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Intraperitoneal injection of JNK- specific inhibitor SP600125 inhibits the expression of presenilin-1 and Notch signaling in mouse brain without induction of apoptosis

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Abstract

Presenilin-1 (PS1) is a multifunctional protein involved in many cellular functions including the processing of type 1 membrane proteins such as β -amyloid precursor protein (APP) and Notch 1 receptor. PS1 acts as the catalytic subunit of the γ -secretase complex, and participates in Notch 1 processing to release Notch intracellular domain (NICD) in the cytoplasm. NICD subsequently migrates to the nucleus and causes Notch signaling by increasing the expression of the Hes1 gene. We have previously shown that inhibition of basal activity of *c-jun*-NH2-terminal kinase (JNK) with JNK-specific inhibitor SP600125 represses the expression of PS1 and γ -secretase activity by increasing p53 level in SK-N-SH cell line *in vitro* (Lee and Das, 2008; Lee and Das, 2010). However, it is largely unknown whether PS1 can be effectively suppressed *in vivo* in adult mouse brains. In this report we showed that intraperitoneal (i.p) injection of JNK-specific inhibitor SP600125 decreased p-JNK level, and reduced PS1 expression by increasing p53 level in adult mouse brains. We also showed that suppression of PS1 expression by SP600125 reduced γ -secretase activity which decreased Notch 1 processing to reduce NICD in mouse brains. Furthermore, inhibition of Notch 1 processing by SP600125 decreased Notch 1 signaling by reducing the expression of the NICD target Hes1 gene in mouse brains without induction of apoptosis. These results provide insights for further study on PS1-mediated reduction of Notch 1 and APP processing for the treatment of Alzheimer's disease.

Keywords

Presenilin-1; JNK; p53; Notch 1; Hes1; Notch signaling

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1. Introduction

Presenilin-1 (PS1) is a multipass transmembrane protein (Dewji et al., 2004; Li et al., 2000; Wolfe et al., 1999) and PS1 mutations have been linked to early onset familial Alzheimer's disease (AD) (Sherrington et al., 1995; Tanzi et al., 1996). PS1 or PS2 is the catalytic subunit of γ -secretase: a multiprotein complex that has also been implicated in the development of AD (Chyung et al., 2005; De Strooper, 2003; Kimberly and Wolfe, 2003; Takasugi et al., 2003). PS1 and PS2 act as a catalyst or may be involved in the structure and metabolism of the complex itself. PS1 or PS2 containing γ -secretase has been implicated in the development of AD because of its role in the cleavage of the β -amyloid precursor protein (APP) and the production of A β peptide which is central to the pathogenesis of AD (De Strooper et al., 1998). Similarly the γ -secretase-mediated processing of the Notch receptor protein, which controls cell-cell communication, has implicated the role of PS1 and PS2 in embryonic development via Notch-mediated signaling pathway (Kopan and Goate, 2000; Shen et al., 1997). Notch 1 undergoes cleavage close to or within its transmembrane domain by PS1/ γ -secretase to release Notch intracellular domain (NICD) to the cytoplasm (Ables et al., 2011; Artavanis-Tsakonas et al., 1999). NICD subsequently translocates to the nucleus and modifies transcription of target genes (Ables et al., 2011; Artavanis-Tsakonas et al., 1999). One of the Notch 1 downstream target genes is Hes1. NICD participates in the activation of Hes1 transcription (Ables et al., 2011; Artavanis-Tsakonas et al., 1999). Hes1 protein is translated in the cytoplasm and then localized in the nucleus to activate pro-neuronal genes (Ables et al., 2011). Regulation of downstream genes by NICD is called Notch signaling. It has been shown that the deletion of the PS1 gene is embryonic lethal and causes defects in brain development due to inhibition of Notch 1 signaling (Shen et al., 1997; Wong et al., 1997). PS1, PS2, and γ -secretase also cleave a variety of other type 1 transmembrane proteins which all release intracellular fragments (ICD) with the ability to interact with transcription co-activators (Koo and Kopan, 2004; Kopan and Goate, 2000). Hence PS1 and PS2 may affect the expression of many genes through intramembrane proteolysis (Thinakaran and Parent, 2004). Therefore, we have studied the transcriptional control of the PS1 gene.

We have identified DNA sequences required for the expression of the human PS1 gene. A promoter region has been mapped in SK-N-SH cells and includes sequences from -118 to +178 flanking the major initiation site (+1) (Pastorcic and Das, 1999; Pastorcic and Das, 2000). The -10 Ets site controls 80% of transcription in SK-N-SH cells. We have previously shown that Ets transcription factors Ets1 and Ets2 bind specifically to the -10Ets element and transactivate PS1 expression in SK-N-SH cells (Lee and Das, 2008; Pastorcic and Das, 2000). p53 has been shown to downregulate the expression of the endogenous PS1 gene (Roperch et al., 1998). We have reported previously that p53 inhibits PS1 transcription without binding to the PS1 promoter (Lee and Das, 2008; Pastorcic and Das, 2000). We also showed that c-jun-NH2-terminal kinase (JNK)-specific inhibitor SP600125 repressed PS1 expression and γ -secretase activity by augmenting p53 level in SK-N-SH cells *in vitro* (Lee and Das, 2008). While it is important to study PS1-mediated reduction of Notch 1 and APP processing for the treatment of Alzheimer's disease, we do not know whether SP600125 would repress PS1 expression and γ -secretase activity *in vivo* in adult mouse brains. In this report, we now show that i.p injection of JNK-specific inhibitor SP600125 also inhibits PS1 expression, γ -secretase mediated Notch 1 processing, and Notch signaling by augmenting total p53 level in mouse brains without induction of apoptosis.

2. Results

2.1 Intraperitoneal (i.p) injection of JNK-specific inhibitor SP600125 reduces phosphorylation of JNK and PS1 protein expression in mouse brains

JNK-specific inhibitor SP600125 binds to JNK to inhibit the phosphorylation of JNK (p-JNK) and subsequently inactivates the function of JNK (Bennett et al., 2001; Bogoyevitch et al., 2010). It has been reported and confirmed that intravenous or intraperitoneal injection of JNK-specific inhibitor SP600125 drastically reduced JNK activity in brain extracts of C57BL/6 mice and had no off target effects of SP600125 (Chen et al.; Gao et al., 2005; Wang et al., 2004). To determine whether basal JNK activity controls PS1 protein expression *in vivo*, mice were treated i.p once a day with 250 μ l of vehicle (45% w/v of 2-hydroxypropyl- β -cyclodextrin in water) control and 250 μ l of SP600125 solution (16 mg/kg/day) respectively, for continuous 14 days. The maximum solubility of SP600125 in the vehicle was determined by us to be 1.92 mg/ml. We also determined that maximum 250 μ l of vehicle or SP600125 solution can be injected to mice without harmful effect. Consequently, we chose to administer maximum amount of SP600125 (16 mg/kg/day) to each mouse. Control and treated mice appeared to have no health problems after 14 days of experiments with the particular dose of SP600125 (16 mg/kg/day). Brains were removed from the animals at day 15 for performing immunofluorescent staining (IFS) and biochemical analysis.

We first examined the levels of p-JNK and PS1 in hemi-brain slices. We performed immunofluorescent staining (IFS) with p-JNK antibody and PS1 antibody on cryosections. As shown in Figure 1, both p-JNK (Figure 1A) and PS1 protein (Figure 1B) levels were reduced significantly in the brains of mice treated with SP600125 compared to controls. Co-immunofluorescent staining of p-JNK and PS1 also suggested that PS1 protein expression was decreased in the area of the brain accompanying with the reduction of p-JNK (Figure 1C). Because IFS could not distinguish different brain regions in detail, we generally looked all the regions of the brain. We could not find obvious difference among different brain regions. To confirm our IFS data, we carried out immunoblot analysis with protein extracts from vehicle-treated control and SP600125-treated mouse cortex because PS1-mRNA, PS1 protein, PS1/ γ -secretase activity are significantly increased in the frontal cortex of late-onset sporadic AD patients relative to controls (Borghi et al., 2010). As shown in Figure 2, i.p injection of SP600125 reduced the levels of p-JNK and PS1 significantly in mouse cortex but the total amount of JNK remained unchanged.

2.2 Administration of JNK-specific inhibitor SP600125 *in vivo* increases p53 level in mouse brains

We tested if administration of SP600125 *in vivo* can increase p53 protein levels in mouse brains. The results from IFS with p53 antibody and p-JNK antibody in cryosections are shown in Figure 3A. p53 protein level was increased more than 2 fold in SP600125-treated mouse brains relative to vehicle-treated controls. On the contrary, p-JNK was reduced substantially in SP600125 treated mouse brain relative to control (Figure 3B). Both p-JNK and p53 proteins were localized in the cytosol (Figure 3B). These *in vivo* data are in agreement with our published *in vitro* data in SK-N-SH cells (Lee and Das, 2010).

2.3 Accumulation of p53 and reduction of PS1 by SP600125 do not cause apoptosis in mouse brains due to constant amount of phosphorylated-p53

JNK-specific inhibitor SP600125 was shown to accumulate non-phosphorylated p53 (Miyamoto-Yamasaki et al., 2007). As increase of p53 and its downstream target proteins are usually involved in increase of apoptosis (Miyamoto-Yamasaki et al., 2007), we want to know whether SP600125-induced decrease of p-JNK and PS1 are related to increase of

apoptosis in the SP600125-treated brain. Furthermore, PS1 is an anti-apoptotic molecule and deletion of the PS1 gene causes defects in brain development due to neuronal apoptosis in fetus (Shen et al., 1997). In order to test if p53 accumulation and reduction of PS1 by SP600125 are associated with apoptosis, we assessed the number of apoptotic cells in the brains of mice treated with vehicle or SP600125 by TUNEL assay. As shown in Figure 4, similar number of apoptotic cells were detected in the brains of mice treated with vehicle or SP600125. Activation and phosphorylation of p53 is often induced by DNA damage and apoptosis (Levine, 1997). DNA damage-induced phosphorylation of p53 occurs at multiple sites *in vivo*, including phosphorylation at serine 15 (Ser15) and serine 20 (Ser20), which lead to a reduced interaction between p53 and its negative regulator, the oncoprotein Mdm2 (Shieh et al., 1997). p53 phosphorylation at threonine 18 (Thr18) is also causally associated with p53-mediated apoptosis (Nakamizo et al., 2008). Therefore, we performed IFS with phospho-p53 (p-p53) antibody (anti-Ser15) in brain cryosections to check whether expression of apoptosis-related p-p53 is increased after treatment of SP600125. As shown in Figure 5, p-p53 (Ser15) protein levels were unchanged in the brains of mice treated with SP600125 or vehicles, and p-p53 was localized in the nucleus. On the contrary, p53 levels were significantly increased in the brains of mice treated with SP600125 compared to the controls, and p53 was localized in the cytosol. (Figure 3A). Therefore, treatment of mice with SP600125 did not increase apoptosis because both TUNEL positive cells and p-p53 were not increased in the SP60012-treated mouse brain tissues. This data also suggests that SP600125 reduces PS1 protein expression by increasing the amount of non-phosphorylated p53 (p53) and without induction of apoptosis in mouse brains.

2.4 Administration of JNK-specific inhibitor SP600125 *in vivo* inhibits Notch 1 processing and signaling in mouse brains

We want to determine whether inhibition of PS1 protein expression by SP600125 also inhibits Notch 1 processing and Notch 1 signaling in adult mouse brains without deleterious consequences. We examined the levels of NICD and Hes1 in brain slices. We performed IFS with NICD antibody and Hes1 antibody on cryosections of mouse brain tissues. As shown in Figure 6, both NICD and Hes1 protein levels were reduced drastically in the brains of mice treated with SP600125. Immunoblot analysis (Figure 7) showed that i.p injection of SP600125 reduced the levels of NICD and Hes1 proteins in mouse cortex compared to controls. Our data also suggest that inhibition of PS1 by SP600125 reduces PS1/ γ -secretase activity and Notch 1 signaling in adult mouse brains without lethal effect or induction of apoptosis.

2.5 Administration of JNK-specific inhibitor SP600125 *in vivo* inhibits Hes1-mRNA expression in mouse brains

We performed RT-PCR to show that i.p injection of JNK-specific inhibitor SP600125 reduced the levels of Hes1-mRNA in mouse cortex compared to controls (Figure 8). This result suggests that SP600125 inhibits Notch 1 signaling by decreasing the transcription of the Hes 1 gene.

Discussion

3.1 Mechanism of repression of PS1 and γ -secretase activity appears to be similar both *in vitro* in SK-N-SH cells and *in vivo* in mouse brains

PS1 is the catalytic subunit of the γ -secretase enzyme which participates in the proteolytic cleavage of several type I membrane proteins including APP and Notch 1. We have shown previously that regulation of PS1 transcription controls γ -secretase activity (Lee and Das, 2010). We have also ascertained the mechanism by which inhibition of PS1 transcription reduces γ -secretase activity in SK-N-SH cells (Lee and Das, 2008; Lee and Das, 2010). We

have shown that p53 downregulates PS1 transcription, PS1 protein expression, and PS1-mediated γ -secretase activity *in vitro* in SK-N-SH cells (Lee and Das, 2008; Lee and Das, 2010). p53 does not bind to the PS1 promoter but inhibits PS1 transcription by protein-protein interaction with Ets1/Ets2 transcription factors resulting in the dissociation of Ets1/Ets2 from the PS1 promoter and repression of PS1 expression (Lee and Das, 2008; Pastorci and Das, 2000). We have also shown that inhibition of basal activity of *c-jun*-NH2-terminal kinase (JNK) by JNK-specific inhibitor SP600125 or JNK1-specific siRNA represses PS1 expression and PS1-mediated γ -secretase activity by increasing the amount non-phosphorylated p53 protein without increasing p53-mRNA levels and without induction of apoptosis *in vitro* in SK-N-SH cells. We have shown that SP600125-mediated inhibition of PS1 expression is very specific for JNK pathway (Lee and Das, 2008). On the contrary, PI3K-specific inhibitor LY294002 and ERK-specific inhibitor PD98059 do not inhibit PS1 expression in SK-N-SH cells ruling out the possible off-target effects of SP600125 (Lee and Das, 2008). In our current study, we show that i.p injection of JNK-specific SP600125 also inhibits PS1 expression and γ -secretase-mediated Notch 1 processing *in vivo* in mouse brains without induction of neuronal apoptosis and deleterious effects. Administration of SP600125 augments the amount of non-phosphorylated forms of p53 protein, and also reduces PS1 expression and γ -secretase activity in mouse brains. Given the correspondence between these results and those previously obtained with SK-N-SH cells in which more mechanistic experiments were possible (Lee and Das, 2008) we conclude that these changes are obtained in a p53-dependent manner. Phosphorylation of p53 at serine 15 (Ser15), threonine 18 (Thr18), and serine 20 (Ser20) is causally associated with p53-mediated apoptosis (Nakamizo et al., 2008; Shieh et al., 1997). Moreover, we could not detect the induction of apoptosis in mouse brains because the amount of p-p53 was unaffected by administration of SP600125.

3.2 Inhibition of JNK by SP600125 stabilizes p53 without induction of apoptosis in mouse brains

The steady state level of p53 is regulated by Mdm2-ubiquitin-proteasome degradation pathway (Morrison and Kinoshita, 2000). Mdm2 is an ubiquitin ligase which binds to p53 to form Mdm2-p53 complex and adds ubiquitin to p53 molecule for degradation (Morrison and Kinoshita, 2000). Certain proteins bind to p53 and increase the stability of p53 by preventing p53 from undergoing ubiquitination via interaction with Mdm2 (Morrison and Kinoshita, 2000). JNK activity determines p53 protein level (Wang and Friedman, 2000). It has been reported that JNK-specific inhibitor SP600125 can upregulate cellular p53 levels (Miyamoto-Yamasaki et al., 2007). SP600125 is an anthrapyrazolone inhibitor which binds to JNK to inhibit the phosphorylation and subsequently blocks the functional activation of JNK (Bennett et al., 2001). Activated JNK (p-JNK) catalyzes the phosphorylation at the NH2-terminus of *c-jun*. Phosphorylated *c-jun* forms heterodimers with phosphorylated *c-fos* to form activated AP-1 transcription factor which regulates the transcription of genes containing AP-1 binding sites in their promoters. Therefore, by binding to JNK, SP600125 inactivates the function of JNK. Anti-sense JNK1 treatment also increased the level of p53 in human fibroblast (Tafolla et al., 2005). JNK1-siRNA increased p53 protein level in human neuroblastoma SK-N-SH cells without increasing p53 transcription (Lee and Das, 2008). Moreover, sustained activation of JNK1 downregulated p53 during apoptosis (Tafolla et al., 2005). It has been reported that JNK directly binds to p53 to form JNK-p53 complex (Fuchs et al., 1998a). By direct binding, JNK also targets p53 for ubiquitin-mediated degradation involving Mdm2-p53 degradation pathway (Fuchs et al., 1998b). Therefore, inactivation of JNK by anti-sense JNK1 or SP600125 would decrease the amount of JNK-p53 and/or Mdm2-p53 complex to increase the steady state level of p53 by preventing p53-degradation in non-stressed cells. On the other hand, JNK also phosphorylates p53 (p-p53) resulting in p-p53 accumulation in non-stressed cells (Fuchs et al., 1998b; Milne et al.,

1995). The accumulated p-53 acts as an activator of genes containing p53-response elements. On the contrary, administration of JNK-specific inhibitor SP600125 increased the total amount of p53 (Figure 3A) but did not alter p-p53 level in the brains of treated mice relative to controls (Figure 5). These data suggest that JNK-specific inhibitor SP600125 may have increased the steady state level of p53 by inhibiting the formation of JNK-p53 and/or Mdm2-p53 complex. Therefore, accumulation of non-phosphorylated p53 may be responsible for compensating the apoptotic cell deaths that would have been otherwise caused by p53-mediated inhibition of PS1 expression and Notch 1 signaling in the brains of mice treated with SP600125.

3.3 Inhibition of Notch signaling and APP processing with SP600125 may have beneficial effect in Alzheimer's disease therapy

The Notch signaling pathway is mostly regarded as a developmental pathway (Ables et al., 2011; Artavanis-Tsakonas et al., 1999). Notch is also a key regulator of adult neural stem cells (Ables et al., 2011). Decrease in Notch activity leads to neuronal stem cell (NSC) proliferation and an increased net number of adult-born neurons because the cell exits the cell cycle and differentiates into neuron (Ables et al., 2011). In addition, Notch signaling plays a crucial role in regulation of migration, morphology, synaptic plasticity, and survival of mature neurons (Ables et al., 2011). Notch activation leads to activation of Hes genes which inhibit NGN3 expression and neurite outgrowth (Ables et al., 2011). Therefore, inhibition of Notch signaling in adult brain leads to increase neurite outgrowth, survival of mature and immature neurons, and restore synaptic plasticity (Ables et al., 2011). PS1/ γ -secretase cleavage is common to both Notch signaling and APP processing. Processing of Notch 1 by γ -secretase generates NICD (Ables et al., 2011) whereas processing of APP by γ -secretase generates A β ₄₀ and A β ₄₂ peptides (Borchelt et al., 1996). A β ₄₂ aggregates faster than A β ₄₀ and produces amyloid plaques in the brains of AD patients resulting in neurodegeneration and cognitive deficits. The amount of A β ₄₀ in C57BL/6 wild-type mouse brain is very low. So we could not accurately determine the amount of A β ₄₀ in wild-type mouse brain using ELISA. Since A β ₄₂ level is very high in the brain of APPTg mouse (Sisodia et al., 1990), JNK-specific inhibitor SP600125 will be tested in APPTg mouse model of AD to determine if it reduces A β ₄₂ as an alternative remedy for Alzheimer's disease.

Processing of Notch was increased in brains of patients with Alzheimer's disease compared to controls. (Berezovska et al., 1998). Thus increased Notch 1 cleavage and Notch 1 signaling exacerbate the pathology of Alzheimer's disease (Ables et al., 2011). Therefore, reducing γ -secretase activity by γ -secretase inhibitors was expected to control Alzheimer's disease. Unfortunately, thus far, γ -secretase inhibitors have not been very successful as potential treatment for Alzheimer's disease. It has been reported that JNK is upregulated in the degenerating neurons of Alzheimer's disease patients compared to controls (Zhu et al., 2001). Therefore, JNK-specific inhibitor SP600125 may potentially reduce JNK activity to prevent neuronal degeneration. Our current study indicates that Notch processing and Notch signaling can be inhibited simultaneously in adult mouse brains by peripheral administration of JNK-specific inhibitor SP600125. SP600125 likely reduces γ -secretase activity and Notch 1 signaling in mouse brains by repressing PS1 transcription via increasing the accumulation of p53. Reduced PS1 expression and Notch 1 signaling by JNK-specific inhibitor should potentially result in apoptosis in mouse brains. It is possible that apoptotic cell deaths caused by p53-mediated reduction of PS1 and Notch signaling may have been compensated by the anti-apoptotic effect of accumulated p53 in the brains of mice treated with SP600125.

Experimental procedures

4.1 Animals and experimental protocols

Three months old adult male C57BL/6 mice (Jackson Laboratory, Bar Harbor, Maine) weighing ~30 g were used. Mice were housed under standardized conditions with free access to a standard chow and water. Mice were divided into two groups with 4 animals in each group. Group 1 was vehicle control. Group 2 was treated with JNK inhibitor SP600125 (LC laboratories, Woburn, MA). Control animals in group-1 (n=4) were given 250 µl of vehicle (45% w/v of 2-hydroxypropyl-β-cyclodextrin; Sigma Aldrich, St Louis, MO) by i.p injection once a day for continuous 14 days. Treated animals in group-2 (n=4) were given 250 µl of SP600125 (16 mg/kg/day of SP600125 in vehicle) by i.p injection once a day for continuous 14 days. Mice were sacrificed on day 15. One hemi-brain from each mouse was frozen for immunofluorescent staining (IFS). The other hemi-brain was used for biochemical studies. For IFS brain tissues were snap frozen with OCT (optimal cutting temperature) compound (Tissue-Tek-Cat. No. 4583; ThermoFisher Scientific, Atlanta, GA) at -70°C. The frozen brain tissue was cut on sagittal plane for sections by cryostat (MICROM HM 525, Thermo scientific, Atlanta, GA). All animal experiments were in compliance with the protocols approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center at Fort Worth, in accordance with guidelines of the NIH.

4.2 Immunoblot analysis

Cortex from mouse hemi-brain (n=3) was homogenized for 30 seconds using a mechanical homogenizer with homogenization buffer (10 mM Tris 7.2, 150 mM NaCl, 5 mM EDTA, 1% Triton X100, 1% sodium deoxycholate, 5 mM sodium orthovanadate, 50 mM NaF,) containing proteinase inhibitors. The homogenate was incubated for 2-3 hrs with shaking at 4°C, sonicated for 10 seconds, and centrifuged at 12,000Xg for 30 minutes. The supernatant (protein extract) was used for determination of protein concentration using Biorad reagent. 40 µg of Protein extract was mixed with equal volume 2X SDS-PAGE loading dye solution containing β-mercaptoethanol and heated for 10 minutes at 90 °C. Proteins were separated by 16% SDS-PAGE and transferred to PVDF membrane at 200 mA for 3 hrs. The membranes were blocked with 2% BSA in TBST (10 mM Tris 7.5, 150 mM NaCl and 0.05% Tween 20) for 2 hrs in room temperature followed by overnight incubation with primary antibodies at 4°C. Following antibodies were used: Anti-PS1 (Cat. No. MAB5232; Millipore, Billerica, MA), anti-phospho-SAPK/JNK (Cat. No. 9255; Cell signaling Tech, Boston, MA), anti-JNK (Cat. No. sc-474; Santa Cruz Biotech, Santa Cruz, CA), anti-activated Notch1 (Cat. No. ab8925; Abcam, Cambridge, MA), anti-Hes1 (Cat. No. ab71559; Abcam, Cambridge, MA), and anti-βActin (Cat. No A5441; Sigma Aldrich Inc, St Louis, MO) The blots were developed by ECL system (Pierce, IL).

4.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Homogenates from mouse cortex (n=3) were centrifuged and cell pellets were used to prepare total RNA using trizol reagent (Invitrogen, CA) according to manufacturer protocol. First strand cDNA was synthesized using oligodT primers and reverse transcriptase (Invitrogen, CA). 34 cycles of PCR were performed with the primers for mouse Hes1 and GAPDH. 250 ng of cDNA was used in PCR for Hes1 and 62.5 ng of the same cDNA was used in PCR for GAPDH. PCR was carried out at 94°C for 30s, 60°C for 30s, 72°C for 60s. PCR products were run on a 5% poly-acrylamide gel, stained with ethidium bromide, and visualized under uv light. The sequences for mouse Hes1 and GAPDH primers are (1) Hes1 forward: 5'-GCCAGTGTCAACACGACACCGG-3' and Hes 1 reverse : 5'-TCACCTCGTTCATGCACTCG-3' (2) GAPDH forward: 5'-AACTTTGGCATTGTGGAAGG-3' and GAPDH reverse : 5'-TGTGAGGGAGATGCTCAGTG-3'

4.4 Immunofluorescent (IF) analysis

For immunofluorescent staining (IFS), each 10µm-thick cryosection was fixed in cold acetone, blocked with 10% donkey serum in TBST, and stained with optimum dilution of primary antibodies, then optimum dilution of fluorochrome-conjugated secondary antibodies. Primary antibodies were anti-presenilin-1 (Cat. No. MAB5232; Millipore, Billerica, MA), phospho-SAPK/JNK (Cat. No. 9255; Cell Signaling Tech, Boston, MA), anti-p53 (Cat. No. sc-98; Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-p53 (Cat. No. 9284L; Cell Signaling Tech, Boston, MA), activated Notch1 (Cat. No. ab8925; Abcam, Cambridge, MA), and Hes1 (Cat. No. ab71559; Abcam, Cambridge, MA). Fluorochrome-conjugated secondary antibodies were Cy3-conjugated donkey anti-mouse IgG (Cat. No. 715-166-151; Jackson Immuno Research, Mill Valley, CA), Cy3-conjugated donkey anti-rabbit IgG (Cat. No. 711-166-152; Jackson Immuno Research, Mill Valley, CA), and Alexa-Fluor-488-conjugated chicken anti-goat IgG (Cat. No. A21467; Invitrogen, Carlsbad, CA). Antibody-stained immunofluorescent samples were mounted by anti-fading aqueous mounting medium containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and covered by cover slips. The magnification indicated in each figure shows that of the objective lens in Nikon Eclipse Ti-U fluorescent microscope. The ratio of % positive staining areas versus % DAPI regions was analyzed by NIH software image-J.

4.5 TUNEL Assay

For TUNEL assay, each 10µm-thick cryosection was fixed in 4% paraformaldehyde, permeabilized with 0.1% TritonX-100 and pH 7.2. Terminal transferase reactions (containing TdT and fluorescein-dUTP) were then performed with the *in situ* Cell Death Detection Kit (Cat. No. 11684795910; Roche, San Francisco, CA) for the TUNEL assay. Labeled samples were mounted by anti-fading aqueous mounting medium containing DAPI and covered by cover slips. The magnification in the figures shows that of the objective lens in Nikon Eclipse Ti-U fluorescent microscope.

4.6 Statistical analysis for IFS and TUNEL assay

For IFS and TUNEL assay, the statistical significance between any two groups was analyzed by unpaired Student's *t*-test. If the F-test comparison of variance was less than 0.05 (i.e. nonparametric distribution), the unpaired *t*-test with Welch's correction was used. Differences were considered statistically significant at values of $p < 0.05$. All measures of variance are presented as SEMs.

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Abbreviations used

AD	Alzheimer's disease
APP	β-amyloid precursor protein
DAPI	4'-6 diamidino 2-phenylindole dihydrochloride
ERK	Extracellular stimulus-regulated kinase
Ets	Avian erythroblastosis virus E26 oncogene homologue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hes1	Hairy and enhancer of split-1

IFS	Immunofluorescent staining i.p, Intraperitoneal
JNK	<i>c-jun</i> -NH ₂ -terminal kinase
Mdm2	Murine double minute 2
NGN3	Neurogenin 3
NICD	Notch intracellular domain p53, Oncoprotein p53
PI3K	Phosphoinositide 3 kinase
PS	Presenilin
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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Highlights

1. Intraperitoneal (i.p.) injection of JNK-specific inhibitor SP600125 decreased the levels of p-JNK and PS1 in the mouse brain in vivo similar to what this laboratory has previously reported using an in vitro cell culture system.
2. Concurrently, injection of SP600125 increased the level of p53 in mouse brain.
3. Suppression of PS1 expression by SP600125 reduced processing of Notch to NICD and expression of the NICD target, Hes1.
4. The SP600125-induced changes observed in mouse brain did not correlate with induction of apoptosis.

Figure 1. Intraperitoneal administration of JNK-inhibitor (SP600125) reduced expression of p-JNK and PS-1 proteins in mouse brains

(A) Immunofluorescent staining results show a significant decrease of p-JNK in SP600125-treated mouse brain. Left panels show representative images of p-JNK (red) and nucleus (blue) on cryosections from mouse brain tissues; right panel shows a summary of ratios of p-JNK/DAPI positive percentage area analyzed by Image-J software from 4 mice in each group. Each symbol represents one animal. (B) Immunofluorescent staining results show a significant decrease of PS1 in SP600125-treated mouse brain. Left panels show representative images of PS1 (red) and nucleus (blue) on cryosections from mouse brain tissues; right panel shows a summary of ratios of PS-1/DAPI positive percentage area analyzed by Image-J software from 4 mice in each group. Each symbol represents one animal. (C) A representative co-staining images of PS1 (red) and p-JNK (green) against counterstaining of DAPI (blue) provides the same information as A and B single staining. Scale bar = 50 μ m.

Figure 2. Immunoblot analysis to show that intraperitoneal injection of JNK-specific inhibitor SP600125 decreases p-JNK and PS1 expression in mouse cortex
(A) Control extracts (40 μ g) (C1, C2, C3) from the cortex of vehicle treated 3 mice and 40 μ g extracts (S1, S2, S3) from the cortex of SP600125 treated mice were subjected to immunoblot analysis with anti-p-JNK, anti-JNK, and anti-PS1. Same protein samples (C1, C2, C3, S1, S2, S3) were run on 4 separate gels ($n=3$). Four blots were developed by chemiluminescence and protein gel bands in four blots were quantified using Labworks Image Analysis Software and for statistical analyses. Arrows mark the positions of p54 subunit of JNK1/3 (total JNK), as well as p54 and p46 subunits of p-JNK. Arrows also mark the position of 45 kDa full length (PS1 FL) and 20 kDa carboxyterminal fragment (PS1CTF) of mouse PS1. **(B)** Data were analyzed by prism software (Graphpad software Inc) and presented as means + SEM. Comparison was made between two groups by unpaired Student's *t*-test. A probability was considered to be significant with less than 0.05. p-JNK (sum total of p54 and p46), and PS1CTF were normalized with total JNK (p54). Bar graphs represent percent changes of p-JNK in SP600125-treated mice compared to vehicle-treated controls. Statistical significance was denoted by asterisks. *** $P<0.002$. **(C)** Statistical analysis is same as **(B)**. Bar graphs represent percent changes of PS1CTF (20 kDa) in SP600125 treated mice compared to vehicle treated controls. Statistical significance was denoted by asterisks. ** $P<0.01$.

Figure 3. Intraperitoneal administration of JNK-specific inhibitor (SP600125) elevated expression of total p53 protein in mouse brains

(A) Left panels: representative immunofluorescent staining results show a significant increase of total p53 (red) in SP600125-treated mouse brain tissues (low panels). Right panel: a summary of ratios of p53 (red)/DAPI (blue) positive percentage area analyzed by Image-J software from 4 mice in each group. Each symbol represents one animal. (B) A representative co-staining images of p53 (red) and p-JNK (green) shows increased total p53 and decreased p-JNK in the same SP600125-treated mouse brain tissue (bottom panels) compared to vehicle-treated controls (top panels). Scale bar = 50 μ m.

Figure 4. TUNEL assay did not show significant difference of apoptotic cells between SP600125 treated and control mouse brains

Top panels: Images show a representative merged fluorescent staining result of TUNEL positive cells (green) and DAPI counterstaining (blue) on cryosections from mouse brain tissues of control and treated groups. Bottom panels: A summary of ratios of TUNEL (green)/DAPI (blue) positive percentage area analyzed by Image-J software from 3 mice in each group. Scale bar = 50 μm .

Figure 5. p-p53 expression did not show significant difference between SP600125 treated and control mouse brains

Top panels: Images show a representative immunofluorescent staining of p-p53 (red) and DAPI (blue) on cryosections from mouse brain tissues. The left panels in each sample show p-p53 (red) single staining, while the right panels in each sample show p-p53 and DAPI merger. Bottom panels: A summary of ratios p-p53 (red)/DAPI (blue) positive percentage area analyzed by Image-J software from 3 mice in each group. Scale bar = 50 μ m.

Figure 6. Inhibition of NICD and Hes1 in the brain of mouse treated with JNK-specific inhibitor SP600125

(A) Immunofluorescent staining results show a significant decrease of NICD in SP600125-treated mouse brain tissues. Top panel shows images of a representative merged immunofluorescent staining of NICD (red) and DAPI (blue) on cryosections of mouse brain tissues. Bottom panel shows summarized ratios of percentage of NICD positive area versus percentage of DAPI regions analyzed by NIH software image-J. Each symbol represents one animal. (B) Immunofluorescent staining results show a significant decrease of Notch target Hes1 gene in SP600125-treated mouse brain tissues. Top panel shows images of a representative merged immunofluorescent staining of Hes1 (red) and DAPI (blue) on cryosections of mouse brain tissues. Bottom panel shows summarized ratios of percentage of Hes1 positive area versus percentage of DAPI regions analyzed by NIH software image-J. Each symbol represents one animal. Scale bar = 50 μm.

Figure 7. Immunoblot analysis to show that intraperitoneal injection of JNK-specific inhibitor SP600125 decreases NICD and Hes1 expression in mouse cortex

(A) Immunoblot analysis shows a significant reduction of NICD and Hes1 expression in the cortex of mice treated with SP600125. Control extracts (40 μ g) (C1, C2, C3) from the cortex of vehicle-treated 3 mice and 40 μ g extracts (S1, S2, S3) from the cortex of SP600125-treated mice were subjected to immunoblot analysis with anti-NICD, anti-Hes1, and anti- β Actin. Same protein samples (C1, C2, C3, S1, S2, S3) were run on 4 separate gels ($n=3$). Four blots were developed by chemiluminescence and protein gel bands in four blots were quantified using Labworks Image Analysis Software and for statistical analyses. Positions of 80 kDa NICD, 16 kDa Hes1, and 42 kDa β -Actin are marked. (B) Data were analyzed by prism software (Graphpad software Inc) and presented as means + SEM. Comparison was made between two groups by unpaired Student's t -test. A probability was considered to be significant with less than 0.05. NICD was normalized with β -Actin. Bar graphs represent percent changes of NICD in SP600125 treated mice compared to vehicle treated controls. Statistical significance was denoted by asterisks. *** $P<0.003$. (C) Statistical analysis is same as (B). Bar graphs represent percent changes of Hes1 in SP600125 treated mice compared to vehicle treated controls. Statistical significance was denoted by asterisks. *** $P<0.002$.

Figure 8. RT-PCR analysis to show that intraperitoneal injection of JNK-specific inhibitor SP600125 decreases Hes1-mRNA expression in mouse cortex

(A) Hes1-mRNA level was measured in the cortex of mice treated with vehicle (C1, C2, C3) or SP600125 (S1, S2, S3) by RT-PCR. RT-PCR products were run on 5% polyacrylamide gel and visualized by ethidium bromide staining. DNA gel bands were quantified using Labworks Image Analysis Software. RT-PCR products (C1, C2, C3, S1, S2, S3) from three different RT-PCR with same first strand cDNA were run on 3 separate gels ($n=3$). DNA bands in three gels ($n=3$) were quantified using Labworks Image Analysis Software and for statistical analyses. Positions of Hes1 (309 bp), and GAPDH (599 bp) are marked. M represents DNA size marker. (B) Data were analyzed by prism software (Graphpad software Inc) and presented as means + SEM. Comparison was made between two groups by unpaired Student's *t*-test. A probability was considered to be significant with less than 0.05. Hes1 was normalized with GAPDH. Bar graphs represent percent changes of Hes1-mRNA in SP600125-treated mice compared to vehicle-treated controls. Statistical significance was denoted by asterisks. *** $P<0.0012$.