$, $p = 0.035$; -1.98, $p = 0.047$ and -1.98, $p = 0.024$, respectively) (Fig. 2a). Regarding Kyn metabolites in CSF of PD patients, non-parametric analyses showed strongly reduced KA levels compared to control ($Z = -3.0$, $p = 0.003$). For the LNAAs, the analyses indicated reduced Phe and a trend toward reduced Val in serum of PD patients compared to control ($Z = -2.88$, $p = 0.004$ and $Z = -2.11$, $p = 0.035$, respectively) while no significant differences between PD and control for CSF were observed (Fig. 2b).

After adjustment for age and sex, KA levels in serum of PD patients were significantly reduced (adjusted marginal mean 33.3 nM/L 95% CI [28.5, 39.0] vs. 44.3 nM/L 95% CI [38.3, 51.2], $p = 0.010$). Adjustment had no major effects on the other analyses.

For AD patients, the analyses showed a trend towards reduced XA in serum of AD patients compared to control ($Z = -2.41$, $p = 0.016$) (Fig. 2a). Similar as in PD patients, KA levels were strongly reduced in CSF of AD patients when compared to control subjects ($Z = -3.0$, $p = 0.003$). Concentrations of LNAAs in serum and CSF were not different between AD patients and controls (Fig. 2b).

When adjusting for age and sex, XA concentrations in serum of AD patients were significantly reduced (adjusted marginal mean 12.0 nM/L 95% CI [9.5, 15.2] vs. 18.7 nM/L 95% CI [15.0, 23.3], $p = 0.007$). Adjustment did not influence other analyses.

Table S2 provides an overview of the median concentrations of the metabolites and statistical details. We also analyzed ratios between Kyn metabolites. These analyses revealed a reduced KA/QA ratio in CSF of PD patients compared with control ($Z = -3.52$, $p < 0.001$). In addition, trends were found towards a reduced KA/QA ratio in CSF of AD patients ($Z = -2.20$, $p = 0.028$), a reduced KA/QA in serum of PD patients ($Z = -2.1$, $p = 0.037$) and a reduced XA/3-Hk ratio in serum of PD and AD patients when compared with control subjects ($Z = -2.16$, $p = 0.031$ and $Z = -2.48$, $p = 0.013$, respectively). Neither PD or AD was associated with changes in the Kyn/Trp ratio when comparing serum or CSF levels to control.

Transporter-mediated brain uptake of Kyn not altered in PD and AD

To establish whether altered transporter-mediated brain uptake of Trp, Kyn or 3-Hk could contribute to changes in cerebral Kyn pathway activity, we then analyzed whether indices for this type of brain uptake were different between groups (Fig. 2c). The analyses indicated no differences with regard to the ratios Trp/LNAA, Kyn/LNAA and 3-Hk/LNAA in serum for PD ($Z = -0.21$, $p = 0.838$; $Z = -1.81$, $p = 0.070$ and $Z = -0.71$, $p = 0.477$, respectively) or AD

($Z = 0.00$, $p = $ not calculated; $Z = -0.50$, $p = 0.621$ and $Z = -0.21$, $p = 0.838$) compared with control.

Medication use does not influence Kyn pathway metabolite levels in PD and AD

Previous studies have shown that the use of medication such as antidepressants and levodopa can influence Kyn metabolite concentrations (Myint et al. 2011; Ara and Bano 2012; Kocki et al. 2012; Gibney et al. 2014; Eskelund et al. 2017; Leppik et al. 2018). Because medication data were not available for all patients, we first conducted sensitivity analyses to assess whether availability of medication use was associated with alterations of Kyn pathway metabolites. For these analyses we used the same multivariate models as described above (thus controlling for disease group, age and sex). These models indicated that availability of medication data was not associated with changes in Kyn metabolites and LNAA in serum and CSF. Because of significant differences between disease groups, patients were then stratified according to diagnosis (control, PD, AD).

The use of antidepressants was associated with a trend towards increased QA levels in serum in control subjects and an increased Kyn/LNAA ratio ($Z = 2.1$, $p = 0.032$ and $Z = 2.6$, $p = 0.007$, respectively) suggesting increased peripheral Kyn pathway activity. In PD patients, administration of antidepressants, antipsychotics, levodopa, and dopamine agonists was not associated with differences in metabolite concentrations or transport ratios in serum and CSF. Moreover, there were no differences when comparing PD patients free of dopaminergic drugs ($n = 6$), patients using either levodopa or a dopamine agonist ($n = 8$ and $n = 1$, respectively) or patients using a combination of both types of drugs ($n = 5$). In AD patients, use of antidepressants, antipsychotics and anticholinesterase drugs was not associated with changes in metabolite concentrations or transport ratios in serum and CSF. For all these analyses, the groups did not significantly differ regarding age and sex.

Association between serum and CSF Kyn metabolites

To investigate the potential of serum Kyn metabolites in predicting cerebral Kyn metabolite production, we used linear regression analyses to establish the associations between serum and CSF concentrations of Kyn metabolites (Fig. 3a). The analyses indicated a strong positive association between serum and CSF levels of Kyn and QA [$F(1, 88) = 62.9$, $p < 0.001$ and $F = 93.4$, $p < 0.001$, respectively]. Concentrations of the other Kyn metabolites in serum, 3-Hk, KA, and XA, were also associated with their corresponding concentrations in CSF ($F = 17.2$, $p < 0.001$; $F = 17.2$, $p < 0.001$ and $F = 15.7$, $p < 0.001$, respectively). The concentration of Trp in serum was not associated with CSF Trp content. Interaction analyses indicated that these associations were not different for control, PD or AD.
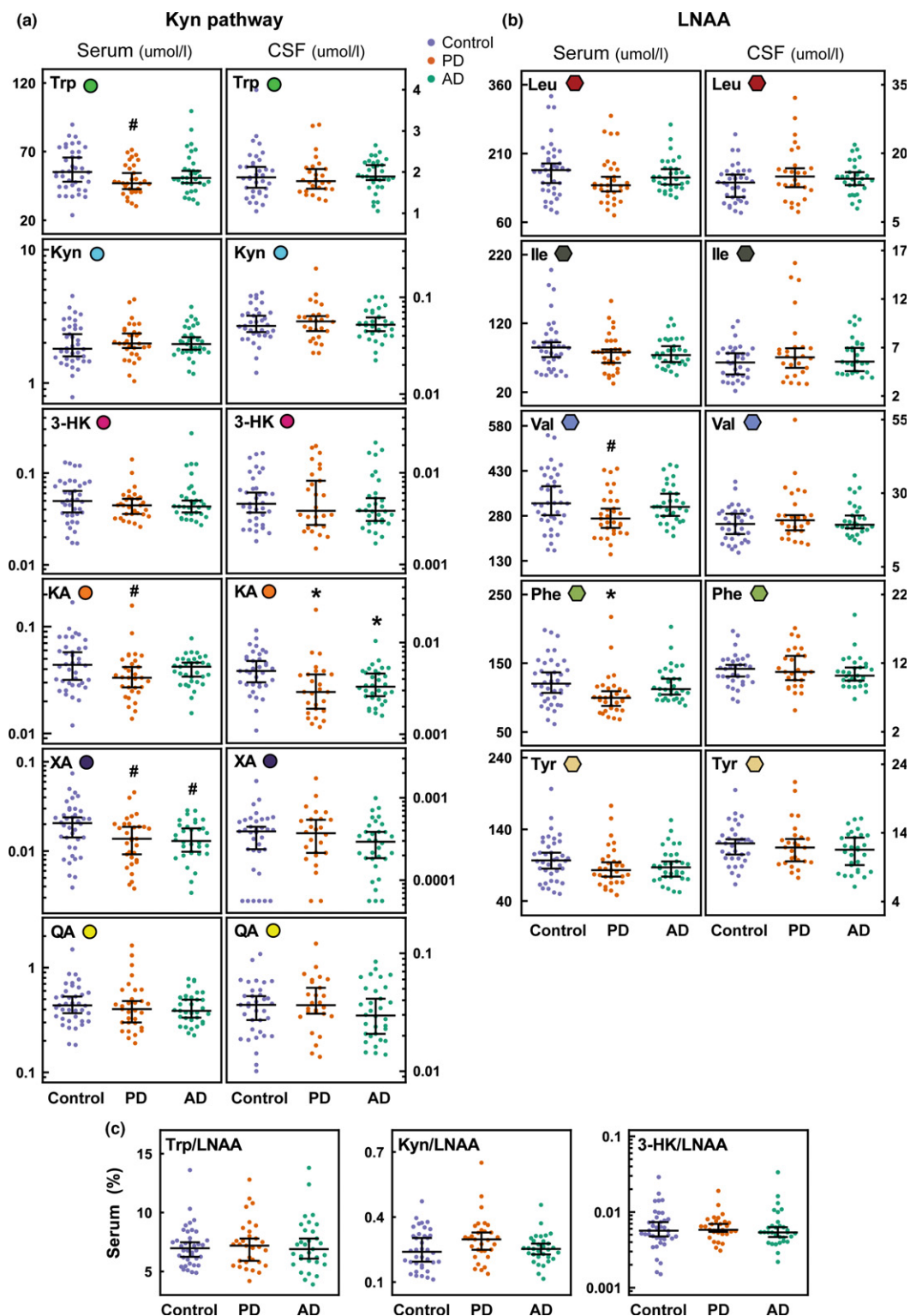
Fig. 2 Specific alterations in central Kyn pathway activity in PD and AD but no evidence of altered transporter-mediated Kyn brain transport. (a) Scatter plots showing serum and CSF concentrations for Trp and Kyn metabolites, LNAA (b) and indices for transporter-mediated brain uptake of Trp, Kyn and 3-HK (c) in control (green), PD (orange), and AD (purple) patients (for serum: $n = 38$, 31 and 32–33, respectively; for CSF: $n = 34$–35, 26, and 29–32, respectively). Median and estimated 95% confidence intervals are provided. Log scales are used in case of skewed distribution. $*p < 0.01$, $\#p < 0.05$ for Mann–Whitney U tests comparing control to PD or AD. Abbreviations as in Fig. 1. LOQ, limit of quantification.
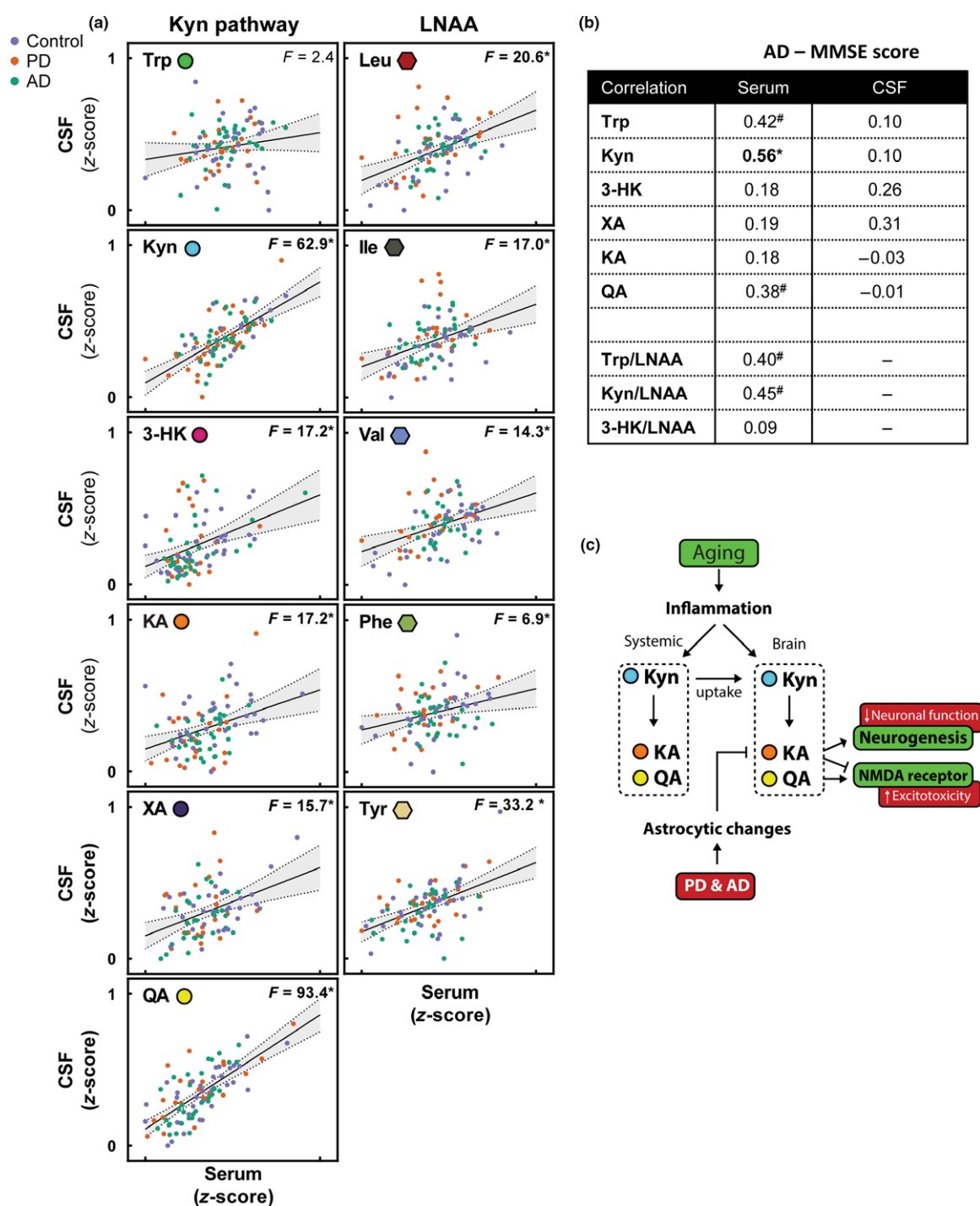
Fig. 3 Peripheral Kyn pathway metabolites as biomarkers for brain Kyn pathway activity and in AD disease severity. (a) Scatterplot showing the relationship between serum and CSF metabolite concentrations (standardized scores) for healthy controls (n = 34), PD (n = 24), and AD (n = 32) patients. The best-fit line from linear regression (including 95% confidence interval) as well as the F-value is provided to compare models. *$p < 0.01$ for F-tests. (b) Table that shows correlation coefficients between Kyn metabolites (in serum or CSF) and AD disease severity measured by MMSE (with a lower score indicating more severe disease). *$p < 0.01$, #$p < 0.05$ for Spearman's rank correlation coefficient. (c) Hypothetical model of findings. Aging is associated with increased Trp metabolism toward the Kyn pathway. This increases brain Kyn content either directly or as a result of increased systemic uptake. This causes increased production of Kyn metabolites including KA and QA. In PD and AD, cerebral KA content seems to be lower. This could be associated with altered NMDA receptor activity and impact the course of these diseases. Abbreviations as in Fig. 1. MMSE, mini-mental state examination; NMDA, N-methyl-D-aspartate.

Regarding the LNAA, except for Phe, all LNAA (Leu, Ile, Val, Tyr) concentrations in serum were associated with their corresponding CSF concentrations [$F(1, 84) = 20.6$, $p < 0.001$; $F = 17.0$, $p < 0.001$; $F = 14.3$, $p < 0.001$ and $F = 33.2$, $p < 0.001$] (Fig. 3a). Interaction analyses indicated that the associations were not different between control, PD and AD, although there was a trend towards an altered association between serum and CSF concentrations of Phe. Stratified regression analyses revealed that the serum Phe was only significantly associated with CSF Phe in control subjects [$F(1, 31) = 10.6$, $p = .003$] and not in PD and AD patients [$F(1, 22) = 4.0$, $p = 0.059$ and $F(1, 27) = 1.34$, $p = 0.260$, respectively].

Correlation between Kyn pathway metabolites and AD severity

Finally, analyses were performed to study the correlation between Kyn metabolites and disease severity of AD. These analyses showed a positive correlation between serum Kyn concentration and MMSE scores in AD patients (Spearman's correlation coefficient = 0.52, d.f. = 23, $p = 0.007$), indicating a negative correlation between Kyn in serum and disease severity (Fig. 3b). The analyses indicated a trend towards a similar negative correlation between Trp, QA, Trp/LNAA, and Kyn/LNAA and the inversed MMSE score.

Discussion

Because of its roles in modulating neuroinflammation and neuronal excitotoxicity, the Kyn pathway could be a promising therapeutic target in age-related neurodegenerative diseases such as PD and AD. To gain further insight into the role and prognostic potential of this pathway in age-related neurodegeneration, we investigated indices of peripheral and central Kyn metabolism and indicative measures of transporter-mediated brain uptake by analyzing Kyn metabolites and LNAAs in serum and CSF of persons with PD and AD and age-matched control subjects.

In our cohort, aging was most strongly associated with increased serum and CSF concentrations of Kyn, KA, and QA. These results are in accordance with previous studies (Gramsbergen et al. 1992; Heyes et al. 1992; Braidy et al. 2011; Theofylaktopoulou et al. 2013; de Bie et al. 2016; Giil et al. 2017) and suggest that the Kyn pathway is activated during aging in mammals. There is strong evidence that indicates that aging is associated with a low-grade inflammatory phenotype (Salminen et al. 2012). As inflammation is a key activator of the Kyn pathway (Schröcksnadel et al. 2006; Badawy 2017), the age-related Kyn pathway activation observed in our study could be related to changes in immune activation and functioning. Importantly, our analyses indicated that aging was not differentially associated with Kyn pathway metabolites in patients with PD and AD. In addition, and largely in accordance with other studies (Heyes et al.

1992; Giil et al. 2017; Chang et al. 2018), Kyn and QA – the metabolites that increase most strongly with aging and with (neuro)inflammation – in serum and CSF did not differ from age-matched controls. Apart from KA, changes in Kyn pathway metabolite levels therefore seem to constitute a physiological phenotype of aging in PD and AD and are not part of an 'accelerated aging-phenotype' that is thought to contribute to neurodegeneration (Wyss-Coray 2016). Studying the role of the Kyn pathway as a biomarker of (brain) aging, preferably in a longitudinal setting, and deciphering the cell-specific age-related changes of Kyn pathway activity in the brain [as previously performed in macrophages (Minhas et al. 2019)] could be interesting directions for future research.

In line with previous reports (Giil et al. 2017; Chang et al. 2018), our analyses showed trends toward reduced serum concentrations of Trp, KA, and XA in PD and reduced XA in AD. However, our results are not consistent with studies describing reduced KA levels in serum of AD patients (Heyes et al. 1992; Hartai et al. 2007; Gulaj et al. 2010). These discrepancies are likely explained by age differences between control subjects and patients in many of the previously described studies. This can have a big impact on analytic outcomes [as demonstrated recently (Giil et al. 2017)]. We therefore recommend controlling for the effect of age and, preferably, the use of larger sample sizes when studying Kyn metabolites.

Our analyses revealed a strong reduction in KA concentrations in CSF of PD and AD patients compared to age-matched controls. This is in line with evidence of reduced post-mortem KA concentrations in a range of brain regions of PD patients (Ogawa et al. 1992), reduced KA concentrations in CSF of PD and AD patients (Heyes et al. 1992) and reduced cerebral KA levels in mouse models of AD (Zwilling et al. 2011) although others showed no changes in KA concentrations in CSF in a small cohort of AD patients (Wennstrom et al. 2014). In theory, reduced KA levels in the brain could result from reduced Kyn bioavailability or increased flux of Kyn through the 3-Hk/QA branch of the Kyn pathway. However, as Kyn levels in serum and concentrations other Kyn metabolites in CSF did not differ between the diseased and controls, it seems less likely that these mechanisms majorly contribute to reduced KA content in CSF of PD and AD patients in our cohort. We also showed that altered transporter-mediated brain uptake of Trp or Kyn does probably not contribute substantially to the observed reduction in KA. Reduced cerebral KA production in PD and AD could also be the consequence of cell-specific changes in the brain. Astrocytes are considered the primary source of extracellular KA concentrations (Guillemin et al. 2001; Kiss et al. 2003) and studies in rodents showed that kynurenine aminotransferase II (KATII) – the enzyme that converts Kyn to KA – was strongly localized to astrocytes (Guidetti et al. 2007; Herédi et al. 2017; Song et al. 2018). In response to activated microglia, astrocytes can change their molecular

behavior during diseases of the brain (Liddelow *et al.* 2017). Whereas astrocytes normally have a supportive function in the brain, these reactive astrocytes are thought to contribute to neurotoxicity in PD and AD (Ben Haim *et al.* 2015). We speculate that reduced KA concentrations in CSF of PD and AD patients in our cohort could reflect reduced (astrocytic) KAT activity, which could be part of the reactive changes that occur in astrocytes during neurodegeneration.

A recent study that investigated the expression of KATII in the adult rat brain provided compelling evidence that KA might play a role in neuronal development in adult life (Song *et al.* 2018). Making use of quantitative *in situ* hybridization, the investigators revealed high astrocytic KATII expression in the subventricular zone, the rostral migratory stream and the hippocampus, regions that are crucial for adult neurogenesis and trafficking. The authors reasoned that KATII-expressing astrocytes are possibly involved in regulating neuronal proliferation and differentiation in the adult brain by modulating local activation of NMDA and the α7nACh receptors (Song *et al.* 2018). Reactive astrocytes – as described above – are thought to play a role in hampering neurogenesis in neurodegenerative diseases (Cassé *et al.* 2018). Taken together, it is tempting to speculate that reduced local KA production as a result of reactive changes in astrocytic functioning could contribute to diminished neurogenesis in neurodegenerative diseases. On the other hand, high levels of KA in the frontal cortex are thought to contribute to schizophrenia and elevated KA can hamper neurogenesis and cause cognitive defects (Forrest *et al.* 2015; Erhardt *et al.* 2017). Future studies should point out whether reduced region-specific KA production, possibly as a phenotype of reactive astrocytes, could influence neurogenesis during PD and AD.

Additionally, increased production of QA in favor of KA could contribute to PD and AD by chronically activating the NMDA receptor, increasing glutamate levels and inducing excitotoxicity (Maddison and Giorgini 2015). Indeed, excessive activation of the NMDA receptor is common in neurodegenerative diseases and NMDA receptor-antagonists are used for symptomatic treatment in PD and AD (Lipton 2006). In line with others (Heyes *et al.* 1992), we did not find evidence of increased production of QA in the brains of PD and AD patients. This suggests that, instead, reduced KA production could play a more prominent role in NMDA receptor overactivation in PD and AD. Indeed, pharmacologic or genetic strategies that increased KA in the brain were proven to reduce glutamate release (Moroni *et al.* 2005; Pocivavsek *et al.* 2011) and enhancing KA levels in the brain – by blocking the enzyme that converts Kyn to 3-Hk – improved symptoms and reduced neuropathology in animal models of PD and AD (Grégoire *et al.* 2008; Zwilling *et al.* 2011). Reduced KA concentrations in CSF of PD and AD patients could thus be an indicator of chronic NMDA receptor activation and consequent neurotoxicity. In this regard, the balance between NMDA receptor activation and inhibition in PD and AD could be further disturbed during aging as QA levels rise.

The use of medication is another factor that has been proposed to contribute to Kyn pathway disturbances in PD and AD. For example, levodopa-induced dyskinesia (LID), a side-effect of dopaminergic replacement therapy, was associated with reduced KA levels in serum of PD patients compared to controls, untreated PD patients and levodopa-treated patients not suffering from LID (Havelund *et al.* 2017). Risk of developing LID is highest in younger patients (< 59 years old) and increases with the duration and dose of the levodopa treatment (Kumar *et al.* 2005; Prange *et al.* 2019). Our PD cohort predominantly consisted of older individuals with newly diagnosed (and treated) PD. In line with this, LID was only reported in one patient. Occurrence of LID is thus unlikely to have had a major influence on our results. Furthermore, clinical and preclinical models have demonstrated that antidepressants and anti-psychotic drugs, which are commonly used by PD and AD patients, can modulate Kyn pathway activity (Myint *et al.* 2011; Ara and Bano 2012; Kocki *et al.* 2012; Gibney *et al.* 2014; Eskelund *et al.* 2017; Leppik *et al.* 2018). Our results demonstrated that that the use of medication was not involved in the reduced KA content observed in CSF of PD and AD patients. To further address the value of the Kyn pathway in diagnosis or prognosis of PD and AD, future studies should, however, make use of a longitudinal setup to dissect the effect of symptomatic treatments and disease progression on Kyn metabolites – preferentially with the inclusion of levodopa measurements.

Finally, we demonstrated that Kyn and QA in serum are strongly related to their respective concentrations in CSF and that Kyn and QA in serum are negatively correlated with disease severity in AD. Although of a very preliminary nature, these data could guide further investigation into the potential of the Kyn pathway in tracking the progress of age-related pathologies of the brain. In this respect, it would be interesting to longitudinally study whether the rate at which Kyn metabolites increase with age is predictive of cognitive deterioration or neurodegenerative disease.

Strengths of this study are that we performed analyses in a relatively large number of subjects and included well-characterized patients. For example, approximately 40% of the AD subjects were neuropathologically confirmed. In addition, we assessed Kyn metabolites in time-linked serum and CSF samples of both PD and AD patients allowing us to detect shared and disease-specific Kyn pathway alterations. To the best our knowledge, this is the first study in PD and AD that analyzed LNAAs in serum to construct estimates of cerebral Kyn transport. However, our results should be interpreted with care because of the observational and retrospective nature of this study. An important limitation of our study is that we were not able to adjust our analyses for possible confounding variables such as body weight, renal function, and markers of immune activation because of missing clinical data

(Theofylaktopoulou *et al.* 2013). In addition, correlating Kyn metabolites with other measures of symptom severity and disease progression (e.g. using the Unified Parkinson Disease Rating Scale) could have been insightful but, unfortunately, these data were unavailable. Finally, we recommend the use of larger sample sizes to better detect small differences in Kyn metabolite concentrations, especially when studying these in an elderly population.

In conclusion, we showed that Kyn and QA concentrations similarly accumulate with aging in serum and CSF of control subjects and patients with neurodegenerative diseases. We found that levels of KA in CSF were strongly reduced in patients with PD and AD, and demonstrated for the first time that differences in transporter-mediated Kyn uptake are unlikely to majorly contribute to cerebral Kyn pathway disturbances in these diseases. We speculate that the combination of age- and disease-specific changes in cerebral Kyn pathway activity could be implicated in reduced neurogenesis and increased excitotoxicity that are shared hallmarks of PD and AD (Fig. 3c). These results could guide fundamental and clinical studies to explore the role of KA in the pathophysiology of PD and AD and suggest that the Kyn pathway has potential as a marker of disease progression or as a therapeutic target in neurodegeneration.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Multivariate analysis of kynurenine pathway and large-neutral amino acids in Alzheimer's and Parkinson's disease patients.
Table S2. Concentrations of kynurenine pathway metabolites and large-neutral amino acids in serum and CSF of Alzheimer's and Parkinson's disease patients.

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