CHAPTER 4

PERSPECTIVE

Brain derived neurotrophic factor (BDNF), the most abundant of the neurotrophins in the nervous system, is recognized as playing an important role in the survival, differentiation and outgrowth of select peripheral and central neurons during development and in adulthood. BDNF is also involved in activity-dependent synaptic plasticity and cognitive functions. As the mutation in the prodomain of BDNF causes dysfunction of BDNF transport and release, which subsequently leads to a number of neurological and psychological disorders such as anxiety and depression disorders, the understanding of how BDNF is transported is critical for understanding brain function in relation to human neurological and psychological diseases. This thesis has identified the current knowledge gap in which we do not know how BDNF-containing vesicles are carried anterogradely and retrogradely along axons. From previous studies, BDNF vesicular transport is found to be altered in HD. The down-regulation of BDNF transport from cortical neurons to striatal neurons results in the degeneration of striatal neurons. This effect on BDNF plays an essential role in the pathogenesis of HD (Altar et al., 1997; Gauthier et al., 2004; Zuccato et al., 2001). It is already known that the scaffold protein Htt which binds HAP1 is involved in axonal transport (Li and Li, 2005). Down-regulation of molecular motor complex accessory molecules including p150Glued and HAP1 reduces the transport of BDNF. The presence of mutant Htt interrupts BDNF transport, whereas wild type Htt could act as an antiapoptotic factor by rescuing this axonal transport (Ferrer et al., 2000; Gauthier et al., 2004; Zuccato et al., 2001). However, these studies leave a

number of questions to be answered. For example, as the Gauthier' study uses GFP protein as a reporter which is fused to the C-terminal domain of BDNF, it is not known whether the transported molecule is the full-length BDNF or cleaved mature BDNF. It is also not known which molecular motor accessory proteins BDNF binds to and how BDNF/BDNF-containing vesicles are trafficked within axons and dendrites. The mechanism of how those molecules interact with each other and then control BDNF transport remains to be investigated.

In the present study, we have made significant progress in understanding how BDNF is transported by the following findings. We first find that both mature BDNF and the prodomain bind HAP1, which is known to interact with kinesin light chain (McGuire et al. 2006; Rong et al. 2006) and the dynactin subunit p150Glued (Engelender et al. 1997) using an affinity purification and proteomic approach. The presence of HAP1 in the binding complex was further confirmed by Western blot, suggesting HAP1-A binds to mature BDNF and HAP1-B mainly binds to the prodomain. As HAP1 is involved in both anterograde and retrograde transport by kinesin and dynein motors, this finding would strongly suggest a role of HAP1 in BDNF transport. The direct GST pull-down assays using recombinant proteins showed that mature BDNF does not directly interact with HAP1 but the prodomain and full-length proBDNF do directly interact with HAP1. The biochemical data indicates HAP1 may act as an accessory molecule carrying BDNF along microtubules. The cotransfection

assays with both HAP1 and prodomain plasmids or mature BDNF plasmids showed that HAP1 is almost completely colocalized with prodomain and proBDNF but only partially colocalized with mature BDNF. The partial colocalization of HAP1 and mature BDNF was abolished by the presence of mature BDNF antibodies, indicating that HAP1 is colocalized with internalized mature BDNF. HAP1 is almost completely colocalized with labelled exogenous BDNF and the colocalization was abolished by the antibody to p75NTR or with 100x concentrations of unlablled BDNF. Furthermore, the internalization of labelled BDNF into the cortical neurons was abolished by the deletion of HAP1. The specific interaction between HAP1 and the prodomain of BDNF was further supported by the data from sucrose-gradient fractionation assays of brain homogenates from wild type and $HAP1^{-/-}$ mice. In the wild type mice brain homogenates, proBDNF is present in nuclei, synaptic vesicles and cytoplasm. The distribution pattern was altered in $HAP1^{-/-}$ mouse brain homogenates where no proBDNF was detected in the vesicle-enriched and synaptosome-enriched fraction (LP2), but proBDNF was increased in P1, S1 and P3 fractions. The data suggest that HAP1 is required for the transport of BDNF into synaptic vesicles. Indeed, the axonal transport of BDNF in the crushed sciatic nerve and spinal cord of $HAP1^{-/-}$ mice was completely abolished but the overall expression of BDNF in $HAP1^{-/-}$ mice did not change. From these data we conclude that HAP1 directly interacts with the prodomain of BDNF and plays an essential role in the anterograde transport of BDNF. We can also draw the conclusion that HAP1 plays a role in the internalization and

retrograde transport of mature BDNF by interacting with other unknown mechanisms.

This thesis has also defined how mutant Htt may play a role of in the axonal transport of BDNF. We have investigated roles of mutant Htt in the interaction between HAP1 and proBDNF and prodomain using three different approaches, First we used the PC12 cell lysate containing mutant polyQ Htt and examined the binding capacity of proBDNF and prodomain with HAP1 by GST pull-down assay. The results clearly demonstrated that mutant Htt significantly reduced the binding of proBDNF and the prodomain to HAP1. The data also suggest that mutant Htt may interfere with the axonal transport of BDNF by competitively binding to HAP1 or disrupting the interaction between HAP1 and proBDNF/prodomain. In the second approach, we used brain homogenates from both normal control and Huntington's disease patients to examine levels of proBDNF in the immunoprecipitated complex. HAP1 antibody was used to immunoprecipitate equal amount of samples from HD and control brains. In the immunoprecipitated samples, we did not detect any mature BDNF but did detect proBDNF, sortilin and possibly prodomain oligomers. We found that the levels of proBDNF and sortilin were significantly reduced in HD brain compared to the control brain. These data indicate that the mutant Htt in the HD brain may interfere with HAP1 and proBDNF thus reducing the binding of the complex. In the third approach, we carried out the GST-pull down assay of HAP1:proBDNF (prodomain) with the incubation mixture in the presence of

HD brain homogenate or control brain homogenate. The levels of proBDNF and prodomain pulled down in the HD brain homogenate were the lowest among all assays, indicating the mutant Htt and/or other proteins in the HD brain can interrupt the interactions between HAP1 and proBDNF. The data from these three different assays support the conclusion that mutant Htt may interfere with the binding of proBDNF to HAP1 and thus reduce the axonal transport of proBDNF carried by HAP1.

The V66M mutation in the prodomain of BDNF causes retardation of BDNF transport and release (Egan et al. 2003; Numata et al. 2006). This thesis tried to see whether the reduction of transport and release of BDNF by the mutation is due a reduced interaction of the prodomain with HAP1. We have examined the interactions of the mutant prodomain in different conditions: 1) in a normal condition in which the level of pull-down by GST-HAP1 is compared with the wild type prodomain; 2) the pull-down levels of mutant prodomain were examined in the presence of PC12 cell lysate containing polyQ Htt or normal Htt; 3) the pull-down levels of mutant prodomain were examined in the presence of brain homogenate of HD and controls. We found that at the basal level, the V66M mutation reduced the pull-down level by 60%, indicating that V66M mutant prodomain has less binding affinity to HAP1 than that of wild type prodomain. Mutant polyQ Htt in the PC12 cell lysates and in the HD brain further reduced the pull-down level of V66M prodomain by 19 % and 13% respectively. These data suggest that the reduction in axonal transport and

release of V66M allele is likely due to the reduced interaction between HAP1 and the prodomain.

In this thesis, the role of HAP1 in the release of proBDNF was also examined. To examine basal release and activity-dependant release of proBDNF, we cultured cortical neurons from P1 HAP1$^{-/-}$ mice and wild type mice. We used two approaches: release of proBDNF from the natively cultured neurons and release of proBDNF from plasmid-transfected cultured neurons. We found there was no significant difference in the basal release of proBDNF from HAP1$^{-/-}$ and from wild type mice. However, the stimulated release of proBDNF from HAP1$^{-/-}$ mice cortical neurons was abolished. The release of proBDNF from plasmid-transfected cortical neurons was also abolished in HAP1$^{-/-}$ mice. This data demonstrated that HAP1 is an essential molecule required for activity-dependant release of proBDNF.

Our current study has provided evidence for the mechanism of how BDNF is transported and released. In summary, the molecular motor accessory protein HAP1 directly interacts with the BDNF prodomain and indirectly with the mature BDNF and plays an essential role in the anterograde and retrograde transport and activity-dependent release of BDNF. However, the mature BDNF transport mechanism awaits further investigation. To answer the question of how mature BDNF indirectly binds to HAP1, we need to study whether HAP1 interacts with the BDNF receptor (TrkB or p75NTR or sortilin) or endosome

components. We could prepare the endosome membrane proteins first and then repeat the affinity purification and 2D-gel technique to collect mature BDNF binding protein. In this way, we may find out the binding proteins of mature BDNF on the endosome membrane and solve the problem of how mature BDNF interacts with HAP1. Furthermore, it is worth investigating the identity of other proteins affinity-purified from the mature BDNF and prodomain BDNF columns. Those proteins could be subunits of dynein/kinesin complex. The understanding of all parts of transport vehicles will help to elucidate the full picture of transport mechanism.

On the other hand, the research on proBDNF transport is not finished and many questions remain to be addressed. For example, what epitopes of BDNF interact with HAP1 and vice versa? How is proBDNF released from the HAP1/proBDNF complex? What is the relationship between HAP1 and sortilin? Does the HAP1/proBDNF complex interact with p150Glued and/or kinesin light chain? Mapping the prodomain BDNF-HAP1 interaction is an important issue. The HAP1 GST-pull down assays suggest that the middle portion of HAP1 (amino acids 280-445) is the domain interacting with prodomain BDNF (amino acids 1-141). The prodomain BDNF can be divided into different “boxes” to test the interaction domain, such as Box1 (amino acids 19-43), Box2 (amino acids 44-75), Box3 (amino acids 76-102) and Box4 (amino acids 103- 141) which are based on the mapping of BDNF-sortilin interaction (Chen et al., 2005). We also could search for a three-dimensional

structure of prodomain BDNF, HAP1 and prodomain BDNF/HAP1 complex using high-resolution X-ray crystallographic structure techniques to reveal a putative binding model. After the identification of the interacting epitope in HAP1, we can test roles of appropriate point mutations of HAP1 in the axonal transport of proBDNF. After this, the mechanism of how PolyQ Htt interrupts the binding of prodomain with HAP1 needs to be further studied. Our GST-pull down assays suggest that PolyQ Htt decreases the binding of prodomain BDNF with HAP1. It is possible that the PolyQ Htt has much higher binding affinity to HAP1 and the PolyQ expansion blocks the binding site of prodomain BDNF. Besides this, how prodomain BDNF dissociates with HAP1 after transport to the nerve terminal and is released from nerve terminals or dendrites is also an interesting issue. BDNF is released via constitutive and regulated pathways depending on whether the release occurs spontaneously or in response to stimuli. Whether depolarization or increased intracellular calcium levels will change the structure of HAP1 or prodomain BDNF and then change their binding affinity is not known. Alteration in pH or interaction with other proteins in releasing vesicles in response to stimuli may also alter the binding affinity of the prodomain to HAP1.