Brief Communications

Presynaptic Defects Underlying Impaired Learning and Memory Function in Lipoprotein Lipase-Deficient Mice

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Lipoprotein lipase (LPL) is predominantly expressed in adipose and muscle where it plays a crucial role in the metabolism of triglyceride-rich plasma lipoproteins. LPL is also expressed in the brain with highest levels found in the pyramidal cells of the hippocampus, suggesting a possible role for LPL in the regulation of cognitive function. However, very little is currently known about the specific role of LPL in the brain. We have generated a mouse model of LPL deficiency which was rescued from neonatal lethality by somatic gene transfer. These mice show no exogenous and endogenous LPL expression in the brain. To study the role of LPL in learning and memory, the performance of LPL-deficient mice was tested in two cognitive tests. In a water maze test, LPL-deficient mice exhibited increased latency to escape platform and increased mistake frequency. Decreased latency to platform in the step-down inhibitory avoidance test was observed, consistent with impaired learning and memory in these mice. Transmission electron microscopy revealed a significant decrease in the number of presynaptic vesicles in the hippocampus of LPL-deficient mice. The levels of the presynaptic marker synaptophysin were also reduced in the hippocampus, whereas postsynaptic marker postsynaptic density protein 95 levels remained unchanged in LPL-deficient mice. Theses findings indicate that LPL plays an important role in learning and memory function possibly by influencing presynaptic function.

Introduction

Lipoprotein lipase (LPL) is a key enzyme hydrolyzing plasma triglyceride carried in very low density lipoproteins (VLDLs) and chylomicrons (CMs). The major sites of expression of LPL are in adipose tissue and skeletal muscle (Eckel, 1989; Goldberg, 1996; Preiss-Landl et al., 2002). Prior studies revealed the presence of LPL mRNA and activity in the brain in several mammalian species, including rat, mouse, and rabbit (Brecher and Kuan, 1979; Goldberg et al., 1989; Yacoub et al., 1990). The highest expression of LPL was found in the hippocampus, where LPL mRNA and protein levels were ~2-fold higher than other brain regions.

Within the hippocampus, CA1 and CA3 pyramidal neurons have been shown to have the most abundant LPL mRNA by *in situ* hybridization (Yacoub et al., 1990).

Neuronal cell lines expressing high levels of active LPL display significant neurite extensions in the presence of VLDL and higher survival rate when exposed to oxidized VLDL (Paradis et al., 2003, 2004). However, considering that the enzymatic function of LPL is to hydrolyze triglyceride (TG) in VLDLs and CMs, and that only high density lipoprotein (HDL)-like lipoproteins circulate in the brain, it is likely that LPL has a nonenzymatic function in the brain parenchyma (Demeester et al., 2000; Koch et al., 2001).

We sought to investigate the role of LPL in the brain, specifically its role in learning and memory. Behavioral studies have not been performed in adult LPL gene-targeted mice because of neonatal lethality (Sattler et al., 1996). By rescuing through somatic gene transfer of a beneficial mutant form of LPL, we have generated viable adult LPL-deficient mice (Ross et al., 2005). In the present study, we report a significant impairment in learning and memory in LPL-deficient animals and demonstrate alterations in presynaptic morphology. Our findings demonstrate that LPL plays a role in cognitive function in the CNS.

Materials and Methods

Detailed methods can be found in online supplemental Methods, available at www.jneurosci.org as supplemental material. LPL-deficient mice with C57BL/6J background were rescued by intramuscular administra-
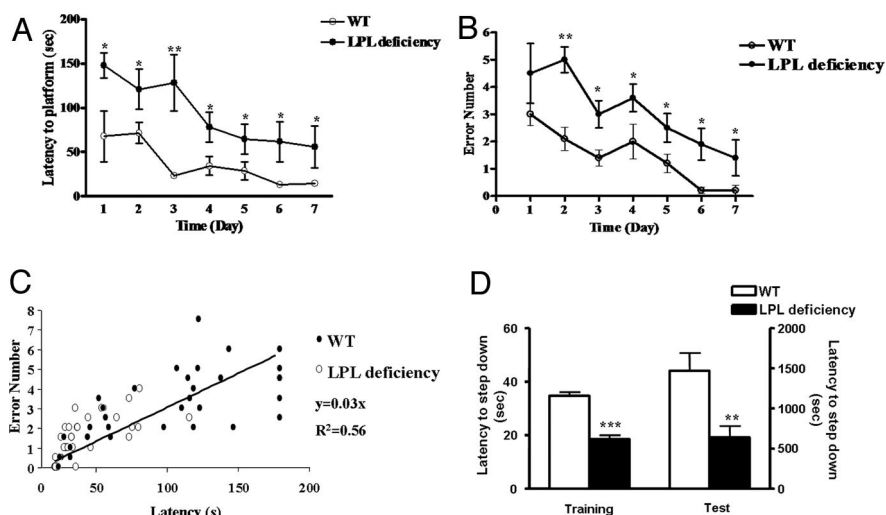
tion of an adenoviral vector coding a human LPL mutant, LPL^{S447X}. Learning and memory were examined by both a water maze and step-down passive avoidance test. Sections of the brain were examined by Nissl staining for morphological evaluation and also stained by immunofluorescence with antibodies against synaptophysin and neurofilaments (Chui et al., 1999). Quantification was performed by image analysis. Ultrastructure of synapses in hippocampus was examined by transmission electron microscopy. Vitamin E content in plasma and brain was assayed chemically. Western blot of neurofilaments, synaptophysin, and postsynaptic density protein 95 (PSD-95) were done with corresponding antibodies and quantitated against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For evaluation of neuronal apoptosis, brains were either stained by TUNEL or assayed by a DNA ladder experiment. Expression of mouse and human LPL in the brain was detected by real-time reverse transcriptase (RT)-PCR. The results were expressed as mean ± SEM. The statistical significance of differences between the two groups was evaluated with unpaired Student's t test, and p values <0.05 were regarded as significant.
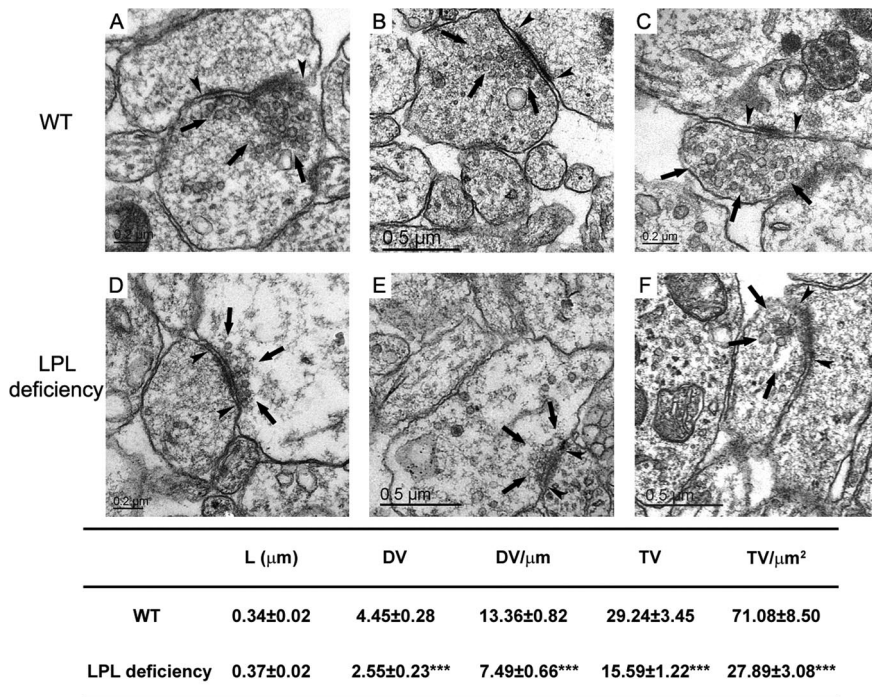

Figure 1. Impaired hippocampal learning and memory in LPL-deficient mice. **A**, Latency to find the platform in water maze escape task. **B**, Frequency of entries into the no-exit arms. **C**, Correlation between latency and error number. **D**, Retention times in step-down passive avoidance task (left) and training trial. Data were expressed as mean ± SEM, $n = 6$ for each group. Statistical significance was determined using the Student's t test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Results

We have previously shown that LPL activity in plasma of adult LPL-deficient mice was almost undetectable (<7% of normal) (Zhang et al., 2008). As the result, plasma TG levels in these mice were 40 times higher than wild-type (WT) control mice (5658.3 ± 1250 and 137.8 ± 30.3 mg/dl, $p < 0.001$) (supplemental Table 1, available at www.jneurosci.org as supplemental material). Although plasma total cholesterol increased nearly fourfold, the HDL-cholesterol was significantly decreased in these LPL-deficient mice (278.1 ± 58.7 vs 65.2 ± 7.1 and 4.2 ± 0.3 vs 42.1 ± 2.3 mg/dl; $p < 0.01$, respectively). There was no significant difference in glucose levels between the two groups of mice, but the body weight of LPL-deficient mice was ~14% lower than WT (supplemental Table 1, available at www.jneurosci.org as supplemental material).

To rule out the potential presence of human LPL expression in the brain as the result of intramuscular adenoviral gene delivery, Human LPL mRNA and protein from brains were assessed by RT-PCR and immunofluorescence with a monoclonal antibody against human LPL. As shown in supplemental Figure 1, available at www.jneurosci.org as supplemental material, by RT-PCR, a single 573 bp band corresponding to mouse LPL was easily seen in WT brain (supplemental Fig. 1A, left three lanes, available at www.jneurosci.org as supplemental material) but not in LPL-deficient mice (supplemental Fig. 1A, right three lanes, available at www.jneurosci.org as supplemental material). Human LPL mRNA and protein were undetectable in the brains in both LPL-deficient and WT mice (supplemental Fig. 1B,C, available at www.jneurosci.org as supplemental material).

Hippocampus-dependent learning and memory of LPL-deficient mice were studied in both water maze performance and step-down passive avoidance test. In water maze test, during training sessions (day 1 and 2), LPL-deficient mice spent significantly longer time than WT mice to find the terminal escape platform (147.9 ± 14.1 vs 67.7 ± 28.7 and 121.0 ± 18.6 vs 71.6 ± 9.7 s; $p < 0.05$, respectively) (Fig. 1A). No differences in the number of entries into the nonexit arms were found on day 1 of training between the genotypes (4.5 ± 1.1 vs 3.0 ± 0.4; $p = 0.24$). The number of no-exit arm entries was, however, significantly increased in LPL-deficient mice on day 2 of training (5.0 ± 0.5 vs 2.1 ± 0.4; $p < 0.001$). From day 3 to day 7, both the latency to escape platform and the frequency of entries into the no-exit arms in LPL-deficient mice were significantly increased than in WT mice. There was a significant correlation between the latency to escape platform and the error numbers (Fig. 1A–C). No changes in swim speeds were observed on any of the days of the training period (85.66 ± 10.28 vs 87.56 ± 8.620 mm/s; $p = 0.89$). This suggests that the increase in latency to platform is most likely attributable to an impairment in navigational strategy in LPL-deficient mice. In fact, the number of entries into the nonexit arms which did not include the escape platform was increased in LPL-deficient mice (Fig. 1B). We further used step-down inhibitory avoidance task to study the role of LPL in learning and memory. As shown in Figure 1D, a significant decrease in the latency to step-down in LPL-deficient mice was observed in the training trial (18.5 ± 1.5 vs 34.7 ± 1.4 s; $p < 0.001$). Similarly, a shortened retention time was observed in LPL-deficient mice (639.8 ± 140.9 vs 1467 ± 221.4 s; $p < 0.01$).

To search for the structural basis underlying the behavioral deficits associated with cognitive functions in LPL-deficient mice, we examined the neuronal counts, synaptic structure, and evidence for apoptosis in the hippocampus. There was no significant difference in neuronal numbers, subcellular structures, and neuronal apoptosis, as determined via light microscopic and transmission electron microscopy image analysis, and TUNEL plus DNA ladder assay (supplemental Figs. 2, 3, available at www.jneurosci.org as supplemental material). However, a severe depletion of synaptic vesicles in presynaptic terminals of LPL-deficient mice was observed (Fig. 2). Quantitatively, the numbers of total vesicles per terminal (TV) and docked vesicles (DV; defined as the vesicles within 50 nm from the plasma membrane in an active zone) were significantly reduced (TV: 15.6 ± 1.2 vs 29.2 ± 3.5, $p < 0.001$; DV: 2.55 ± 0.23 vs 4.45 ± 0.28, $p < 0.001$, respectively) when compared with controls. The number of vesicles per terminal (DV per micrometer) and docked vesicles per

	L (μm)	DV	DV/μm	TV	TV/μm²
WT	0.34±0.02	4.45±0.28	13.36±0.82	29.24±3.45	71.08±8.50
LPL deficiency	0.37±0.02	2.55±0.23***	7.49±0.66***	15.59±1.22***	27.89±3.08***

Figure 2. Ultrastrucural changes of synapses from WT and LPL-deficient neonatal mice detected by transmission electron microscopy. Synaptic vesicles within the presynaptic part are highlighted by arrows; the edges of the active zone/postsynaptic density complexes are marked by arrowheads. **A–C** are representative photos showing the total and docked synaptic vesicles in the hippocampal terminals of WT mice, whereas **D–F** are those of LPL-deficient ones. The table below shows the morphological analysis of synapses in CA3 regions of hippocampi from WT and LPL-deficient mice. L, Active zone length (μm); DV, number of docked vesicles per active zone; DV/μm, number of docked vesicles per micrometer of active zone length; TV, total vesicle number per terminal; TV/μm², total vesicle number per micrometer square of synapse area. Numbers are mean ± SEM; 30 synapses from each mouse were analyzed in WT group ($n = 4$); 50 synapses from each mouse were analyzed in LPL deficiency group ($n = 4$). ***$p < 0.001$.
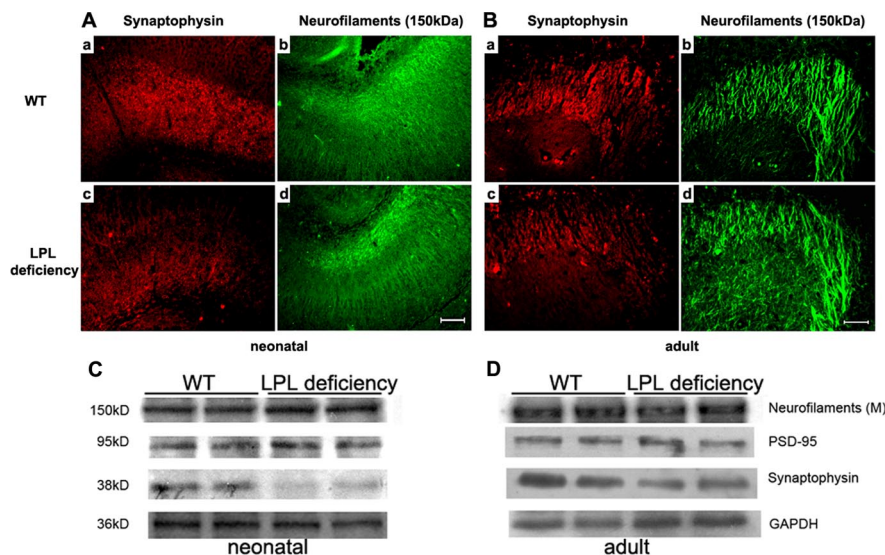
Figure 3. Immunofluorescence of presynaptic vesicles and neurofilaments in hippocampi of LPL-deficient and WT mice. Paraffin-embedded sections were immunostained and quantitated for presynaptic vesicle marker synaptophysin and cytoskeleton marker neurofilaments M. **A**, From neonatal mice ($n = 5$ for WT and $n = 3$ for LPL deficiency); **B**, from adult mice ($n = 6$ for WT and $n = 3$ for LPL deficiency). Scale bar, 50 μm. Western blotting detection of synaptophysin, PSD-95, and neurofilament M (150 kDa) levels in the hippocampus from WT and LPL-deficient mice. **C, D**, The hippocampal protein homogenates of neonatal (**C**) and adult (**D**) mice were subjected to SDS-PAGE, electrotransferred to PVDF membranes, and detected with antibodies against neurofilaments, PSD-95, and synaptophysin. Quantitation of blots was done by image analysis against GAPDH.

square micrometer of synapse area (TV per micrometer square) were all reduced ($p < 0.001$) accordingly.

Immunofluorescence and Western blotting were used to examine the levels of synaptic markers including presynaptic synaptophysin, postsynaptic PSD-95 as well as neurofilament-M. In immunohistochemical analysis, synaptophysin levels in the hippocampus of both adult (20% of control; $p < 0.001$) and neonatal (24% of control; $p < 0.001$) LPL-deficient mice were significantly decreased, whereas neurofilament-M levels were similar, compared with WT mice (Fig. 3A,B). Western blotting showed similar results with significant reduction of synaptophysin levels in the hippocampus of both neonatal (33% of control; $n = 4$ for both WT and LPL deficiency; $p < 0.001$) and adult (58% of control; $n = 4$ for both WT and LPL deficiency; $p < 0.01$) LPL-deficient mice, whereas no change of neurofilament-M or PSD-95 was observed, as shown in Figure 3, C and D.

To detect the effect of LPL on brain lipids, we analyzed vitamin E content in plasma and brains in LPL-deficient and WT mice. Marked accumulation of vitamin E in plasma was noticed in adult LPL-deficient mice (Fig. 4A) (64.2 ± 9.7 vs 4.5 ± 0.3 μg/ml; $p < 0.001$), whereas the levels of vitamin E in the hippocampus were dramatically decreased (Fig. 4B) (1.4 ± 0.2 vs 9.2 ± 2.7 ng/mg; $p < 0.001$). The contents of vitamin E in the brains of the LPL-deficient fetus were also significantly lower than in WT fetus (Fig. 4C) (1.19 ± 0.17 vs 3.05 ± 1.05 ng/mg; $p < 0.05$). These findings indicated a possible role of LPL in the transport of vitamin E from the periphery to the CNS.

Discussion

The current study was undertaken to investigate the role of LPL in the CNS *in vivo*. Specifically, in the present study, we have used viable adult LPL-deficient mice and demonstrated that these mice display impaired hippocampus-dependent learning and memory, indicating an important role of LPL in cognitive function. In search for the structural basis for such dysfunction, no obvious difference in neuronal counts or neuronal apoptosis could be observed. However, ultrastructural analysis revealed severe depletion of synaptic vesicles at presynaptic terminals of hippocampal neurons in LPL-deficient mice. Consistent with this, decreased levels of the presynaptic marker synaptophysin were also detected.

The reduction of synaptophysin and

synaptic vesicles in the hippocampus in LPL-deficient mice could be involved in the impairment of learning and memory function, as demonstrated in other animal models and in human studies (Bamji et al., 2003; Tanemura et al., 2006). Neuronal synapses are essential for memory formation and maintenance. Neurotransmitter release at neuronal synapses is mediated by the synaptic vesicle through fusion and exocytosis at active zones (Sudhof, 2004; Rohrbough and Broadie, 2005). Recent discoveries have made researchers focus on a wider consideration of synaptic vesicle cycle lipids and lipid-based regulatory mechanisms rather than only on protein mechanism (Rohrbough and Broadie, 2005). It is shown that some kinds of lipids interact with lipid-binding proteins and have effects on the synaptic vesicle cycle. In the process of vesicle exocytosis, phosphatidylinositol-4,5-bisphosphate-DAG-mediated signaling and lipid–protein interactions have regulating effects in several synaptic vesicle cycle stages, such as vesicle priming and fusion (Martin, 2001; Wenk and De Camilli, 2004). Also, cholesterol is enriched in secretory and synaptic vesicles as well as the exocytic domains of neurosecretory cell plasma membranes (Deutsch and Kelly, 1981; Michaelson et al., 1983; Benfenati et al., 1989; Lang et al., 2001). Cholesterol binds to synaptophysin and modulates the interaction with synaptobrevin, which is one of the essential vesicular SNARE proteins, and this function of cholesterol helps vesicle trafficking (Thiele et al., 2000; Mitter et al., 2003).

Although the function of LPL in the brain is not clearly understood, several previous in vitro studies have suggested several possible functions for LPL in the CNS. Paradis et al. (2004) reported that active LPL promoted VLDL-stimulated differentiation of Neuro-2A cells, indicating the enzymatic activity of LPL may play a role in the CNS, although VLDL is normally not present in CSF. LPL was also found to regulate the uptake of vitamin E from chylomicrons in both patients with LPL deficiency (Traber et al., 1992) and LPL transgenic mice (Sattler et al., 1996). Indeed, in cultured porcine brain capillary endothelial cells, LPL is found to be crucial for transport of vitamin E across the blood–brain barrier (BBB). Moreover, a 25% reduction in the cerebral concentration of vitamin E was observed in LPL-deficient neonates (Goti et al., 2002). Others used dietary vitamin E-deficient Wistar rats and found the synapse-to-neuron ratio decreased by 30%, and both the numerical density of the synapses and total synaptic area decreased significantly as well, suggesting that decreases in vitamin E intake could significantly affect the structural dynamics of hippocampal cholinergic synapses (Fattoretti et al., 1995). By using fluorescent and EPR spin probing techniques in investigation of rat brain synaptosomal membranes, Erin et al. (1986) observed stabilization of synaptosomal membranes by α-tocopherol, suggesting a possible mechanism for the effect of vitamin E on biological membranes. In addition, synaptophysin, one of the presynaptic vesicle membrane proteins, was significantly increased in the brains of rats treated with vitamin E (Ferri et al., 2006). In addition, the study of behavioral changes in rats with vitamin E deprivation showed the slightly impaired spatial concept formation and significant impaired relearning ability in these animals (Sarter and van der Linde, 1987), whereas the research in older persons demonstrated a significant association of vitamin E intake, from foods or supplements, with
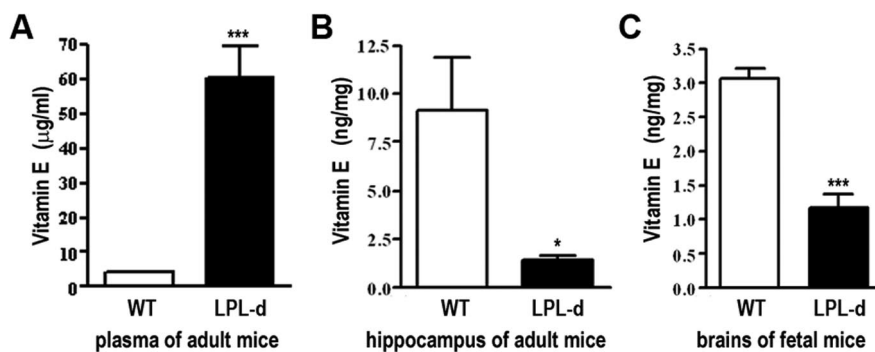
Figure 4. Vitamin E contents in plasma and the brain. Vitamin E was extracted and measured by commercial kit. **A**, Plasma vitamin E contents in adult mice (n = 6 for both WT and LPL deficiency). **B**, Vitamin E levels in hippocampus of adult mice (n = 4 for each group). **C**, Vitamin E levels in the brain in fetal mice (n = 6 for each group). *p < 0.05, ***p < 0.001.

less cognitive decline with age (Morris et al., 2002), indicating the possible relation between low level of vitamin E in brain and memory impairment. In the present study, we observed a sixfold decrease in vitamin E levels in the hippocampus of adult LPL-deficient mice and a 60% reduction in vitamin E in cerebral regions of the LPL-deficient fetus, as well as a significant accumulation of vitamin E in the plasma.

Thus, impaired transport of vitamin E across the BBB in these LPL-deficient mice is most likely responsible for the observed presynaptic vesicle reduction and subsequent memory impairment in LPL-deficient mice. Further investigation in this area is certainly warranted to explore the precise mechanism(s) in details.

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