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Generation and characterization of mice harboring a conditional *CXCL12* allele

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ABSTRACT The chemokine CXCL12 has important functions in immune and central nervous systems. Moreover, a global disruption of *CXCL12* in mice results in perinatal lethality. To circumvent this impediment and provide a tool for analyzing CXCL12 functions in specific organ systems, we have generated a mouse line harboring a *loxP*-site flanked exon 2 of *CXCL12*. A germ line deleter, β-*actin::cre* was used to remove a *CXCL12* exon 2 and subsequently systemic *CXCL12* exon 2 deficient embryos were generated. These mutant embryos showed a marked depletion of *CXCL12* transcript. As expected from the global mutant phenotype, our mutants were also characterized by highly irregular cerebellar cytoarchitecture of the external granule layer as well as altered radial migration of midbrain dopaminergic neurons. Importantly, migration of the pontine grey nucleus (PGN) was derailed and remarkably resembled the global mutant phenotype of the CXCL12 receptor – CXCR4 in this system. Despite the fact that CXCL12 signaling can be mediated through receptors other than CXCR4, our results indicate a monogamous relationship between the CXCL12 ligand and CXCR4 receptor in controlling PGN migration. Our findings further expand on the understanding of CXCL12 function in PGN development. Moreover, phenotypic similarities between our mutants and mice harboring a global *CXCL12* disruption support the validity of our line. Importantly, these results strongly suggest that our conditional *CXCL12* line can be used as a powerful tool to manipulate CXCL12 signaling and function *in vivo*.

KEY WORDS: *SDF1 floxed, pontine grey nucleus, external granule layer, midbrain dopaminergic neuron*

Introduction

Chemokines are a class of small molecules that were initially found to function as leukocyte attractants in the immune system (Baggiolini, 1998). Apart from this system, chemokines also impact hematopoiesis, lymphopoesis, angiogenesis and neuron migration in the central nervous system (CNS) (Bodea *et al.*, 2014; Ma *et al.*, 1998; Nagasawa *et al.*, 1996; Sánchez-Alcañiz *et al.*, 2011; Wang *et al.*, 2011; Yang *et al.*, 2013).

One of the chemokine ligands, stromal cell-derived factor 1 (SDF-1; CXCL12) binds receptors CXCR4 and CXCR7 (Balabanian *et al.*, 2005; Oberlin *et al.*, 1996). In the developing CNS, CXCL12 regulates neuron migration and proliferation (Sánchez-Alcañiz *et al.*, 2011; Wang *et al.*, 2011; Zou *et al.*, 1998). For instance, the cerebellum of *CXCR4* or *CXCL12* mutant mice is characterized by ectopically located Purkinje cells and irregular external granule layer (EGL), which protrudes into the cerebellar parenchyma (Ma *et al.*, 1998; Vilz *et al.*, 2005). In the midbrain, CXCR4 is expressed in postmitotic midbrain dopaminergic (mDA) neurons, whereas CXCL12 is expressed in meninges. Consistently, a global disruption of *CXCR4* or *CXCL12* transiently alters radial migration of mDA neurons wherein CXCL12/CXCR4 signaling appears to modulate the migration efficiency of mDA neurons (Bodea *et al.*, 2014; Yang *et al.*, 2013). In the hindbrain, the pontine grey nucleus (PGN) neurons migrate anteriorly beneath the pial surface and settle down in the

Abbreviations used in this paper: CXCL12; chemokine (C-X-C motif) ligand 12; CXCR4, chemokine (C-X-C motif) receptor 4; CXCR7, atypical chemokine receptor 3; ECN, external cuneate nucleus; EGL, external granule layer; LRN, lateral reticular nucleus; mDA, midbrain dopaminergic; PGN, pontine grey nucleus.

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ventral anterior hindbrain, in contrast to posterior migration of the lateral reticular nucleus (LRN) and external cuneate nucleus (ECN) neurons (Rodriguez and Dymecki, 2000). These migrating neurons express CXCR4, whereas CXCL12 is expressed in surrounding meninges. Consistent with this expression pattern, *CXCR4* mutants are characterized by derailed PGN neuron migration and reduced size of this nucleus in contrast to the intact posterior migration of the LRN and ECN (Vilz *et al.*, 2005; Zhu *et al.*, 2009).

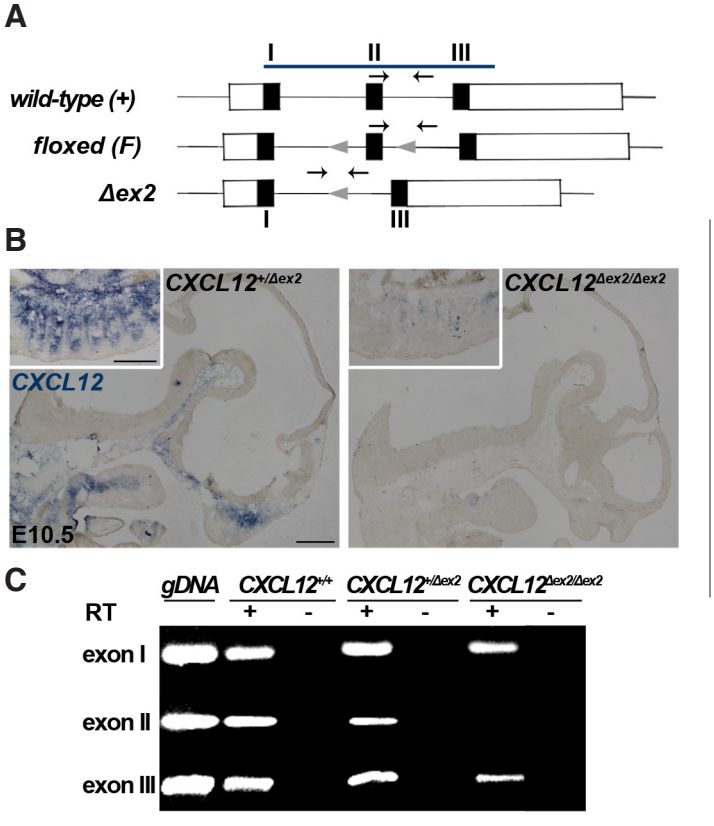
Here, we generated a mouse line bearing a floxed exon 2 of the *CXCL12* gene. Mutants with a conditional deletion of both alleles show aberrant development of the cerebellum, mDA neurons, and PGN that is strikingly similar to phenotypes in corresponding structures of global *CXCL12* or *CXCR4* mutants.

Results

Generation of mice bearing a conditional CXCL12 allele

Analyses of global *CXCL12* mutants generated by insertional mutagenesis are restricted to embryonic development due to perinatal lethality (Ma *et al.*, 1998; Nagasawa *et al.*, 1996; Zhu *et al.*, 2009; Zhu *et al.*, 2002). Accordingly, we sought to generate a conditional *CXCL12* allele that can be used as a tool to target its specific developmental and postnatal functions and characterized this line based on established neural phenotypes of mice with disrupted CXCL12 signaling.

CXCL12 binds its cognate receptor CXCR4 through exon 2 sequences (Crump *et al.*, 1997). Thus, removing this exon should prevent any corresponding downstream signaling. Accordingly, we positioned *loxP* sites to flank exon 2 (Figs. 1A, S1, and S2). Furthermore, we reasoned that homozygous, $CXCL12^{\Delta ex2/\Delta ex2}$ mutant embryos lacking both alleles of exon 2 should recapitulate a global *CXCL12* mutant phenotype (Nagasawa *et al.*, 1996). To generate these mutants, we first crossed floxed *CXCL12* exon 2 animals ($CXCL12^{F/+}$) to a germ line deleter β*actin::cre*. Resulting animals heterozygous for an exon 2 deletion ($CXCL12^{+/\Delta ex2}$) were then intercrossed to generate $CXCL12^{\Delta ex2/\Delta ex2}$ embryos (Fig. S3A). All animals and embryos were genotyped by *PCR* analysis (Fig. S3B). To address if a deletion of exon 2 of the *CXCL12* gene results in a loss-of-function mutation, we performed *RNA in situ* hybridization using a *CXCL12* riboprobe (Fig. 1A; dark blue line).



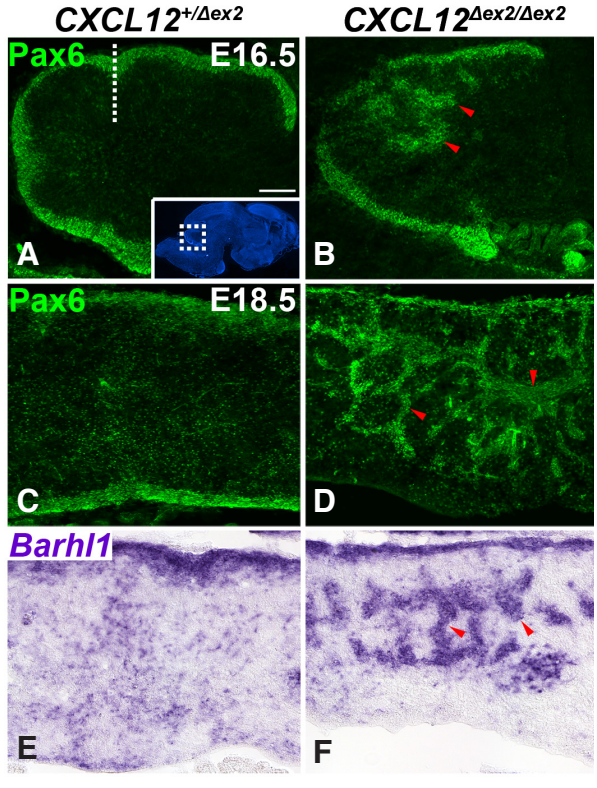


Fig. 1 (Left). Generation of the *CXCL12* floxed line. **(A)** *Schematic of the* CXCL12 *genomic locus. Exons (I-III) and introns are depicted by black boxes and solid lines, respectively. The 5′UTR and 3′UTR are indicated by open boxes. A position of the primers used for genotyping (Supp. Fig. S3B) is indicated by black arrows. Grey arrowheads indicate* loxP *sites. The dark blue line indicates the position of the* CXCL12 *riboprobe used in (B).* **(B)** *RNA* in situ *hybridization on sagittal sections for* CXCL12. *Somite regions of the corresponding genotypes are shown in insets. Anterior is to the right, posterior to the left.* **(C)** *RT-PCR analysis of control and mutant samples. gDNA, genomic DNA; RT, reverse transcriptase. Scale bars, 500 μm.*

Fig. 2 (Right). $CXCL12^{\Delta ex2/\Delta ex2}$ embryos exhibit aberrant cerebellar organization. *Immunofluorescent labeling for Pax6* **(A-D)** *and RNA* in situ *hybridization using a* Barhl1 *riboprobe* **(E,F)** *reveal a disorganized external granule layer (EGL) in mutant* **(B,D,F)** *(red arrowheads), in comparison to control embryos* **(A,C,E)** *in sagittal* **(A,B)** *and coronal sections* **(C-F)**. **(A)** *The dashed white line indicates the relative axial level of coronal sections shown in (C–F). Inset in (A) is a DAPI labeled sagittal section in which the white dotted box indicates the approximate region shown in (A,B) at higher magnification. Anterior is to the right, posterior to the left. Scale bar, 100 μm.*

In E10.5 control embryos, *CXCL12* is expressed in cranial (Fig. 1B left) and somite regions (inset in Fig. 1B left). In sharp contrast, in mutant embryos *CXCL12* expression is markedly decreased but not completely absent in corresponding regions (Fig. 1B right and inset). We have also performed RT-PCR analyses using primers positioned in each of three *CXCL12* exons in *CXCL12*$^{+/+}$, *CXCL12*$^{+/\Delta ex2}$ and *CXCL12*$^{\Delta ex2/\Delta ex2}$ samples (Fig. 1C). Our analyses revealed that no transcript is produced from exon 2 in *CXCL12*$^{\Delta ex2/\Delta ex2}$ mutants. However, we have detected some mutant transcript(s) generated from exons 1 and 3.

CXCL12Δ$^{ex2/\Delta ex2}$ *mutant embryos are characterized by disrupted cerebellar cytoarchitecture*

Since CXCL12 is required for normal cerebellar development (Ma *et al.*, 1998; Zhu *et al.*, 2002), we sought to examine this structure in our *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos using known EGL molecular markers. To do so, we performed *RNA in situ* hybridization using a *Barhl1* riboprobe and immunofluorescent analyses using Pax6 antibodies. In E16.5 and E18.5 control embryos, the EGL is peripherally localized (Figs. 2A and 2C/2E, respectively). In sharp contrast, the mutant EGL appears to extend into the cerebellar parenchyma apparent at both embryonic stages (Figs. 2B, 2D, and 2F).

Initial radial migration of mDA neurons is altered in CXCL12$^{\Delta ex2/\Delta ex2}$ *embryos*

CXCL12 global mutant mice exhibit altered initial radial migration of mDA neurons. Accordingly, we examined this phenomenon in our *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos by using antibodies against the postmitotic mDA neuron marker, tyrosine hydroxylase (TH). In control embryos, upon exiting the ventricular zone around E10.5-11.5, young postmitotic mDA neurons emerge in the intermediate zone and transition to the mantle zone where they start expressing TH (Fig. 3A). In contrast, *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos were characterized by some mDA neurons localized within the intermediate zone, indicating that these neurons were setback along their radial migration path (Fig. 3B). As development proceeds, at E13.5 in control embryos, mDA neuron radial migration becomes prominent throughout the rostrocaudal extent of the ventral midbrain midline (Fig. 3 C,E,G, respectively). In contrast, radial migration of mDA neurons in *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos deviated from the midline and some mDA neurons appeared to be ectopically positioned in the middle region of the mantle zone at all axial levels (Fig. 3D, 3F and 3H). This initial obstruction to mDA neuron radial migration seems to exist transiently since these misplaced migrating neurons become less evident at later embryonic stages (E18.5) as apparent by similarities in the formation of the Substantia Nigra pars compacta (SNpc) and Ventral Tegmental Area (VTA) in control and mutant embryos (Fig. 3I and 3J, respectively).

CXCL12$^{\Delta ex2/\Delta ex2}$ *mutant embryos show derailed migration and disrupted formation of the PGN*

Aberrant PGN migration in global *CXCR4* mutants led us to examine formation of this structure in our *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos. In control animals at E14.5, newly arrived Pax6+ and *Barhl1+* PGN neurons begin to settle in the anterior ventral hindbrain. En route to their final destination, PGN neuron migration is also detected in more caudal regions of the hindbrain localized beneath the pial surface. In contrast, numbers of PGN neurons appear to be substantially reduced in the anterior ventral hindbrain of *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos. Concomitantly, mutant migration appears to be derailed wherein PGN neurons are ectopically located at the midline

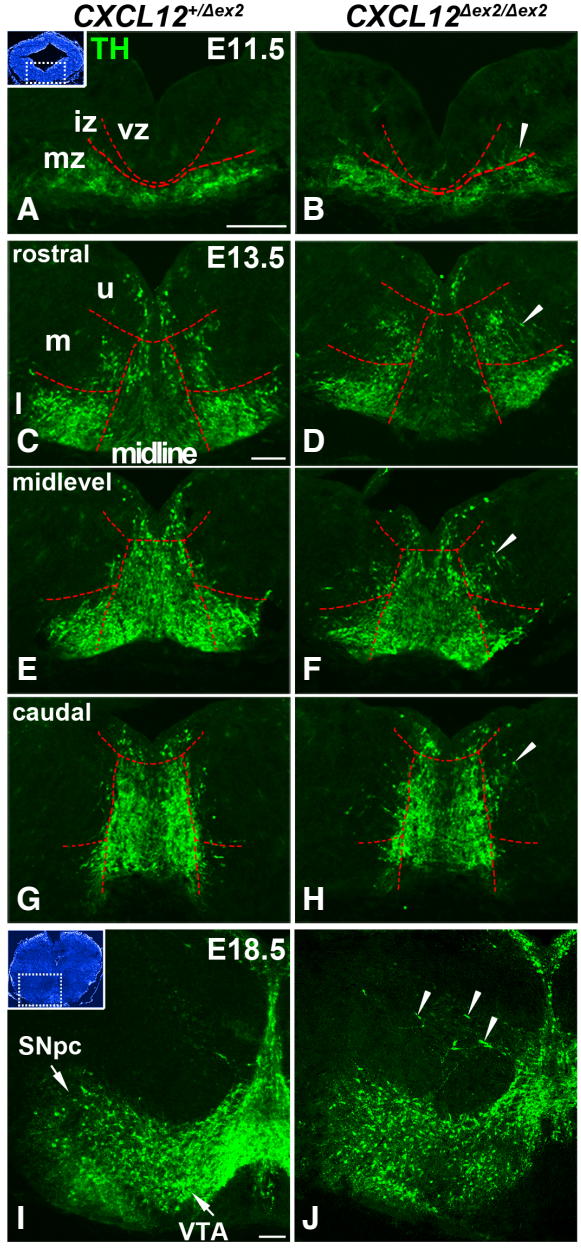


Fig. 3. Altered radial migration of mDA neurons in *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos. *Midbrain coronal sections were labeled for tyrosine hydroxylase (TH).* **(A,B)** *Red dotted lines indicate a separation between ventricular (vz; TH–), intermediate (iz; TH–) and mantle (mz; TH+) zones. Arrowhead depicts TH+ neurons ectopically positioned in the mutant iz.* **(C–H)** *The TH+ mz was partitioned into upper (u), middle (m) and lower (l) regions by red dotted lines. Arrowheads indicate increased numbers of TH+ neurons in the middle (m) region of the mz in mutants compared to controls.* **(I,J)** *Substantia Nigra pars compacta (SNpc) and Ventral Tegmental Area (VTA) are apparent in controls* **(I)** *while mutant embryos show a few misplaced TH+ neurons* **(J)** *(see arrowheads). Insets in (A,I) are DAPI labeled coronal midbrain sections in which the white dotted boxes indicate approximate regions shown at higher magnification in (A-H) and (I,J), respectively. Scale bar, 100 μm.*

and enter the caudal hindbrain parenchyma (Fig. S4). This early disruption of PGN neuron migration resulted in a drastic reduction of the size of the PGN at E16.5 (Fig. S5).

PGN, ECN, and LRN are prominent structures in control embryos at E18.5 (Fig. 4A, 4E and 4G, respectively). Consistent with phenotypes in younger mutant embryos (Figs. S4 and S5), a drastic reduction of the PGN is maintained at E18.5 (Fig. 4B). Moreover, migrating mutant neurons appear to ectopically enter the hindbrain parenchyma becoming stalled at the midline (Fig. 4D and 4F, arrowheads). Despite these hindrances to PGN neuron migration, ECN and LRN appear to be intact in C*XCL12*$^{\Delta ex2/\Delta ex2}$ embryos (Fig. 4F and 4H), reminiscent of phenotypes reported in *CXCR4* mutants (Vilz *et al.*, 2005; Zhu *et al.*, 2009).

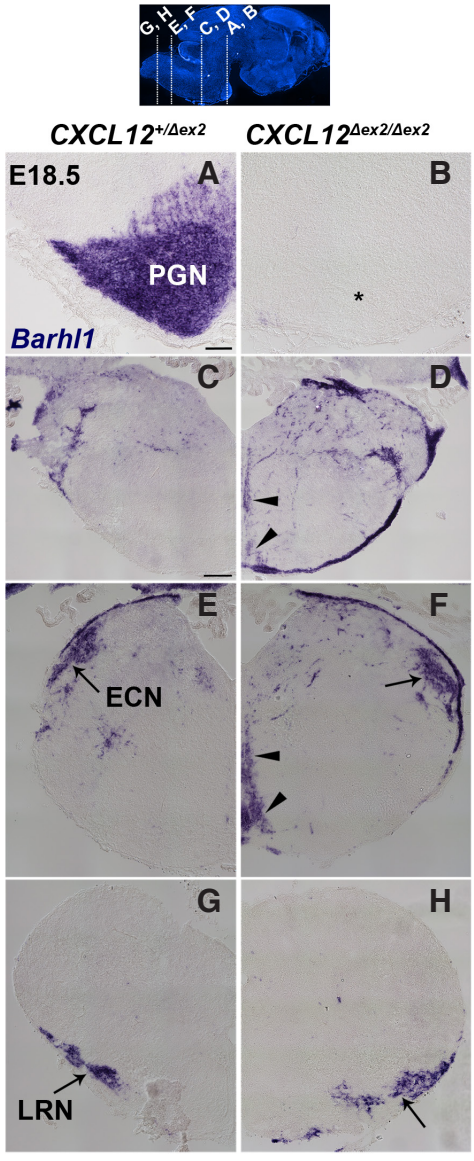


Fig. 4. CXCL12 is required for proper migration of pontine grey nucleus (PGN) neurons. *Coronal hindbrain sections were labeled with a* Barhl1 *riboprobe. Control embryos display prominent PGN anteriorly* **(A)** *and external cuneate nucleus (ECN) along with lateral reticular nucleus (LRN) posteriorly (see arrows in* **(E,G)** *respectively). Mutant* CXCL12$^{\Delta ex2/\Delta ex2}$ *embryos exhibit a drastically reduced PGN (see asterisk in* **(B)***) and ectopically localized PGN neurons at the hindbrain midline (see arrowheads in* **(D,F)***). In contrast, the ECN and LRN seem to be unaffected in the mutants (arrows in* **(F,H)** *respectively), when compared to controls* **(E,G)** *respectively. The upper image is a DAPI labeled sagittal section in which white dotted lines indicate approximate positions of coronal sections shown in (A-H). Anterior is to the right, posterior to the left. Scale bars, 100 μm (A,B) and 200 μm (C–H).*

Discussion

In this report, by using our newly developed *CXCL12* floxed line, we have generated *CXCL12*$^{\Delta ex2/\Delta ex2}$ mutants that were characterized by a marked reduction of *CXCL12* transcript. Though our RT-PCR analyses indicated a presence of mutant transcripts, the observed *CXCL12*$^{\Delta ex2/\Delta ex2}$ mutant phenotypes either matched or complemented those that have been previously established for the CXCL12/CXCR4 system. Thus, it is conceivable that although mutant transcripts could be transiently produced, our floxed line is capable of generating a *CXCL12* loss-of-function mutation *in vivo*.

Although previous *CXCL12* lines have also been generated with *loxP* sites flanking exon 2 (Ding and Morrison, 2013; Greenbaum *et al.*, 2013; Tzeng *et al.*, 2011), our line was characterized specifically based on neural phenotypes established in the literature. Moreover, we have recapitulated global *CXCL12* mutant phenotypes in the developing CNS such as disorganized EGL structure in the cerebellum and negatively affected radial migration of mDA neurons confirming previously proposed functions of the CXCL12/CXCR4 axis in these systems. Importantly, we reveal that CXCL12 is required for proper migration of PGN neurons *in vivo*. Furthermore, the striking similarities between PGN phenotypes observed in our *CXCL12*$^{\Delta ex2/\Delta ex2}$ embryos and global *CXCR4* mutants suggest a monogamous relationship between the CXCL12 ligand and its receptor CXCR4 in PGN development.

Taken together, presented results strongly suggest that our newly developed *CXCL12* floxed line can be used as a powerful tool to generate a *CXCL12* loss-of-function mutation and manipulate CXCL12 signaling *in vivo*. With the plethora of available *cre* drivers, conditional mutagenesis of *CXCL12* could provide for a more detailed understanding of the role of this chemokine in the development of numerous biological systems and processes.

Materials and Methods

Generation of mice harboring a CXCL12 floxed allele

A *CXCL12* floxed exon 2 mouse line was generated by homologous recombination (inGenious Targeting Laboratory, Inc., Ronkonoma, NY, USA; Supplementary Text and Illustrations). Mice were maintained and sacrificed according to the protocols approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

PCR, RT-PCR, RNA in situ hybridization and immunofluorescence

These analyses were performed as previously described (Joksimovic *et al.*, 2009; Supplementary Text and Illustrations). At least three embryos of each genotype were analyzed for each experiment.

Acknowledgments

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