J Neural Transm (2009) 116:897–903
DOI 10.1007/s00702-009-0238-5

BASIC NEUROSCIENCES, GENETICS AND IMMUNOLOGY - ORIGINAL ARTICLE

Gene polymorphisms in prodynorphin (*PDYN*) are associated with episodic memory in the elderly

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Received: 13 February 2009 / Accepted: 30 April 2009 / Published online: 26 May 2009

Abstract Cognitive functions show large variation in elderly people and are substantially heritable. Animal studies revealed that dynorphins influence cognition and memory, especially in aged animals. Thus, we tested the effect of four SNPs (rs7272891, rs1997794, rs2235751 and rs910080) and the VNTR promoter polymorphism in the prodynorphin gene (*PDYN*) on episodic memory and verbal fluency in a large ($n = 1619$) sample of elderly people (mean age: 80 ± 3.39 years; range 75–90 years) recruited through the German study on ageing, cognition and dementia in primary care patients (AgeCoDe). We found that carriers of the minor alleles of rs1997794 ($P < 0.002$) and rs910080 ($P < 0.005$) presented with higher episodic memory scores than homozygote carriers of the major allele. Also, a three marker haplotype including these two SNPs and rs2235751 was associated with better episodic memory scores. Verbal fluency scores were non-significantly better in carriers of these respective alleles. Thus, our results suggest a role of *PDYN* gene variations in determining memory function also in elderly humans.

Keywords PDYN · Episodic memory · Verbal fluency · Aging · Genetic association

Introduction

In the aged population the inter-individual variance of cognitive performance and abilities is very high: some elderly perform as well or even better as younger individuals, while others suffer from severe cognitive disabilities (Ardila 2007). The causes of these differences are unknown. However, understanding of the mechanisms influencing cognitive performance in the elderly might help

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to develop preventive therapeutic strategies for cognitive disabilities.

Behavioral genetic evidence suggests that genetic factors have a strong impact on memory functions across the life span, with a heritability of about 50% (McClearn et al. 1997). Moreover, recent studies in humans described different genes possibly associated with memory function, i.e. gene variations in *BDNF*, *KIBRA*, *HTR2A* and *APOE* (Miyajima et al. 2008; Nilsson et al. 2006; Papassotiropoulos et al. 2006; Wagner et al. 2008). Animal studies have identified additional genes and these might be candidate genes for an influence on cognitive performance in humans as well. One of these genes is Prodynorphin (*PDYN*), the gene encoding dynorphin A, dynorphin B and Big dynorphin. Dynorphins are endogenous opioid neuropeptides activating the kappa-opioid receptor (KOR) and are widely expressed in the central nervous system (CNS). They serve as neuromodulators and are involved in neuroendocrine signaling. Dynorphins play an important physiological role in memory acquisition (Kuzmin et al. 2006) and pain processing, and are thought to influence addiction by regulating the activity of the reward system.

Dynorphins possess neuroprotective functions in the CNS (Itoh et al. 1993). However, under pathophysiological conditions, these peptides act excitotoxic largely through actions at glutamate receptors (Hauser et al. 2005). Furthermore, Dynorphin A levels are increased in the brains of Alzheimer's disease patients and there is a correlation with neuritic plaque density in those brains, suggesting a role of dynorphins in neurodegenerative disorders (Yakovleva et al. 2007).

Animal studies revealed that activity of dynorphin signaling is important for learning and memory. The expression of *PDYN* in the hippocampus of aged animals is increased, suggesting that dynorphin signaling may have a higher impact on cognitive abilities in aged compared to younger animals (Kotz et al. 2004). Moreover, learning deficient rats present with higher hippocampal *PDYN* expression than unimpaired control animals (Jiang et al. 1989). Finally, aged but not young *PDYN* knockout mice perform better in the spatial water maze task than similarly aged wild-type mice (Nguyen et al. 2005).

Several polymorphisms in *PDYN* have been investigated mainly for their association with addiction in humans (Ray et al. 2005; Xuei et al. 2006); however, functional studies are rare. The longer alleles (alleles 3 and 4) of a variable number of tandem repeat (VNTR) promoter polymorphism of *PDYN* were reported to be associated with an increased chloramphenicol acetyltransferase (CAT) activity of the reporter (Zimprich et al. 2000), but the biological consequences of this finding are still unknown. In addition, the rare alleles of other PDYN polymorphisms in the 3′ region of the gene were associated with decreased PDYN mRNA expression in human brain tissues (Yuferov et al. 2008). These data suggest that PDYN gene variants might influence gene expression and thus PDYN function also in humans.

The current study aimed to examine the role of *PDYN* polymorphisms on episodic memory and verbal fluency in an aged general population.

Materials and methods

Human subjects

Subjects were members of a population-based cohort of subjects aged 75–90 years, participating in the AgeCoDe study, recruited through general practitioners in six German cities. The design of the AgeCoDe study is given in more detail elsewhere (Jessen et al. 2007; Luck et al. 2007). Because a number of medical conditions can significantly affect cognitive performance, we reduced such nuisance variables for the present study by excluding subjects with known health issues. Therefore, subjects were not included into this study if they met one of the following criteria: visual or auditory impairments possibly limiting the cognitive evaluation, neurological disorders (e.g. Parkinson Disease, seizures, transitory ischemic attacks, stroke, closed head injury with more than 30 min unconsciousness), diabetes, current depression (Geriatric Depression scale ≥ 6) or intake of tricyclic antidepressants, poor motivation during testing, first language other than German, harmful use of alcohol or alcohol addiction, or medication with benzodiazepines. A total of 1,600 subjects were excluded because of any of these possibly confounding medical conditions, and 1,619 largely healthy elderly subjects (mean age: 80.0 ± 3.39 years; $n = 1{,}077$ females, $n = 542$ males) were included in the present study. The ethics committees of the participating centers approved the study. Written informed consent was obtained from all participants.

Neuropsychological testing

All subjects were assessed at their home by trained research assistants with a battery of tests. These include a structured interview for the assessment of dementia (SIDAM), the word list learning task (episodic memory) and the category fluency task of the CERAD battery (Welsh et al. 1994). The total number of words recalled during the three learning trials (maximum = 30), the delayed recall trial (maximum = 10) and the number of hits corrected for the number of false alarms in the recognition trial (maximum = 10) were summed up to give an overall measure of verbal episodic memory. Aggregating these variables into a single

score was justified by high intercorrelations between the constituent variables (Cronbachs Alpha = 0.788) and allowed us to limit the number of statistical comparisons and hence the probability of false positive results. In addition, the number of correct items named during 60 seconds for the category animals was used as a measure of verbal fluency. Semantic and phonemic verbal fluency tasks require executive control and rely on frontal brain activation in old subjects (e.g. Meinzer et al. 2009). Therefore, the category fluency task was included to address the possible specificity of *PDYN* effects.

Genotyping

Leukocyte DNA was isolated from whole blood with the Qiagen® blood isolation kit according to the instructions of the manufacturer (Qiagen, Hilden, Germany). We investigated four SNPs and the VNTR promoter polymorphism in *PDYN* (Ray et al. 2005; Zimprich et al. 2000). Two SNPS (rs7272891 and rs1997794) are located in the 5′-UTR of the gene, rs2235751 in intron 2 and rs910080 in the 3′-UTR of exon 4 (Fig. 1).

SNPs were determined by using the SNPstream system (Beckman Coulter) according to the instructions of the manufacturer. Primers used for genotyping are given in Table 1.

The VNTR polymorphism was determined by PCR using the following primers: fwd: CCTGTGTATGGAG AGGCTGAG and rev: CACCAGGCGGTTAGGTAGAG. The forward primer was Fam labelled for fragment size analysis using Genescan (Applied Biosystems) and analyzed using Genotyper software (Applied Biosystems). The corresponding products had a length of 279 bp (allele 1, frequency: 1.8%), 347 bp (allele 2, frequency: 29.0%), 414 bp (allele 3, frequency: 67.7%), 482 bp (allele 4, frequency: 1.4%) and 550 bp (allele 5, frequency 0.6%). Following a functional study (Zimprich et al. 2000), which revealed that the longer *PDYN* VNTR alleles 3 and 4, in comparison to the "short" alleles 1 and 2, result in increased CAT activity, we subdivided the VNTR alleles in two classes of "short" (S) alleles, including alleles 1 and 2, and "long" (L) alleles, including alleles 3 and 4. *APOE* genotyping was performed according to standard procedures (Hixson and Vernier 1990).

Statistical analysis

Normal distribution of the data was verified and allowed data analysis by parametric tests. Thus, the effect of single alleles on episodic memory and verbal fluency was evaluated by using ANCOVA (SPSS14), including the *APOE*4 allele, gender, age and education as covariates.

Haplotype association of *PDYN* gene variants with episodic memory and verbal fluency was tested by using Haplo.score with the R software (Schaid et al. 2002). Haplotypes were calculated with FAMHAP16 (Becker and Knapp 2004), a software program estimating haplotype frequencies using the expectation-maximization algorithm. For the confirmation of the association of *PDYN* haplotype on episodic memory and verbal fluency from Haplo.score analysis we performed ANCOVA including the identified haplotype and the covariates *APOE*4 allele, gender, age and education. With our sample size of 1,606 subjects, the power to detect even small ($d = 0.2$) effects of *PDYN* alleles on cognitive measures exceeded 99% [determined with G*POWER 3 (Faul et al. 2007)]. Since our primary hypothesis investigates a possible role of *PDYN* gene variants in episodic memory, the Alpha-error was set to 0.01 (0.05 divided by 5 genetic variants), irrespective of the linkage disequilibrium between the *PDYN* variants examined.

Results

No deviation from the Hardy–Weinberg disequilibrium could be shown for any SNP ($P \geq 0.04$).

In the whole study population the mean scores for episodic memory and verbal fluency were 33.6 ± 6.60 words and 20.3 ± 5.53 words, respectively.

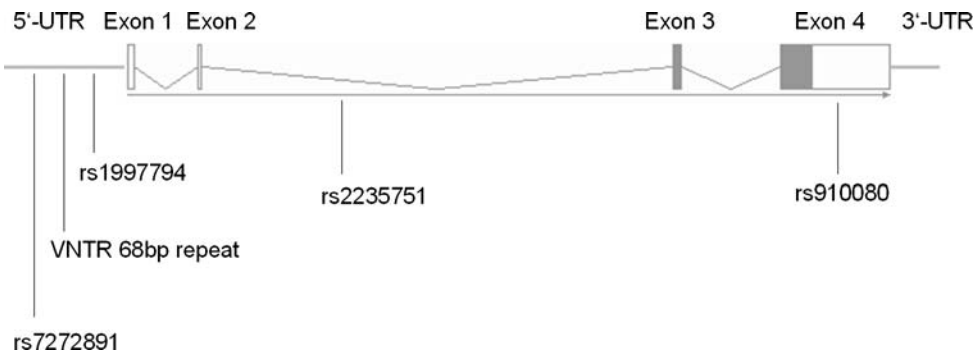

Fig. 1 Schematic representation of the *PDYN* gene, localization of the identified gene variants

Table 1 Primers for genotyping of *PDYN* SNPs by SNP-stream

rs-Number	Primers	Base pairs (bp)
rs910080	F-Primer: TCCTTGATCATTTAAGCATTCA	132
	R-Primer: TTGAAGAACTGACTTCCTGAACT	
	S-Primer: *AATAAGCTCACCACCGTCAA*TCATGTTGGTACCCTGGACAGTGCC	
rs2235751	F-Primer: AAGCAACTGGCCCCATTC	108
	R-Primer: AAAAGGTAAATCCCAAGGCA	
	S-Primer: *GCAACATAAGACCGCTCAA*CCCCTGAGCCCTTTCTAGTTGCCTGG	
rs1997794	F-Primer: TGAGAGCAGTCAGAGCACAG	98
	R-Primer: TTCCTCCTCCACATCCCT	
	S-Primer: *AAGTACCACGTCAACGTCAC*GTCACGAAGAGAAGCCTATTGTGTC	
rs7272891	F-Primer: CTCAGATCCTGAAGCTGCA	108
	R-Primer: CAGTCAGACCCCTTGTGC	
	S-Primer: *GGCTATGATTCGCAATGCT*TGGACCCACAGCCTCACTTTCCTTCT	

5′-Tag sequences

Two out of the five *PDYN* gene variants investigated were associated with episodic memory in the ANCOVA controlling for APOE, gender, age and education as covariates. We found that carriers of the minor alleles of rs1997794 and rs910080 presented with higher episodic memory scores than homozygote carriers of the major allele (rs1997794: $P = 0.002$, rs910080: $P = 0.005$, Table 2). A similar trend was observed for rs2235751 ($P = 0.020$), while rs7272891 and the VNTR polymorphism did not show an association with memory. Similar but non-significant associations with verbal fluency were revealed for rs1997794 ($P = 0.032$, Table 3), rs2235751

Table 2 Association of *PDYN* polymorphisms with episodic memory (sum of CERAD test battery correct immediate and delayed free recall trials, maximum score = 50). Statistical analysis was performed using ANCOVA including the APOE4 allele, gender, age and education as covariates (effect of the APOE4 allele, gender, age and education was $P < 0.001$ in all analyses). Means adjusted for these covariates are shown

Polymorphism	*n*	Mean ± SE	*F*	*df*	*P*
RS7272891			C-allele carrier vs. non-carrier		
CC	1,330	33.36 ± 0.24	0.02	1	0.892
CG	266	33.79 ± 0.42			
GG	10	33.16 ± 2.00			
68 bp VNTR			L-allel carrier vs. non-carrier		
L-allele carriers[a]	1,395	33.51 ± 0.24	0.02	1	0.963
Non-carriers	156	33.53 ± 0.54			
RS1997794			C-allele carrier vs. non-carrier		
TT	657	32.88 ± 0.29	10.03	1	0.002
CT	728	33.76 ± 0.29			
CC	218	34.32 ± 0.46			
RS2235751			G-allele carrier vs. non-carrier		
AA	907	33.07 ± 0.27	5.45	1	0.020
AG	591	33.70 ± 0.31			
GG	105	34.44 ± 0.64			
RS910080			G-allele carrier vs. non-carrier		
AA	867	33.03 ± 0.27	7.74	1	0.005
AG	626	33.79 ± 0.30			
GG	117	34.52 ± 0.61			

[a] Alleles 3 and 4 of the VNTR polymorphism were combined as long (L) alleles

Table 3 Association of *PDYN* polymorphisms with verbal fluency in elderly subjects. Statistical analysis was performed using ANCOVA including the APOE4 allele, gender, age and education as covariates (effect of the APOE4 allele, age and education was $P < 0.001$ in all analyses, effect of gender was $P < 0.06$ in all analyses). Means adjusted for these covariates are shown

Polymorphism	*n*	Mean ± SE	*F*	*df*	*P*
RS7272891			C-allele carrier vs. non-carrier		
CC	1,330	20.91 ± 0.20	1.66	1	0.199
CG	266	21.16 ± 0.35			
GG	10	18.80 ± 1.67			
68 bp VNTR			L-allel carrier vs. non-carrier		
L-allele carrier[a]	1,395	20.98 ± 0.20	0.67	1	0.415
Non-carrier	156	20.62 ± 0.45			
RS1997794			C-allele carrier vs. non-carrier		
TT	657	20.61 ± 0.25	4.63	1	0.032
CT	728	21.10 ± 0.24			
CC	218	21.46 ± 0.39			
RS2235751			G-allele carrier vs. non-carrier		
AA	907	20.71 ± 0.22	3.34	1	0.068
AG	591	21.03 ± 0.25			
GG	105	22.09 ± 0.53			
RS910080			G-allele carrier vs. non-carrier		
AA	867	20.72 ± 0.23	3.11	1	0.078
AG	626	21.17 ± 0.25			
GG	117	21.25 ± 0.51			

[a] Alleles 3 and 4 of the VNTR polymorphism were combined as long (L) alleles

($P = 0.068$) and rs910080 ($P = 0.078$). Again, rs7272891 ($P = 0.199$) and the VNTR polymorphism ($P = 0.415$) were unrelated to verbal fluency. As expected, presence of the APOE-ε4 allele, higher age and lower education were all strongly associated with poorer memory and reduced verbal fluency ($P < 0.001$). In a dose-related fashion, carriers of the APOE-ε4 allele performed worse. Females had better episodic memory ($P < 0.001$) and marginally higher verbal fluency ($P < 0.06$). Such gender differences have been noted before, including studies with the CERAD test battery (e.g. Chandler et al. 2005; Collie et al. 1999).

We explored whether the significant association of rs1997794 and rs910080 with episodic memory would be particularly pronounced for immediate memory and learning (trials 1–3 of the CERAD battery) or with delayed memory (Recall and Recognition trials of the CERAD), as immediate and delayed memory may be selectively associated with gene variants (e.g. Wagner et al. 2008). However, rs1997794 and rs910080 were associated both with immediate memory (rs1997794, $P = 0.003$, rs910080: $P = 0.034$) as well as with delayed memory measures (delayed recognition rs1997794, $P = 0.004$, rs910080: $P = 0.002$, delayed recall, rs1997794, $P = 0.051$, rs910080: $P = 0.056$).

The LD plot structure for all *PDYN* gene variants is given in Fig. 2. We restricted our further haplotype analysis to the three variants nominally ($P < 0.05$) associated with episodic memory (rs1997794, rs2235751 and rs910080). Haplo.score identified a three marker *PDYN*

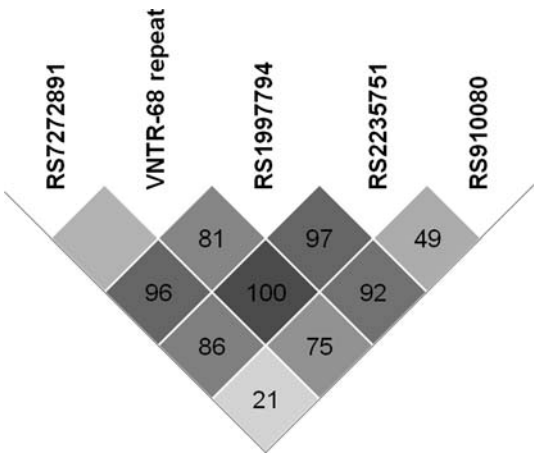

Fig. 2 Linkage disequilibrium (LD) structure of *PDYN* gene variations. The number at the intersection of each pair of SNPs represents the Pairwise D values between two SNPs. The VNTR polymorphism is included in the LD via carrier status of the long (L) allele

haplotype (C/G/G) which was associated with higher episodic memory scores in carriers of this haplotype in comparison to non-carriers ($P = 0.013$). Verbal fluency was not significantly related to this haplotype ($P = 0.14$). The effect of this haplotype on episodic memory was confirmed in the ANCOVA ($P = 0.019$, Table 4), while verbal fluency again was not significantly associated ($P = 0.197$).

Discussion

Our data suggest that *PDYN* polymorphisms might be associated with memory, especially episodic memory, in healthy elderly subjects. Thus, our study provides first evidence for a role of dynorphins in human memory.

Animal studies revealed that dynorphin expression increases in aged rats and mice and causes impairment of memory function (Jiang et al. 1989; Nguyen et al. 2005). In line with this, increased Dynorphin A levels have been observed in the brain's of Alzheimer's disease patients (Yakovleva et al. 2007). In addition it was shown that dynorphin reduces long-term potentiation at the CA3 synapses in hippocampal mossy fibres and by this inhibits synaptic transmission and neural plasticity (Weisskopf et al. 1993). Thus, increased Dynorphin levels might be associated with decreased memory function in rodents, but also in humans.

In our study we found a significant association of the rare alleles of two *PDYN* polymorphisms with a better measure of episodic memory, which required encoding and retrieval of recently learned words, while the association with verbal fluency failed to reach significance. We note that the same SNPs related to memory also appeared to be related to verbal fluency, with the minor allele carriers doing better in both tasks. Because verbal fluency requires recall from semantic memory, this might suggest that not only the formation of, but also the retrieval from memory may be influenced by dynorphins.

Table 4 Association of *PDYN* three marker haplotype C/G/G including SNPs rs1997794, rs2235751 and rs910080 with episodic memory and verbal fluency in elderly subjects. Statistical analysis was performed using ANCOVA including the APOE4 allele, gender, age and education as covariates. Means adjusted for these covariates are shown

Parameter	*n*	Mean ± SE	*F*	*df*	*P*
Episodic memory			Carrier vs. non-carrier		
C/G/G carrier	436	33.31 ± 0.33	5.51	1	0.019
Non-carrier	1,183	32.49 ± 0.22			
Verbal fluency			Carrier vs. non-carrier		
C/G/G carrier	436	21.21 ± 0.29	1.66	1	0.197
Non-carrier	1,183	20.83 ± 0.21			

The two SNPs which were associated with episodic memory in our study were rs1997794, located in the 5′-UTR (promoter region) and rs910080, which is located in the 3′-UTR region of the *PDYN* gene. In a large family study by Xuei et al. (2006), these SNPs have also been found to be associated with alcohol dependence, whereas rs2235751, which revealed only a trend for association in our study, also in this study showed only a weak association. In our study, subjects with harmful use or with alcohol addiction, according to the judgment of the subjects' GP, had been excluded. Furthermore, all subjects had been asked about current and lifetime alcohol intake, but in additional analyses we found no association of these variables with any of the *PDYN* polymorphisms examined (data not shown). Therefore, we think that the memory effect of the genetic variation in *PDYN* is not mediated through alcohol consumption differences in our sample.

Functional studies on *PDYN* polymorphisms are rare. However, a just recent study showed, that *PDYN* polymorphisms, rs910080 (also investigated in our study), rs910079 and rs2235749, in the 3'region of the gene and the corresponding haplotypes were associated with altered PDYN mRNA expression in human brain tissues (Yuferov et al. 2008). The authors report that these SNPs were in almost complete LD ($D' = 1.0$, $r^2 = 1$); haplotype analysis revealed that the PDYN haplotype combining the rare alleles of these SNPs was associated with a reduced expression of PDYN. As outlined before, animal studies revealed evidence for an improved memory function if levels of dynorphins are low. In line with this, our study revealed that carriers of the rare allele of rs910080, which has been associated with reduced PDYN expression (Yuferov et al. 2008), presented with higher scores for episodic memory. Thus, previous and our data support the hypothesis that PDYN gene variations might be associated with altered memory function, possibly due to an altered PDYN gene expression and thus altered dynorphin levels.

We are aware, that our study has several limitations. We investigated only four PDYN SNPs and the VNTR polymorphism, and included only SNPs, which revealed evidence for association in the ANCOVA, also in our haplotype analysis. This might have lead to an overestimation of the association of PDYN gene variants with memory. However, our study was performed with a large, representative sample that allowed a cross-sectional association design. The quality of our study population is verified by the replication of the finding that the *APOE4 allele*, a gene with established relevance for dementia and cognitive decline, in our study also showed a gene-dose effect on episodic memory as has been described previously in old subjects (70–85 years) in a population based sample in Sweden (Nilsson et al. 2006), with homozygous ε4 carriers performing worst.

In summary, our study provides first evidence that genetic variation in *PDYN* may be associated with memory in elderly humans. However, while being biologically plausible, this novel finding requires replication in independent large cohorts. The underlying mechanisms of *PDYN* function on memory will have to be explored in further studies.

Acknowledgments This study was supported by the German Competence Network on Dementia (CND) and was funded by the German Federal Ministry for Education and Research (grants CNDD: 01 GI 0710 Hamburg, 01 GI 0711 Bonn, 01 GI 0712 Mannheim, 01 GI 0713 Düsseldorf, 01 GI 0714 Leipzig, 01 GI 0715 München, 01 GI 0716 Hannover, 01 GI 0717 Bremen). We thank Beisa Burnic, Christine Frahnert-Ledschbor, Sandra Schmitz and Anne Schulz for skillful technical assistance.

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