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MINI REVIEW

Glycobiology of the synapse

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Synapses are the fundamental units of connectivity that link together the nervous system. Lectin studies from 30 years ago suggested that specific glycans are concentrated at neuromuscular synapses in the peripheral nervous system and at excitatory synapses in the brain. Subsequent studies have confirmed that particular glycan structures are localized at these synapses, including polysialic acid, high mannose, the cytotoxic T cell antigen, and forms of heparan sulfate. Though the role of these molecules in synapse formation and function is still poorly understood, there is increasing evidence that the function of agrin, a synaptogenic factor in neuromuscular formation, is modulated by several glycans. In addition, the recent generation of ST8SiaIV null mice strongly suggests a role for polysialic acid in synaptic plasticity in the some regions of the central nervous system.

Key words: acetylcholine receptor/agrin/glutamate receptor/ long-term potentiation/polysialic acid

Introduction

Most neurobiologists do not consider glycobiology in their thinking about synapses. Many studies of synaptic development and function are done using methods in which glycan composition is either completely ignored or not considered. Thus it would seem that most students of the synapse at least passively subscribe to the view that glycans are not central to understanding synapses. Accordingly, of the past thousand papers with the words *synapse* or *synaptic* in their title, only three have glycobiology as their primary topic. This is understandable, given the history of the field. As described in this article, two vast bodies of literature support the notion that carbohydrates are not critical for understanding synapses.

Many studies have shown that one of the primary events in synapse formation, the localization of postsynaptic neurotransmitter receptors, involves protein–protein interactions between the cytoplasmic tails of receptors and cytoplasmic anchoring factors (for review, see Sanes and Lichtman, 1999a; Sheng and Pak, 2000). Examples of such anchoring proteins have now been found for almost every family of neurotransmitter receptor. Even synaptic proteins that are not receptors but are highly glycosylated, such as densin 180, a sialomucin (Apperson *et al.*, 1996), and syndecan-2, a heparan sulfate proteoglycan (Ethell and Yamaguchi, 1999), have cytoplasmic peptide anchoring motifs that likely are important for their localization to synaptic areas.

Another group of studies support the notion that glycosylation is not important for neurotransmitter receptor function. Addition of such glycosylation inhibitors as tunicamycin or direct mutagenesis of N-linked glycosylation sites on nicotinic acetylcholine receptors (AChRs) show that although all subunits of the nicotinic AChR contain N-linked glycans and that these sites are by and large important for receptor assembly and stability (Merlie *et al.*, 1982; Prives and Bar-Sagi, 1983; Blount and Merlie, 1990; Gehle and Sumikawa, 1991; Ramanathan and Hall, 1999), they are not required for receptors to conduct ions in response to acetylcholine (Gehle and Sumikawa, 1991). Interestingly, glycosylation governs the resistance of cobra and mongoose AChRs to the paralytic effects of α-bungarotoxin and so may be functionally important from an evolutionary perspective (Kreienkamp *et al.*, 1994). Both α-amino-3-hydroxy-5-methyl-4isoxazole-proprionic acid (AMPA) and N-methyl-D-aspartate (NMDA) types of glutamate receptor subunits also have multiple N-linked glycosylation sites (see Everts *et al.*, 1997). As with AChRs, inhibition of N-linked glycosylation can have a profound effect on cell surface expression of some glutamate receptor subunits (NR1 in particular; Everts *et al.*, 1997), but in general the loss of N-linked sites shows that they are not required for ionic conductance (Everts *et al.*, 1997). Ligand binding and lectin-induced inhibition of desensitization, however, can be altered by glycosylation of some glutamate receptors (Thio *et al.*, 1992; Everts *et al.*, 1997; for review see Standley and Baudry, 2000). Thus although there are always some exceptions to the rule, the general conclusion from studies on neurotransmitter receptors is that glycosylation may affect receptor stability or folding, as in other glycoproteins, but has no intrinsic effect on activity.

In this review, I will discuss the evidence that there are indeed synaptic glycans and cover recent studies that suggest that glycans do play important roles in synapse formation and synaptic plasticity. Although these examples are few, their precedent should lead to a more thorough examination of this issue in the future.

Synaptic glycans: are there unique ones?

Because of the role of glycan structures as receptors or co-receptors for many viruses and bacteria (Gagneux and Varki, 1999), carbohydrate expression patterns are rarely phylogenetically conserved; however, the neuromuscular junction (NMJ) is an

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exception. The NMJ is the synapse formed between the nerve terminal of a motor neuron whose cell bodies reside in the spinal cord or hindbrain and a skeletal muscle fibers (for review see Sanes and Lichtman, 1999a). It is the synapse that ultimately controls voluntary movement, and defects in its formation are lethal from birth. Lectin staining studies, combined with enzymatic characterization by glycosidases, have shown that terminal β-linked GalNAc is confined to the neuromuscular junction of birds, frogs, fish, chicks, rats, mice, and humans and is highly concentrated (though not exclusively so) in Torpedo and lamprey (Sanes and Cheney, 1982; Scott *et al.*, 1988).

A number of anticarbohydrate monoclonal antibodies that recognize terminal β-linked N-acetylgalactosamine (GalNAc) as part of its structure stain the NMJ in rodents (Martin *et al.*, 1999). Several of these recognize the cytotoxic T cell (CT) carbohydrate antigen (Lefrancois and Bevan, 1985). The CT antigen in rodents is very similar to the Sda/Cad blood group antigen in humans (Conzelmann and Lefrancois, 1988) and can generally be defined as GalNAcβ1,4[NeuAcα2,3]Galβ1(3GalNAcα or 4GlcNAcβ-). Two CT antigens are expressed at the NMJ in rodents and these are defined by the CT1 and CT2 monoclonal antibodies. CT1 and CT2 are differentially distributed on the pre- and postsynaptic membrane; this subsynaptic localization is maintained to a large degree in rodent cell line models for motor neurons and skeletal muscle (Martin *et al.*, 1999). Antibodies to carbohydrate structures with terminal β1,3 linkages, including antigloboside antibodies and stage-specific embryonic antigen 3, also stain the NMJ (Scott *et al.*, 1988; Martin *et al.*, 1999). Based on immunocytochemistry and immunoblotting, a GalNAc O-phosphotransferase is also expressed at the NMJ (Balsamo *et al.*, 1986; Scott *et al.*, 1990). Thus at least three types of GalNAc linkages may be concentrated at this synapse. Unlike the brain, where biochemical purification of synaptic fractions is feasible, there are no studies on direct glycan sequencing of neuromuscular glycoproteins. Thus although all of these studies point to the importance of GalNAc at this synapse, there is in fact no direct proof of their existence.

Using antiphage antibodies absorbed against heparan sulfate proteoglycans of varying composition, Jenniskens *et al.* (2000) have identified unique anti–heparan sulfate antibodies that stain the rodent NMJ. Antigen recognition by these phages depended on the presence of $GlcNSO_3$ and ester-linked sulfate groups. Though most antibodies isolated recognize the entire muscle basal lamina, several are highly concentrated at the NMJ. These appear to recognize neural antigens, as they did not recognize postsynaptic specializations in cultured muscle cells. Though the glycosaminoglycan (GAG) structures these antibodies identify are not known, they are especially intriguing, given the fact that almost all synaptic cleft proteins at the NMJ either bind heparin (Gesemann *et al.*, 1996; Pall *et al.*, 1996; Fischbach and Rosen, 1997; Talts *et al.*, 1999) or are themselves heparan sulfate proteoglycans (Anderson and Fambrough, 1983; Noonan *et al.*, 1991; Tsen *et al.*, 1995; Zhou *et al.*, 1997; Peng *et al.*, 1995).

Excitatory glutaminergic synapses in the brain have been studied by similar methods to those applied to the NMJ but have had the added advantage of the ability to isolate synaptosomes, a purified synaptic fraction, by differential detergent extraction and centrifugation. Matus and colleagues first described concentrated binding of concanavalin A (ConA) to excitatory brain synapses in the rodent, suggesting that high mannose structures are concentrated at brain synapses (Matus *et al.*, 1973; Cotman and Taylor, 1974). *Ricin communis* agglutinin binding is also concentrated in glutaminergic synapses (Bittiger and Schnebli, 1974; Kelly *et al.*, 1976), as are some heparan sulfate moities (Ethell and Yamaguchi, 1999). ConA binding is present both in the pre- and postsynaptic membrane as well as in the synaptic cleft (Wood and McLaughlin, 1976). Using a synaptosome preparation, Gurd and colleagues have sequenced high mannose structures in synaptic junctions isolated from rat brain (Gurd and Fu, 1982). The presence of high mannose is consistent with measurements of glycan composition showing a high concentration of mannose and a relative dearth of sialic acid in postsynaptic densities (Churchill *et al.*, 1976). High mannose oligosaccharides (Man9–Man5) have been shown by sequencing to be present on both the NMDA and AMPA types of glutamate receptors (Clark *et al.*, 1998). In both receptor subtypes, about 50% of the neutral glycan chains were oligomannosidic structures. Although Man5 was the predominant high mannose structure found on all receptors, a significant portion, about 40%, was Man6–9 (Clark *et al.*, 1998). Indeed, the extent of mannosylation appears to be developmentally regulated; the Man8/Man5 ratio is high at postnatal day 10 and is significantly reduced at day 28 (Fu and Gurd, 1983). Changes in lectin binding and radiolabeling studies also suggest that developmental changes in fucose and sialic acid incorporation into synaptic proteins occur in the postnatal period (Fu *et al.*, 1981; Cruz and Gurd, 1983; Stanojev and Gurd, 1987). Given that all glutamate receptors have numerous sites (between 4 and 12) for N-linked glycosylation and may also have O-linked sites (see Everts *et al.*, 1997), regulation of their glycosylation is likely to be complex. This is especially true given that glycoprotein synthesis could occur locally in the dendrites just beneath synapses (Villaneuva and Steward, 2001). Thus proteins synthesized at the synapse could be glycosylated in a manner different from the same proteins produced in the neuronal cell body.

Polysialic acid (PSA) can become synaptic by virtue of its expression on neural cell adhesion molecule (NCAM), a cell adhesion molecule found at some synapses. The synaptic localization of PSA, however, is complex. PSA is not always present at synapses where NCAM is present and can be absent from NCAM-positive synapses as well (Seki and Arai, 1999). The fact that PSA may exist on other brain molecules, such as the voltage-gated sodium channel α subunit, may also contribute to such differential distribution patterns (Zuber *et al.*, 1992).

Last, there is little evidence for the presence of uniquely presynaptic glycans. The presence of keratan sulfate on the synaptic vesicle protein SV2 (Scranton *et al.*, 1993) and synaptic staining by anti-GAG phage antibodies (Jenniskens *et al.*, 2000) suggest that certain GAGs could be localized to the presynaptic membrane, and the finding that botulinum toxin C2 binds complex and hybrid N-linked glycans suggests a concentration of particular N-linked moeties as well (Eckhardt *et al.*, 2000a). Both PSA and high mannose are also likely to be present on presynaptic proteins at some synapses but can also be present in postsynaptic regions, as can the CT antigen, though a variant of this structure appears to be primarily presynaptic (Martin *et al.*, 1999).

Glycans in neuromuscular formation: agrin

Agrin, a highly glycosylated heparan sulfate proteoglycan (Tsen *et al.*, 1995), was the first nerve-derived molecule shown by reverse genetics to be essential for synapse formation; mice lacking agrin fail to make NMJs and die at birth due to an inability to breathe (Gautam *et al.*, 1996). Agrin is made in different splice forms by the motor neuron and the skeletal muscle (Ferns *et al.*, 1993). The motor neuron-derived form is secreted and binds muscle membrane complex that includes the transmembrane tyrosine kinase, MuSK (Glass *et al.*, 1996). Neural agrin activates muscle-specific kinase (MuSK) activity, leading to a signaling cascade that ultimately aggregates post-synaptic AChRs under the nerve terminal. Mice lacking MuSK also fail to make NMJs and die at birth (DeChiara *et al.*, 1996).

Though it remains unclear exactly how agrin signals, four groups of findings implicate glycans in this process. First, agrin is highly glycosylated protein and glycans on agrin mediate binding to other proteins. Agrin is one half sugar by weight (Tsen *et al.*, 1995). Most of that sugar consists of heparan sulfate chains (Tsen *et al.*, 1995), but agrin can also contain O-linked glycans in its mucin region (Parkhomovskiy *et al.*, 2000) and has sites for N-linked glycosylation (Rupp *et al.*, 1991; Tsim *et al.*, 1992). Both fibroblast growth factor 2 and thrombospondin bind agrin via its heparan sulfate moities (Cotman *et al.*, 1999) and laminin-1 (α1,β1,γ1) and laminin-2 (α2,β1,γ1) bind by both heparan sulfate–dependent and heparan sulfate–independent mechanisms (Cotman *et al.*, 1999). Thus agrin is a synaptic protein organizer by virtue of its myriad interactions with other glycoproteins.

Second, agrin binds to carbohydrates. Recombinant forms of neural agrin that are highly active in inducing AChR clustering bind less well to heparin (Gesemann *et al.*, 1996; Campanelli *et al.*, 1996) and to N-acetyllactosamine (Parkhomovskiy *et al.*, 2000) than do inactive muscle forms. By contrast, at least one fragment of neural agrin binds more strongly to Galβ1,3GalNAcα- (Parkhomovskiy *et al.*, 2000). Thus, there are differences in glycan binding between active and inactive fragments, and these may correlate with differential synaptic distributions of glycan chains.

Third, glycans affect agrin signaling *in vitro*. Galβ1,4GlcNAc and Galβ1,3GalNAc can inhibit the induction of MuSK auto-phosphorylation by agrin. Because agrin likely has these glycan chains on its mucin domain, this finding suggests that agrin could dimerize via such glycans or bind to and activate MuSK, as MuSK also binds Galβ1,4GlcNAcβ- (Parkhomovskiy *et al.*, 2000). Fourth, glycans can affect agrin signaling and AChR clustering on cultured myotubes. Heparin (Wallace, 1990) and sialic acid (Grow and Gordon, 2000) block agrin activity on cultured myotubes, and muscle cells deficient in heparan sulfate biosynthesis have severely reduced AChR clustering and agrin responsiveness (Gordon *et al.*, 1993). In addition, unmasking of Galβ1,4GlcNAc or Galβ1,3GalNAc levels by neuraminidase (Martin and Sanes, 1995; Grow *et al.*, 1999) or α-galactosidase (Parkhomovskiy and Martin, 2000) causes agrin-independent activation of AChR clustering and, in at least some cases, activation of MuSK (Grow *et al.*, 1999). Thus, the differential affinity of neural agrin for glycans, coupled with the differential glycosylation of agrin itself, likely plays a role in its ability to activate MuSK and stimulate synapse formation. Repetition of these experiments *in vivo* will be required, however, for conclusive proof of their role.

Nature may have performed one of these experiments already: the UDP-GlcNAc-2-epimerase/ManNAc kinase, an enzyme involved in sialic acid synthesis, is mutated in recessive hereditary inclusion body myopathy (Eisenberg *et al.*, 2001). Such mutations likely reduce sialic acid content on myofibers. If so, they could be the *in vivo* equivalent of the desialylation studies done on cultured myotubes. Interestingly, one of the hallmarks of this and related disorders is the accumulation of synaptic proteins in inclusion bodies that are located in extra-synaptic regions of the myofiber (Askanas *et al.*, 1998). Thus these "synaptic" inclusions may reflect the increased formation of synaptic membrane in the absence of sialic acid, much as occurs in cells in culture (Martin and Sanes, 1995).

Though glycans may be important in agrin signaling at the neuromuscular junction, agrin is also expressed in almost every region of the brain. It is not clear whether agrin is important for the formation of brain synapses, but early studies of this subject suggest that it is not (Serpinskaya *et al.*, 1999; but see Ferreira, 1999). These studies, however, involve no electrophysiology and do not address agrin's role in synaptic function. Agrin, of course, could play other roles more typical of brain proteoglycans, such as directing axon migration or stimulating glial responses after injury. It is interesting to note in this regard that agrin is the predominant heparan sulfate proteoglycan found in plaques in Alzheimer's disease (Verbeek *et al.*, 1999), a disorder associated with profound synapse loss (Terry *et al.*, 1991). At least one agrin-binding protein, α dystroglycan, is concentrated in brain synapses (Smalheiser and Collins, 2000; Zaccaria *et al.*, 2001). α Dystroglycan has a mucin domain that is glycosylated with an unusual NeuAcα2,3Galβ1,4GlcNAcβ1,2Manα-O-Ser structure in brain (Smalheiser *et al.*, 1998), peripheral nerve (Chiba *et al.*, 1997), and skeletal muscle (Sasaki *et al.*, 1998). Some Lewis X structures also occur in brain (Smalheiser *et al.*, 1998). Though agrin is not likely to be present at many brain synapses, members of another protein family that share homology with agrin, the neurexins, also interact with brain-derived α dystroglycan via its carbohydrate moeties (Sugita *et al.*, 2001). Thus other proteins in the brain may utilize binding partners and perhaps mechanisms similar to those used by agrin at the neuromuscular junction.

Glycans in synaptic plasticity: PSA and others

An increasing number of studies have shown that NCAM, a protein often modified with PSA, has an important role in synaptic plasticity, both in the brain and at the neuromuscular junction. NCAM is required for long-term potentiation (LTP) of synaptic activity in the Schaffer collateral–CA1 and mossy fiber–CA3 regions of the hippocampus (Cremer *et al.*, 1994, 1998), long-term plasticity in *Aplysia* (Mayford *et al.*, 1992), and paired-pulse facilitation at the NMJ (Rafuse *et al.*, 2000). The extent to which PSA contributes to the phenotypes in NCAM-deficient mice is now becoming understood in some cases. PSA is highly expressed in the brain in the perinatal period at the time when most synapses are forming, but it declines markedly during postnatal development except in areas of postnatal neurogenesis (for review see Rutishauser

and Landmesser, 1996). Glycosylation of NCAM with PSA primarily results from the activity of two sialyltransferases, alpha 2,8 sialyltransferase (ST8) SiaII and ST8SiaIV, though ST8SiaIII also has some activity (Angata *et al.*, 2000). STSia8II (or STX) expression is high in embryonic development but diminishes rapidly in the early postnatal period, whereas ST8SiaIV (or PST-1) remains high in adult brain (Kurosawa *et al.*, 1997; Ong *et al.*, 1998).

The recent characterization of mice lacking ST8IV has shed much light on the extent to which PSA controls the effects of NCAM on synaptic plasticity (Eckhardt *et al.*, 2000b). Mice lacking ST8SiaIV show deficits in both LTP and long-term depression (LTD) in the Schaffer collateral–CA1 projections of the hippocampus (Eckhardt *et al.*, 2000b). This work has confirmed earlier studies where treatment of hippocampal slices with endo-neuraminidase inhibited both LTP and LTD in this pathway as well as spatial learning (Becker *et al.*, 1996; Muller *et al.*, 1996). Expression of PSA in neuronal migratory pathways and in early development was unchanged in ST8SiaIV –/– mice, presumably due to the continued expression of ST8SiaII. Because of this, no deficits in the laminar structure of the hippocampus occurred in these mice as had been found in NCAM-deficient mice. The deficit in plasticity appeared to be very specific, as other measures of synaptic transmission, including basal activity and posttetanic potentiation, were normal. In addition, LTP in ST8SiaIV –/– mice was normal in the mossy fiber–CA3 pathway, where PSA is not normally expressed (Seki and Rutishauser, 1998; Seki and Arai, 1999). As LTP is affected in this pathway in NCAM –/– mice, there may be examples where non-PSA-dependent mechanisms are responsible for NCAM function; however, such functions could also be due to altered development.

The exact role of PSA in the mechanism of LTP is still unclear, but several studies suggest a potential role in modulation of AMPA receptors (discussed in Eckhardt *et al.*, 2000b). This is intriguing, given that AMPA receptors are required for LTP. First, colominic acid, a homopolymer of sialic acid, can increase AMPA receptor channel open time (Suppiramaniam *et al.*, 1999). Second, desialylation of hippocampal membanes alters AMPA binding to its receptor (Hoffman *et al.*, 1997). Last, activity rapidly modulates NCAM/PSA and AMPA receptor expression on the cell surface (Muller *et al.*, 1996), and this rapid recycling may be essential for AMPA receptor control LTP (Isaac *et al.*, 1995; Luscher *et al.*, 1999). The finding that brain-derived neurotrophic factor can rescue the deficit in LTP in ST8SiaIV –/– hippocampal slices (Muller *et al.*, 2000), coupled with the fact that NCAM can bind proteoglycans via PSA (Storms and Rutishauser, 1998), also suggests a potential role for PSA in localizing trophic factors via GAGs at the synapse. Of course, because of its potential to destabilize cell–cell interactions, PSA could also serve as a more general "de-adhesive" to allow alterations in synaptic morphology or connectivity. Studies on estrogen-induced morphological changes in the arcuate nucleus (Hoyk *et al.*, 2001) and on environmentally induced plasticity in the hypothalamic-neurohypophysial system (Theodosis *et al.*, 1999) show that PSA is required for changes in synaptic morphology. By contrast, removal of PSA increases ectopic mossy fiber–pyramidal cell synapse formation in the hippocampus (Seki and Rutishauser, 1998). Last, although ST8SiaIV is mostly expressed in adulthood and its absence causes no apparent change in hippocampal structure, alterations in hippocampal development still cannot be ruled out as being the cause of the phenotype. Inducible knockout studies in adult mice will be required to rule out such effects.

Studies using carbohydrates, glycosidases, and anticarbohydrate antibodies suggest that other glycans, such as HNK-1 (Saghatelyan *et al.*, 2000), heparan sulfate (Lauri *et al.*, 1999), fucose (Krug *et al.*, 1994), and high mannose oligosaccharides (Luthi *et al.*, 1994) are essential for LTP. Experiments in the leech also suggest that mannose structures control presynaptic vesicle aggregation in sensory neurons (Tai and Zipser, 1998), and lectin experiments in *Aplysia* suggest that glycans may control the specificity of synaptic connectivity (Lin and Levitan, 1987). Both of these phenomena, like LTP, could pertain to issues of plasticity. Given the long litany of candidate protein mediators put forward as being important in LTP (Sanes and Lichtman, 1999b), one needs to treat the carbohydrate literature here with an equivalent degree of skepticism. Nevertheless, a good case is being made that heparan sulfate, separate from the PSA studies, has a role in hippocampal LTP. Addition of heparitinase (Lauri *et al.*, 1999), soluble heparin-type carbohydrates (Lauri *et al.*, 1999), soluble syndecan-3 (Lauri *et al.*, 1999), and soluble heparin-binding growth-associated molecule (HB-GAM) (Lauri *et al.*, 1998) all inhibit aspects of LTP in the Schaffer collateral–CA1 pathway. In addition, syndecan-3 can be co-purified from the hippocampus with c-fyn, a tyrosine kinase involved in hippocampal LTP and spacial learning (Grant *et al.*, 1992). Last, mice lacking HB-GAM have enhanced hippocampal LTP (Amet *et al.*, 2001). Thus, a body of literature is forming that makes a convincing case that one or more glycans modulate synaptic plasticity in at least some regions of the brain. The creation of mice lacking enzymes involved in GAG synthesis should aid in differentiating the role of proteoglycans from direct roles of GAGs, as has occurred for PSA.

Conclusions

Although the glycobiology of the synapse is still in its infancy, the modulation of agrin signaling by glycans during neuromuscular development and the effect of PSA on hippocampal LTP suggest that glycans will have important roles in synapse formation and synaptic plasticity. In addition, the presence of high mannose at glutaminergic synapses suggests an important role at excitatory synapses in the brain. The combination of improved reagents for detecting and sequencing glycans, coupled with forward and reverse genetic approaches in mice, should allow a more complete characterization of the role of glycans in this very important aspect of neurobiology.

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Abbreviations

AChR, acetylcholine receptor; AMPA, α-amino-3-hydroxy-5-methyl-4isoxazole-proprionic acid; ConA, Concanavalin A; CT,

cytotoxic T cell; GAG, glycosoaminoglycan; GalNAc, N-acetylgalactosamine; HB-GAM, heparin-binding growth-associated molecule; LTD, long-term depression; LTP, long-term potentiation; MuSK, muscle-specific kinase; NCAM, neural cell adhesion molecule; NMDA, N-methyl-D-aspartate; NMJ, neuromuscular junction; PSA, polysialic acid; ST8, alpha 2,8 sialyltransferase.

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