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Cansu Agca, University of Missouri
Diana Klakotskaia, University of Missouri
Todd R. Schachtman, University of Missouri
Anthony Chan, Emory University
James J Lah, Emory University
Yuksel Agca, University of Missouri

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Presenilin 1 transgene addition to amyloid precursor protein overexpressing transgenic rats increases amyloid beta 42 levels and results in loss of memory retention

Cansu Agca¹, Diana Klakotskaia², Todd R. Schachtman², Anthony W. Chan³, James J. Lah⁴ and Yuksel Agca¹*

Abstract
Background: We previously reported the production of transgenic rats (APP21 line) that over-express human amyloid precursor protein (APP) containing Swedish and Indiana mutations. In order to generate a better model for Alzheimer’s disease (AD), the APP21 rat line was used to generate double transgenic line that over-expressed Presenilin 1 (PS1) with L166P mutation in addition to APP transgene (APP + PS1 line).

Results: Thirty-two double transgenic founders were generated and the ultimate transgenic founder was selected based on PS1 transgene copy number and level of amyloid-beta (Aβ) peptide. The APP + PS1 double transgenic rats had 38 times more PS1 in brains compared to APP rats. Behavioral assessment using Barnes maze showed that APP + PS1 rats exhibited a larger learning and memory deficit than APP21 rats. Double transgenic rats also produced more Aβ42. Histological examination of the brains showed that the APP21 rat line displayed neurofibrillary tangles and in contrast, the APP + PS1 line showed chromatolysis in hippocampal neurons and neuronal loss in CA3 region of hippocampus.

Conclusions: Due to the separate segregation of APP and PS1 transgenes in APP + PS1 double transgenic rats, this transgenic line may be a valuable model for studying the effects of various levels of APP and PS1 transgenes on various aspects of brain pathologies associated with the AD phenotype.

Background
Alzheimer’s disease (AD) is one of the most devastating and costly diseases that affect approximately 12% of the population over the age of 65 [2]. Alzheimer’s disease is associated with the over-production and reduced clearance of amyloid-beta (Aβ) peptides [19]. Cleavage of amyloid-β precursor protein (APP) by the presenilin (PS) 1 enzyme results in the release of Aβ peptides, which aggregate and form Aβ plaques in the brain. These Aβ plaques and neurofibrillary tangles are the two primary pathological manifestations of AD in the brain.

Amyloid peptides are produced via the cleavage of APP by T-secretase, a protease complex of four proteins that includes PS1 or PS2, which are both homologous proteins that contain the catalytic site of the enzyme [7]. T-secretase cleaves the β-amyloid protein into two major forms of Aβ polypeptides, Aβ40 and Aβ42. The relative amount of the longer form of Aβ, Aβ42, is particularly critical for AD progression because it is more prone to aggregation than the shorter Aβ40 peptide [4, 10, 23]. In addition, the ratio of Aβ42/Aβ40 correlates with the load of Aβ plaques in the brain [8, 14]. Furthermore, the reduction of PS1 mRNA using siRNA against PS1 reduced Aβ42 in cultured cells [11] which suggests that lower levels of PS1 lead to decreased production of Aβ42.

We previously generated rats that overexpress human APP containing the Swedish and Indiana mutations [1].
The APP21 line expressed high levels of APP, but failed to produce Aβ plaques in the brain. However, intrahippocampal seeding of dilute Alzheimer brain extracts containing aggregated Aβ led to the production of plaques in APP21 rats [20]. This finding indicates that plaque formation depends on environmental challenges, conditions which laboratory rats are not exposed to routinely. In order to increase the pathogenicity of overexpressed APP, we generated double transgenic rats from the APP21 line that over-express both the PS1 and the APP transgenes. Our hypothesis was that the addition of a PS1 transgene would increase more toxic Aβ42 polypeptide and thus, would produce a more suitable model for examining AD.

**Methods**

**Animals, construct design, and preparation of vesicular stomatitis virus glycoprotein pseudotyped lentivirus**

The homozygous APP21-transgenic rat line [1], containing human APP with the Swedish double missense mutation (K595M/N596L) and Indiana single missense mutation (V642F), were super ovulated by administering intraperitoneal (IP) injections of 15 IU pregnant mares’ serum gonadotropins (PMSG) followed by IP injections of 15 IU human chorionic gonadotropin 52 h after PMSG. Twenty-four hours after mating, rats were euthanized using CO₂ and zygotes were collected from the oviducts. Morphologically normal zygotes having two pronuclei and a sperm tail were used for lentiviral vector injections into perivitelline space as described earlier [1]. Lentiviral vector-injected zygotes were then transferred into the oviducts of 8–10 week-old Sprague–Dawley pseudopregnant recipient rats.

**Genomic DNA isolation and polymerase chain reaction**

Genomic DNA from tail-snips was isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Screening of transgenic rats was conducted by PCR using primers annealing the Ubi-C promoter (Forward; TGTCGCTAAATTCTGTCCGGT) and PS1 transgene (Reverse; AGCATTCAAGATTTGAGTCAGGGCC). The PCR reactions were carried out using 50 ng genomic DNA, 100 ng of each primer and 1.5 U Biolase taq (Biorad, Randolph, MA), and amplified PCR products were size separated through a 1 % agarose gel stained with ethidium bromide for visualization.

**ELISA measurement of Aβ40 and Aβ42**

Serum samples were analyzed for Aβ40 and Aβ42, using commercial ELISA kits (Genetics Company, Schlieren, Switzerland). Serum samples were diluted in assay buffer and processed according to the manufacturer’s recommended protocols. Briefly, samples and standards were incubated in capture wells overnight at 4 °C with either a biotinylated Aβ40 or an Aβ42-specific antibody. After several rinses, the enzyme-conjugated detection reagent was added to the wells for 30 min. After additional rinses, wells were incubated with the chromogen solution for 30 min at room temperature and were shielded from light. After the addition of the stop solution, the wells were read for absorption at 450 nm and the Aβ concentration in the samples was calculated based on standard curves.

**Southern blot analysis**

Southern blot analysis was conducted to determine the copy number of the transgene in founder rats. Genomic DNA was digested with EcoRI, which cut the junction of the PS1 and the WRE transgene. The digestion products were size-separated through a 0.8 % agarose gel and transferred to a Genescreen plus membrane (Perkin Elmer, Wisconsin, MA), and amplified PCR products were size separated through a 1 % agarose gel stained with ethidium bromide for visualization.

**Zygote collection, microinjection of lentiviral vector, and embryo transfer**

Homozygous APP21-transgenic female rats (28–30 day-old) were super ovulated by administering intraperitoneal (IP) injections of 15 IU pregnant mares’ serum gonadotropins (PMSG) followed by IP injections of 15 IU human chorionic gonadotropin 52 h after PMSG. Twenty-four hours after mating, rats were euthanized using CO₂ and zygotes were collected from the oviducts. Morphologically normal zygotes having two pronuclei and a sperm tail were used for lentiviral vector injections into perivitelline space as described earlier [1]. Lentiviral vector-injected zygotes were then transferred into the oviducts of 8–10 week-old Sprague–Dawley pseudopregnant recipient rats.
template, Ready-To-Go DNA Labeling Beads (Amersham Biosciences, Piscataway, NJ) and [α-32P]-dCTP (Perkin Elmer, Wellesley, MA). The membranes were prehybridized in 10% dextran sulfate, 6 × saline sodium citrate (SSC), 1% sodium dodecyl sulfate (SDS) for 2 hours and hybridized using the 32P labeled probe overnight before exposure to BioMax MS-1 Autoradiography Film (Kodak, Rochester, NY).

**Northern blot analysis**

Total RNA was isolated using Trisure (Bioline, CA) from founder APP21 + PS1 and APP21 transgenic rats. Brain, heart, kidney, liver, and lung tissues were used in Northern blot analysis. Total RNA was size-separated through 1% agarose gel before transferring to the GeneScreen plus membrane overnight. The membrane was prehybridized in 10% dextran sulfate, 5 × SSPE, 50% formamide, 5 × Denhardt’s, 1% SDS at 42°C for 6 hours prior to hybridization with the PS1 probe overnight. After exposure to BioMax MS-1 Autoradiography Film, membranes were stripped to remove labeled cDNA probe by boiling for 1 hour in 1% SDS and 0.1 × SSC. The intensity of the bands was determined using Kodak 1D v 3.6.3 software (New Haven, CT).

**Behavioral measures**

Behavioral assessment was conducted using APP + PS1 (n = 12), APP (n = 11), and non-transgenic Fischer 344 (n = 12) rats when their average age was approximately 10 months. Prior to behavior studies, each rat was handled at least 3 times to reduce stress due to handling during the behavioral testing.

**Barnes maze**

The maze consisted of a grey circular platform 122 cm in diameter. The platform was surrounded by a wall that was 30.5 cm in height. The maze was elevated 83.8 cm above the floor by a stand. Twenty holes measuring 10.2 cm in diameter were evenly spaced around the perimeter. A rectangular grey escape box (28 cm in length × 12.7 cm wide × 7.6 cm high at the area closest to the maze tapering to 16.5 cm high) could be placed beneath any hole. The escape box included an entry ramp that provided easy entry access for the rat. Black curtains were hung around the maze and above the maze walls to surround the apparatus and ensure that rats could only use the visual cues provided in the maze, rather than the distal cues within the testing room. Proximal cues were more likely to remain constant, within subjects and across subjects, during the course of training. Four visual cues consisting of various shapes (triangle, square, circle, cross) were placed at evenly spaced intervals on the inside of the maze walls. Two 86-W, 120-V floodlights producing 1690 lumens were hung above the platform served to brightly light the maze in order to create a potentially aversive environment to help motivate the rats to escape from the brightly lit, open surface in favor of the dark environment of the escape box. One light hung 68.5 cm on side of the maze while the other hung 137 cm from the other side.

Each rat was assigned an escape hole number under which the escape box was placed on each test trial; assigned hole numbers were alternated across rats to eliminate odor cues for consecutively tested rats. The escape box location remained constant for any individual rat across test trials. Behavioral testing consisted of two pretraining trials on the first day, and 6 evaluation trials (2 trials/day) over a period of 3 days. Each day, the animals were transferred in their home cage from their colony room to the testing room 30 min prior to the start of testing. On the pretraining trial, the rat was preexposed to the goal box (with the goal box placed in the same hole that would be used during subsequent training) by placing the rat in the goal box for 90 s. The rats’ escape from the goal box was prevented by covering the opening to the maze surface with a grey, opaque start box (a 23 cm × 23 cm box). A training trial began by placing the rat under the start box positioned in the center of the platform. After 30 s, the box was lifted and the rat had a maximum of 5 min (300 s) to locate and enter the escape box. Latency (time it took for the rat to find and enter the escape box) and total errors (nose-pokes into non-escape holes) were recorded. If the rat did not enter the escape box within 5 min, it was gently guided there by the experimenter’s hand. After 30 s, the rat was removed from the escape box and returned to its home cage. Rats were allowed to rest in their home cage in the testing room for 30 min before starting their second daily trial. The platform and escape box were cleaned after every trial with a 20% ethanol solution. After the third day, testing abated for 3 days, after which retention was evaluated for two additional trials (1 day), conducted exactly like those just described. The following day, the rats were given reversal training in which the rats were given training exactly like those of original acquisition, except that the escape box was located in a new location (in the opposite quadrant). Reversal training continued for a total of 3 days.

**Histopathology**

Nine of the female rats [3 of each: APP + PS1, APP, and wild type (WT)] were euthanized at 18–19 months of age and their brains were collected. Tissues were fixed in 10% formalin for 48 h prior to paraffin embedding. Hematoxylin and eosin staining was used to determine histological changes. The stained slides were analyzed using a Nikon Eclipse E600 microscope (Melville, NY) and an Olympus DP72 Camera (Center Valley, PA).
Statistical analysis
Statistical analysis for PS1 mRNA expression, serum Aβ levels, and behavioral analyses were performed using general linear models in SAS (SAS 9.3, SAS Institute Inc., Cary, NC, USA). For the Barnes maze, the average of each animal’s daily score was used in the analysis of overall performance during acquisition and reversal training. Comparisons of daily performance were analyzed separately and differences were considered significant at \( p < 0.05 \) for all analyses.

Results

APP21-PS1 double transgenic founders
Forty-five rats were born from the lentiviral vector which contained PS1 transgene-injected embryos and 32 of them were PCR positive for the PS1 transgene. Southern blot results showed that transgene copy number ranged between 1 and 5 for each PS1 transgenic founder rat (Fig. 1a). Serum Aβ42 ranged between 25 and 479 pg/ml in the founder animals at 4 weeks of age and transgene copy number positively correlated with serum AB42 levels in founder animals (\( r = .54 \)). Serum Aβ40 and Aβ42 levels at 8 weeks of age were also determined in founder animals (Fig. 1b). Due to the segregation of multiple integration sites in subsequent generations of transgenic animals, founder animals with one copy transgene were used for breeding. Founders with one PS1 transgene copy and relatively high serum Aβ42 levels were considered in order to determine the ultimate founder animal. Founder 964 had one of the highest serum Aβ42 (196 pg/ml) levels as well as Aβ42/Aβ40 levels (0.28) at 8 weeks of age. Furthermore, the F1 generation from founder 964 had the highest serum Aβ42 levels and had a relatively large-sized litter. Thus, founder 964 was used as the transgenic founder for generation of APP + PS1 transgenic rats.

Northern blot analysis of various tissues from the founder APP + PS1 transgenic animals showed that brain, heart, kidney, liver, and lung had 38, 8, 19, 15, and 15 fold more PS1 mRNA (\( P < 0.01 \)), respectively, compared to the APP21 rat line. Serum Aβ40 and Aβ42 levels at 8 weeks were positively correlated with PS1 mRNA levels in APP + PS1 founders (Table 1). Statistical analysis of the PS1 expression of founder animals was done by grouping the animals based on their 8 wk serum Aβ42 levels. The groups comprised of rats with Aβ42 serum levels of less than 100 pg/ml (<100), between 100 and 149 pg/ml (100–149), between 150 and 200 pg/ml (150–200), and greater than 200 pg/ml (>200), as well as APP21 rats that did not have the PS1 transgene. PS1 expression in tissues increased significantly (\( p < 0.05 \)) as serum Aβ42 levels increased (Fig. 2; Additional file 1: Table 1) with the exception of the liver, in which PS1 levels were not significantly different.

Subsequent generations of APP + PS1 double transgenic rat line
The APP21 line has been previously bred as a homozygous transgenic line. However, homozygous APP + PS1 rats failed to produce pups. Thus, the APP + PS1 double transgenic line was retained as homozygous for APP transgene and hemizygous for PS1 transgene.

Serum Aβ40 and Aβ42 levels were determined in F1 generation pups at 30 days of age and in F2 generation pups at 50 days of age (Fig. 3; Additional file 2: Table 2). The Aβ42 levels increased with age in both APP and APP + PS1 lines and both F1 and F2 generation APP + PS1 rats had significantly greater (\( p < 0.05 \)) Aβ42 levels compared to APP rats. Serum Aβ42/Aβ40 levels were approximately two times greater in the APP + PS1 rats compared to the APP rats for both F1 and F2 generations (\( p < 0.001 \)), but serum Aβ42 levels were similar between PS1 + APP and APP rats and did not change with age.

Behavior changes among AP + PS1, PS and fischer wild type rats

Barnes maze

There were no statistical differences in performance between male and female rats, thus the data were collapsed over sex for all of the behavioral analyses.

Acquisition
Overall, APP + PS1 rats made significantly more errors compared to WT rats (\( p = 0.04 \)) during acquisition training, but there were no group differences in latency (\( p > 0.05 \); Fig. 4a). More specifically, there were no statistical differences in performance among the groups on acquisition days 1 and 2 (\( ps > 0.05 \)), however, on day 3, APP + PS1 rats made significantly more errors compared to both APP and WT rats (\( p < 0.001 \)) and had significantly longer latencies compared to WT rats only (\( p = 0.02 \); Fig. 4d). Overall, these results suggest that the APP + PS1 rats had a larger learning deficit in the Barnes maze task than the APP rats.

Retention
After the retention interval, APP + PS1 rats made numerically more errors (\( p = 0.1 \)) and had significantly longer latencies (\( p = 0.01 \)) than both the APP and WT rats (Fig. 4b), implying a larger memory deficit in the APP + PS1 rats. These differences in memory retention may be due to the larger learning deficit in APP + PS1 animals during acquisition training.

Reversal
Overall, APP + PS1 rats made significantly more errors (\( p = 0.04 \)) and had significantly longer latencies (\( p = 0.03 \)) compared to both the WT and APP rats during reversal training (Fig. 4c). More specifically, there were no significant differences on days 1 and 2 of reversal training.
training ($p > 0.05$), but on day 3, APP + PS1 rats made significantly more errors ($p < 0.01$) and had significantly longer latencies ($p = 0.04$) than both the APP and WT rats (Fig. 4e). These results suggest deficits in reversal learning in the APP + PS1 rats. Additional file 3: Table 3 contains the dataset of the Barnes Maze.

**Histopathology of brain**

The hippocampal pyramidal cell layer of **Cornu Ammonis** (CA) 1 and CA2 regions of the APP transgenic rats contained many necrotic neurons and neurofibrillary tangles (Fig. 5a, d, and g). Mineralization was also evident as indicated by the rough cell membrane observed in some of the neurons in the hippocampus and the cortex in both the APP and the APP + PS1 rats. In addition to neurons containing flame-shaped neurofibrillary tangles, hippocampus also contained remnants of neurofibrillary tangles that were embedded in the neuropil (ghost tangles) in the APP rats (Fig. 5d, g). Neurofibrillary tangles were present in viable cells that had nuclei as well as neurons with no visible nuclei (Fig. 5). The CA3 region also contained necrotic neurons and neurofibrillary tangles in both the APP and APP + PS1 rats (Fig. 5j, k).

Both the APP and APP + PS1 rats had fewer pyramidal neurons in the CA3 region compared to WT, but...
The APP + PS1 animals had more severe neuronal loss in the CA3 region (Fig. 5b). The hippocampal pyramidal cell layer in the APP + PS1 rats had many neurons with central and segmental chromatolysis, showing eccentric displacement of the nucleus and loss of Nissl substance with the exception of the margins of the cell body (Fig. 5h, k). Both the APP and the APP + PS1 brain cortex contained neurons with various degrees of degeneration that ranged from neurons with condensed Nissl substance to dark necrotic neurons and ghost tangles (Fig. 5p, q). The brain cortex of the APP + PS1 animals had larger areas with dark stained neurons, which indicated more severe neuronal necrosis (Fig. 5q).

**Discussion**

The production of transgenic rats using lentiviral vectors is an efficient method for gene transfer. However, the major drawback of lentiviral vectors is the integration of the transgene into multiple sites of the genome. Multiple copies of the transgene result in high levels of expression, but segregation in subsequent generations drastically decreases transgene expression. In this study, the founder animal was selected among the animals that had only one copy of the transgene and those that also had the greatest serum Aβ42 levels and Aβ42/Aβ40 ratio.

Serum Aβ42 and Aβ40 levels were positively correlated with PS1 mRNA expression, indicating that the addition...
of the PS1 transgene contributed to the production of both Aβ42 and Aβ40. Furthermore, the greater correlation efficiencies for Aβ42 and PS1 mRNA expression indicate preferential cleavage of Aβ42 by the PS1 enzyme. Transfection using PS1 resulted in an approximately 1.6 fold increase in Aβ42/Aβ40 levels [16] in cell cultures. Similarly, the APP + PS1 transgenic animals had approximately a twofold Aβ42/Aβ40 increase compared to the APP rats. The level of PS1 mRNA expression varied by tissue with the highest PS1 expression in the brain and kidney, while the heart and liver had lower expression. Tissue specific expression was also observed in the APP transgenic rats [1].

Histologic examination of the brains showed that both the APP and the APP + PS1 animals exhibited pathological abnormalities typically associated with AD. Interestingly, the APP rats had large hippocampal sections containing neurons with neurofibrillary tangles. The hippocampus of the APP + PS1 rats also contained neurofibrillary tangles, but these were more sporadic compared to what was observed in the APP rats. Interestingly, neuronal loss was much greater in the CA3 region of the APP + PS1 rats. Another difference between the hippocampus in APP and the APP + PS1 animals was that neurons in the CA1 and the CA2 regions of the APP + PS1 animals had chromatolysis. Chromatolysis is
caused by axonal injury in motor neurons [18] and it has been associated with neurototoxicity and AD [5, 15, 22], but it is not reported as widely as the presence of neurofibrillary tangles or Aβ plaques.

A previously reported rat model for AD, the TgF344-AD model [6], was found to produce plaques at 15 months. The difference in plaque production between the TgF344-AD and the APP + PS1 rats could be due to the fact that the PS1 transgene in the TgF344-AD had Δ exon 9 mutation, as opposed to the L166P mutation form the PS1 transgene in the TgF344-AD had Δ exon 9 mutation, as opposed to the L166P mutation form the PS1 transgene that was used in these APP + PS1 rats. On the other hand, the behavioral characterization of the APP + PS1 rats in this current study revealed learning and memory deficits similar to what has been found in TgF344-AD rats. In addition, previous research has found that an AD mouse model that did not develop plaques even as late as 30 months old also showed memory deficits [9]. Consequently, the presence of Aβ plaques may not always correlate with AD severity. For example, Shankar et al. [21] showed that Aβ dimers are synaptotoxic and disrupted memory, whereas insoluble Aβ plaque cores from Alzheimer’s diseased cortex did not impair long-term potentiation. Furthermore, the number of Aβ plaques in the brain has not been found to correlate with the severity of cognitive impairments in humans or in APP and/or PS transgenic mice [3].

Wild type rats outperformed both the APP and the APP + PS1 rats during the initial learning phase of the Barnes maze test. Additionally, retention of the learned task was poorer in the APP + PS1 rats relative to both the APP and the WT rats. As stated earlier, one of the major differences between the APP + PS1 and the APP rats was that the APP + PS1 rats had neuronal loss in the CA3 region of hippocampus (Fig. 5b shows an APP + PS1 rat with very few neurons in the CA3 region). The CA1 region recodes information from the CA3 region and sets up associatively learned backprojections to the neocortex to allow subsequent retrieval of information to the neocortex [13]. The hippocampal CA3 region and the dentate gyrus also play an important role in the encoding of new spatial information and processing the geometry of the environment [12], which are both crucial to the successful completion of the task. Thus, the differences between the APP + PS1, APP, and WT rats in learning and retention in the task may be related to the differences in the pathology of the hippocampus.

Conclusions

The addition of the PS1 transgene to the APP transgenic rat genome affected serum Aβ42 and Aβ42/Aβ40 levels, brain histopathology, as well as, the cognitive behavior of the double transgenic rats. Although the APP rat brain had many histopathological abnormalities that are often seen in AD brains, their memory did not show significant impairment during the behavioral testing. In contrast, the APP + PS1 rats showed significantly reduced memory retention compared to the APP and the WT rats, in addition to, neuronal loss and necrosis in the hippocampus and brain cortex.

Additional files

Additional file 1: Table 1. contains dataset for Northern blot analysis (see Fig. 2). PS1 mRNA level, serum Aβ42 and tissue are reported in the table.

Additional file 2: Table 2. contains dataset for serum Aβ42, Aβ40 and Aβ42/Aβ40 ratio of F1 and F2 generation transgenic rats (see Fig. 3).

Additional file 3: Table 3. contains dataset for Barnes maze (see Fig. 4). The table includes acquisition day 1, 2 and 3 latency and errors and their averages, retention latency and errors and reversal days 1, 2 and 3 latency and errors and their averages.

Abbreviations

AD: alzheimer’s disease; Aβ: amyloid-beta; APP: amyloid precursor protein; CA: cornu Ammonis; eGFP: enhanced green fluorescence protein; IP: intraperitoneal; pLV: lentiviral vector; PMSG: pregnant mares’ serum gonadotropins; PS1: presenilin 1; SSC: saline sodium citrate; SDS: sodium dodecyl sulfate; UbC: ubiquitin-C; WT: wild type; WRE: woodchuck hepatitis virus post-transcriptional regulatory element.

Authors’ contributions

CA was responsible for genotypic and phenotypic characterization of transgenic rats, statistical analysis of data, colony management, and writing the paper. JIL generated lentiviral vectors and performed ELISA. DX and TRS have conducted rat behavioral assessment and analysis. YA and AWC generated transgenic rats. YA designed the research, and contributed to the analysis of the data. All authors read and approved the final manuscript.

Author details

1 Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Missouri, 1600 East Rollins Street, Room W191, Columbia, MO 65211, USA. 2 Department of Psychological Sciences, University of Missouri, Columbia,
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