Universität Hamburg

Neural cell adhesion molecule NCAM modulates dopamine-related behavior by regulating dopamine D2 receptor internalization in mice (*Mus musculus* Linnaeus, 1758)

DISSERTATION

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ABSTRACT

Dopaminergic transmission plays a critical role in the regulation of locomotion, cognition, reward and emotional behavior, and endocrine secretion. Responses to dopamine are controlled by a family of G protein-coupled proteins, which are classified to D1-like (D1R and D5R) and D2-like (D2R, D3R and D4R) subfamilies. Increased activity of D2 receptor signaling is believed to play an important role in the pathogenesis of schizophrenia, and most antipsychotic drugs exert their functions by blocking D2 receptors. The signaling strength mediated by D2 receptor is extensively regulated by multiple processes, and endocytosis is a major mechanism of D2 receptor signal attenuation. However, the specific molecular mechanisms which modulate D2 receptor endocytosis have remained poorly understood.

Neural cell adhesion molecule (NCAM), belonging to immunoglobulin superfamily, is a glycoprotein highly expressed and first discovered in the nervous system. Increasing evidence indicates that NCAM is related to psychiatric disorders, such as schizophrenia and bipolar disorders. Since both NCAM and D2 receptor are associated with psychiatric disorders, we become interested in a potential functional relationship between NCAM and D2 receptor.

Here, we identified NCAM as a novel binding partner to D2 receptor. Co-immunoprecipitation revealed that NCAM could form complex with D2 receptor in physiological context, furthermore, the NCAM/D2R interaction was enhanced upon dopamine stimulation. Direct interaction was determined by pull down assay with recombinant NCAM and D2 receptor. Moreover, we clarified that the NCAM/D2R interaction was mediated via a short sequence stretch in the third intracellular loop of D2 receptor and the membrane-proximal part of the NCAM intracellular domain.

To investigate the functional effect of NCAM/D2R interaction, subcellular distribution of D2 receptor in NCAM deficient mouse brains was analyzed. NCAM deficiency in mice led to increased D2 receptors at the plasma membrane and reduced

D2 receptors in endosomes and lysosomes with unaltered total D2 receptor expression, indicating NCAM might be involved in the internalization process of D2 receptor. *In vitro* internalization assays and cell surface biotinylation experiment confirmed that NCAM promoted D2 receptor internalization/degradation and reduced cell surface localization of D2 receptor after dopamine stimulation. Furthermore, by *in vivo* pharmacological approach we could show that NCAM deficiency in mice resulted in excessive D2R-mediated signaling and exaggerated activity of dopamine-related behavior, which probably resulted from impaired internalization and increased levels of D2 receptors at the cell surface.

These results demonstrate that, besides its classical function in cell adhesion, NCAM is involved in regulating the trafficking of neurotransmitter D2 receptor and receptor-related behaviors, thus implicating NCAM as an unexpected modulator for dopaminergic system and a potential pharmacological target for dopamine-related neurological and psychiatric disorders.

I. INTRODUCTION

I.1. Neural cell adhesion molecule (NCAM)

Cell adhesion molecules (CAMs) contribute to the establishment of various neuron-neuron and neuron-glial cell interactions from the embryonic age until adulthood, thus play important roles in neurogenesis, neuronal migration, neuritogenesis, neurite outgrowth, axon pathfinding and fasciculation, synaptogenesis and myelination (Rutishauser and Jessell, 1988; Doherty et al., 1990; Doherty et al., 1992; Doherty and Walsh, 1992; Jørgensen, 1995; Fields and Itoh, 1996; Cremer et al., 1997; Schachner, 1997).

One group of cell adhesion molecules is termed as immunoglobulin superfamily, which is characterized by the presence of several immunoglobulin (Ig)-like domains. The Ig superfamily includes several subfamilies, for example: neural cell adhesion molecule (NCAM); the L1 family, such as L1, CHL-1 (Holm et al., 1996), neurofascin (Rathjen et al., 1987); the DCC (deleted in colon cancer) family (Fearon et al., 1990); myelin-associated glycoprotein MAG (Sutcliffe et al., 1983) and P0 (Lemke et al., 1988); GPI-linked cell adhesion molecules including transient axonal glycoprotein TAG-1 (Dodd et al., 1988) and contactin/F3 (Ranscht 1988; Gennarini et al., 1989); molecules contains enzymatic cytoplasmic domains, such as FGF receptor.

I.1.1. Structure and expression of neural cell adhesion molecule

NCAM was the first characterized member of Ig-like adhesion molecule superfamily (Jørgensen and Bock, 1974; Rutishauser et al., 1976). NCAM is encoded by a single copy gene, which is located on chromosome 9 in mice (D'Eustachio et al., 1985) and chromosome 11 in human (Nguyen et al., 1986). In mice NCAM gene contains 26 exons.

It is reported that at least 20-30 distinct isoforms can be generated by alternative splicing and posttranslational modification. There are three major isoforms, termed

NCAM180, NCAM140 and NCAM120 on the basis of their apparent molecular weight (Goridis et al., 1983). NCAM140 and NCAM180 are transmembrane proteins, while NCAM120 is attached to the plasma membrane via a glycophosphatidyl inositol (GPI) linkage (Doherty et al., 1995). NCAM180 differs from NCAM140 by the presence of additional 261 amino acids in the cytoplasmic domain, which is encoded by exon18. The extracellular domains of these isoforms are identical, containing five Ig-like domains and two fibronectin type III homologous (FNIII) repeats (Cunningham et al., 1987) (Fig.I.1). In addition, there are soluble forms of NCAM, which are generated by truncation, proteolysis or shedding (Bock et al., 1987; Olsen et al., 1993). Interestingly, Poltorak and colleagues reported that there is elevation of soluble NCAM in the cerebrospinal fluid in schizophrenia patients (Poltorak et al., 1996).
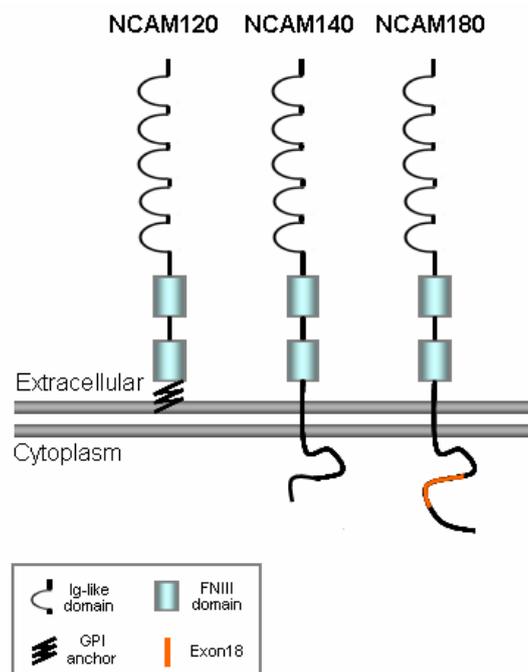
Figure I.1. Structure of NCAM

NCAM120, 140 and 180 are three major isoforms of NCAM. NCAM120 is attached to the plasma membrane via a glycophosphatidyl inositol (GPI) anchor. NCAM140 and NCAM 180 are transmembrane proteins. NCAM180 differs from NCAM140 by the presence of additional 261 amino acids in the cytoplasmic domain, which is encoded by exon18. The extracellular part of NCAM contains five immunoglobulin (Ig)-like domains and two fibronectin type III homologous (FNIII) repeats.

One important characteristics of NCAM is that all NCAM isoforms can carry an unusual carbohydrate, polysialic acid (PSA), which has been found on NCAM and another recognition molecule neuropholin-2 (Rutishauser and Landmesser, 1996; Kiss and Rougon, 1997; Muhlenhoff et al., 1998; Curreli et al., 2007). PSA is a large carbohydrate homopolymer of α-2, 8-linked polysialic acid, which can contain up to 100 residues of sialic acid. The unique structure of this carbohydrate can be recognized by monoclonal antibodies and by a phage-derived endoneuraminidase (endo-N). PSA is attached to the fifth Ig domain of NCAM. This attachment requires the adjacent fourth Ig domain and the first FNIII domain (Nelson et al., 1995). Polysialylation of NCAM is regulated developmentally, decreasing during late embryonic ages and in adulthood. Because of its highly negative charge and large hydration volume, the existence of α-2, 8-linked polysialic acid on NCAM may decrease not only homophilic NCAM interaction, but also heterophilic interactions of NCAM to other molecules. PSA modifies functional properties of the NCAM protein backbone during neural migration, axon targeting and synaptic plasticity (Eckhardt et al., 2000; Angata et al., 2004; Weinhold et al., 2005).

NCAM is expressed during neural tube closure and persists into adulthood in both neurons and glia in nervous system (Edelman, 1985). Three major isoforms show different expression patterns in the nervous system. NCAM120 is predominantly expressed by glial cells and is not detectable in synaptosomal membranes, while the NCAM180 and NCAM140 are primarily expressed by neurons (Schachner, 1997). NCAM140 is localized to migratory growth cones and axon shafts of developing neurons and promotes neurite outgrowth, whereas NCAM180 is enriched in postsynaptic densities of mature neurons and is responsible for the stabilization of cell-cell contacts (Pollerberg et al., 1987; Persohn et al., 1989; Schuster et al., 2001). The different localization contributes to different functions of NCAM isoforms. Besides, NCAM is also expressed in non-neuronal tissues, like muscle (Sanes et al., 1986), heart (Burroughs et al., 1991), pancreas and gonad (Moller et al., 1991).

I.1.2. Homophilic and heterophilic interaction of NCAM

NCAM exerts its function via homophilic interactions in "*cis*" on the same cell and in "*trans*" on different cells or via heterophilic interaction. The mechanism of NCAM homophilic binding is still controversial. Studies with antibodies, peptides and recombinant Ig domains indicate that all five Ig modules are involved in NCAM homophilic binding, particularly, the third Ig module plays an essential role (Zhou et al., 1993; Rao et al., 1994). In contrast, nuclear magnetic resonance (NMR) and other biophysical studies suggest NCAM homophilic binding only takes place between Ig I and Ig II modules (Kiselyov et al., 1997; Atkins et al., 1999; Jensen et al., 1999).

Besides homophilic interaction, increasing evidence show that NCAM has numerous heterophilic binding partners, such as fibroblast growth factor receptor (FGFR) (Williams et al., 1994), glial cell line-derived neurotropic factor (GDNF) (Paratcha et al., 2003), the L1 cell adhesion molecule (Horstkorte et al., 1993), TAG-1 (Milev et al., 1996), prion protein (Schmitt-Ulms et al., 2001) and a variety of extracellular matrix components including various proteoglycans (Cole et al., 1985; Friedlander et al., 1994; Milev et al., 1994).

All molecules mentioned above interact with the extracellular domain of NCAM, on another hand, the intracellular region of NCAM also associates with certain proteins. NCAM180 specific sequence in intracellular domain can interact with cytoskeleton proteins spectrin to stabilize intracellular organelles at nascent synapses (Pollerberg et al., 1986; Sytnyk et al., 2005). Interaction of NCAM to signaling transduction proteins tyrosine kinase p59[Fyn] leads to the downstream signal transduction of NCAM (Beggs et al., 1994). Büttner and his colleagues discovered phopholipase C gamma, LANP, TOAD-64 (turned on after division molecule), syndapin, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) as novel cytosolic binding partners of NCAM by ligand affinity chromatography and glutathione S-transferase (GST) pull down assays (Büttner et al., 2005). In addition, our lab also identified several novel binding partners to the intracellular domain of NCAM, including receptor protein tyrosine kinase TrkB, inwardly rectifying K+ channel subunit Kir3.3 (dissertation of Cassens C, 2008) and adaptor protein AP2 (dissertation of Shetty A, 2008)

.I.1.3. NCAM mediated signal transduction pathways

There are two major signaling pathways mediated by NCAM: FGFR-independent pathway and FGFR-dependent pathway (Fig.I.2).
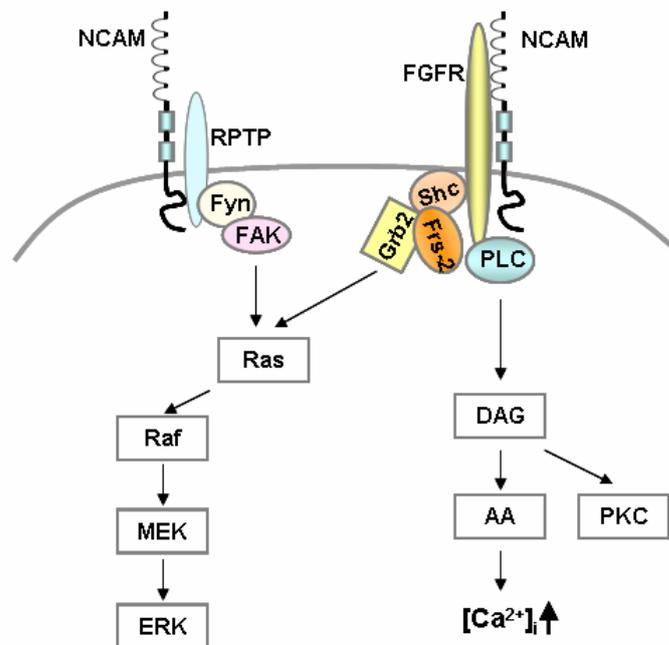
Figure I.2. NCAM-mediated signal transduction pathways.

NCAM mediates signaling transduction via the tyrosine kinase Fyn or direct interaction with the fibroblast growth factor receptor (FGFR). Activation of Fyn mediated by the interaction between NCAM and RPTPα leads to stimulation of FAK, followed by the activation of Ras/Raf/MEK/ERK signaling pathway and thereby modulation of protein transcription and/or changes in cytoskeletal dynamics. NCAM interacts with FGFR and leads to the activation of phospholipase C (PLC), protein kinase C (PKC), changes in the intracellular calcium concentration and stimulation of further downstream targets.

NCAM is known to initiate intracellular signals by direct interaction with and activation of intracellular signaling molecules apart from the interaction with FGFR. It is shown that NCAM can promote neurite outgrowth via the nonreceptor tyrosine kinase Fyn pathway (Beggs et al., 1994; Beggs et al., 1997; Kramer et al., 1999). Activation of Fyn, mediated by the interaction between NCAM and receptor protein tyrosine phosphatase (RPTPα) (Bodrikov et al., 2005), leads to the activation of another nonreceptor tyrosine kinase focal adhesion kinase (FAK), thus activating the

Ras/Raf/MEK/ERK signaling pathway, thereby modulating protein transcription and/or changes in cytoskeletal dynamics and promoting neurite outgrowth (Kolkova et al., 2000; Walmod et al., 2004).

Another pathway is involved in fibroblast growth factor receptor (FGFR). NCAM interacts with FGFR via the acid box motif (Sanchez-Heras et al., 2006), which leads to the activation of phospholipase Cγ (PLCγ)/diacylglycerol (DAG)/arachidonic acid (AA) pathway, protein kinase C (PKC), changes in the intracellular calcium concentration and stimulation of further downstream targets (Hinsby et al., 2004). It was demonstrated that NCAM-mediated FGFR signaling pathway may also link to the Ras/Raf/MEK/ERK signal transduction via adaptor proteins Grb2, docking protein ShcA and FGFR substrate-2 (Frs-2) (Downward, 1996; Hinsby et al., 2004). Thus, the main two pathways mediated by NCAM are not independent and they have cross-talk in between.

It has been show that NCAM appears to be present in lipid raft, detergent-resistant microdomains of the plasma membrane, which plays an important role in the regulation of NCAM signaling. There is indication that lipid raft is essential for NCAM-mediated Fyn/FAK activation. NCAM-mediated Fyn activation is blocked by disruption of raft structures or by exclusion of NCAM from lipid rafts. In contrast, raft-excluded NCAM signals mainly via FGFR (Niethammer et al., 2002).

I.1.4. NCAM deficient mice

Mice with a constitutive disruption of the NCAM gene show morphological changes, including the reduction in the size of the olfactory bulbs and disturbed lamination of mossy fibers in the CA3 region of hippocampus. Long term potentiation has been reported to be reduced in NCAM deficient mice in CA1 (Muller et al., 1996) and CA3 (Cremer et al., 1998). In addition, NCAM deficiency in mice leads to impaired spatial learning and memory (Cremer et al., 1994; 1997) and abnormal synaptic vesicle release at the neuromuscular junction (Polo-Parada et al., 2001; 2004). NCAM deficient mice also show increased aggressive behavior of males toward an unfamiliar

male intruding into their home cage, correlating with an increase in activation of limbic system areas when compared with their wild-type control littermates (Stork et al., 1997). Furthermore, NCAM deficient mice are more anxious and hypersensitive to serotonin 1A receptor agonists (Stork et al., 1999). Recently, Aonurm-Helm discovered that NCAM deficient mice also demonstrates a depression-like phenotype with increased freezing time in the tail-suspension test and reduced preference for sucrose consumption in the sucrose preference test (Aonurm-Helm et al., 2008).

Tomasiewicz and colleagues established another NCAM knockout mice strain, NCAM180 knockout mice, which were designed to knockout only the 180-kDa isoform (Tomasiewicz et al., 1993). It was observed that in the neonatal brain and the adult olfactory bulb other isoforms were expressed except NCAM180, however, in the adult brain both NCAM140 and NCAM180 isoforms were not present. This mouse strain shows a decrease in the size of the olfactory bulb due to impaired cell migration from the subependymal zone to final positions in the olfactory bulb (Tomasiewicz et al., 1993). NCAM180 knockout mice also show anterior ventricle enlargement, hippocampal dentate gyrus thinning, learning deficits in Morris water maze, and impairment in prepulse inhibition of startle (PPI), which is one characteristic of schizophrenia patients (Wood et al., 1998).

I.2. Dopamine D2 receptor

Dopamine (DA), discovered by Arvid Carlsson in 1960, is the predominant catecholamine neurotransmitter in the mammalian brain, where it is involved in the regulation of locomotion, cognition, reward and emotional behavior, and endocrine secretion (Zhou and Palmiter, 1995; Carlsson, 2001). Aberrant dopamine receptor signaling has been implicated in several neurological and psychiatric diseases, such as schizophrenia, Parkinson's disease, depression and drug abuse (Zhou and Palmiter, 1995; Carlsson, 2001; Greengard, 2001; Nestler, 2001).
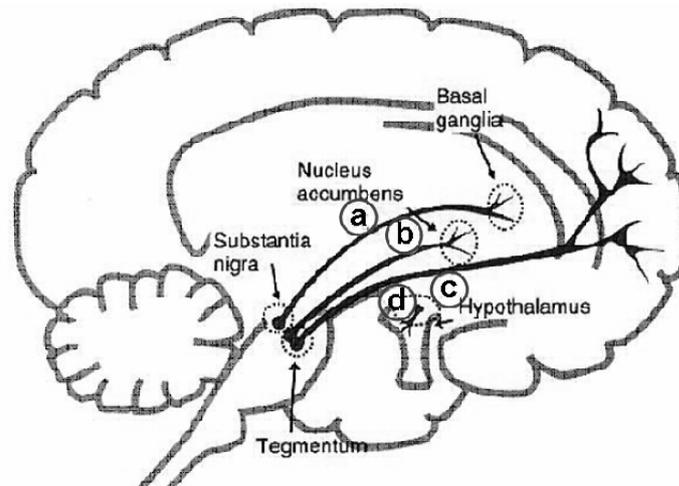
Figure I.3. Dopaminergic pathways

Four major dopamine tracts in brains: (a) nigrostriatal: from substantia nigra to the putamen and caudate nucleus; (b) mesolimbic: from ventral tegmental area to limbic system; (c) mesocortical: from ventral tegmental area to the neocortex; (d) tuberoinfundibular: from arcuate nucleus of hypothalamus to the pituitary stalk.

Dopaminergic neurons are well dispersed among four major tracts in the brain (Fig.I.3) (Iversen and Iversen, 2007). (a) Nigrostriatal tract: it begins in the substantia nigra and extends to the putamen and caudate nucleus. It is involved in the fine tuning of movement. The breakdown of neurons in this pathway is associated with the tremors, rigidity, bent posture, and slowness of movement characteristic of Parkinson's disease. (b) Mesolimbic tract: it begins in the ventral tegmental area and projects to the limbic system. (c) Mesocortical tract: it also stems from the ventral tegmental area, but then projects to the neocortex, mostly to the prefrontal areas of the neocortex. These two pathways are involved in motivated and emotional behavior, and organized thought processes. Excess dopaminergic transmission within these two tracts is associated with disordered thought and inappropriate emotional behavior characteristic of schizophrenia. (d) Tuberoinfundibular tract: these neurons have cell bodies in the arcurate nucleus of the hypothalamus and extend to the pituitary stalk. These cells are involved in the control of hormones secretion.

I.2.1. Structure and expression of dopamine D2 receptor

Dopamine exerts its effect through G protein-coupled dopamine receptor families, which are classified to two subfamilies, D1-like receptor subfamily (D1R, D5R) and D2-like subfamily (D2, D3 and D4) based on functional and pharmacological properties (Spano et al., 1978; Kebabian and Calne, 1979). D1Rs activate adenylate cyclase (AC) via coupling to G_s/G_{olf}, while D2 receptors are linked to $G_{i/o}$ to release $G_{\alpha i/o}$ and $G_{\beta/\gamma}$ subunits to inhibit adenylate cyclase to prevent the production of cAMP and the activation of protein kinase A (PKA) (Seeman and Van Tol, 1994; Missale et al., 1998). D2 receptor shares the common molecular structure with other GPCRs containing a hydrophobic core of seven transmembrane-spanning helices, three intracellular loops, three extracellular loops, and N-terminus outside the cell, and a C-terminus inside the cell.

D2 receptor is widely expressed in the central nervous system, and is preferentially located in the substantia nigra, striatum (which includes the nucleus accumbens shell and core and the dorsal striatum), hippocampus, cortex, ventral tegmental area and olfactory bulb and the pituitary gland (Missale et al., 1998). D2 receptor gene starts expression on E14, by E18 abundant D2 receptor are observed in cell groups similar to adult brain (Schambra et al., 1994). Peak expression of D2 receptor gene occurs on P15, when most dopaminergic synapses are established and become functional, then expression declined slightly (Rani and Kanungo, 2006).

Two isoforms of D2 receptor: long isoform (D2L) and short isoform (D2S) (Dal Toso et al., 1989; Giros et al., 1989) are generated by alternative splicing. D2L differs from D2S by the insertion of 29 amino acids in the third cytoplasmic loop, which is absent in the short isoform. D2S is the likely dopamine autoreceptor which is present in presynaptic bouton and regulate release of dopamine and other neurotransmitters, whereas D2L is primarily a postsynaptic receptor which exerts its functions via intracellular signaling cascade at postsynaptic spines (Khan et al., 1998).

I.2.2. Dopamine D2 receptor-mediated signal transduction pathways

The intracellular signaling of D2 receptor can be mediated via G protein-dependent pathway and G protein-independent pathway.

Binding of dopamine to the D2 receptor leads to the activation of $G_{\alpha i/o}$ protein coupled to its third cytoplasmic loop, which inhibits adenylate cyclase and thereby the activation of protein kinase A. Park and colleagues demonstrate that the interaction of proapoptotic protein Par-4 (prostate apoptosis response 4) and D2 receptor within the third intracellular loop is essential for $G_{\alpha i/o}$–mediated inhibition of cAMP activity (Park et al., 2005). $G_{\beta/\gamma}$ subunit of G protein can also act at a number of intracellular targets, such as ion channels and MAP kinase system upon D2 receptor activation (Choi et al., 1999; Neve et al., 2004) (Fig.I.4).
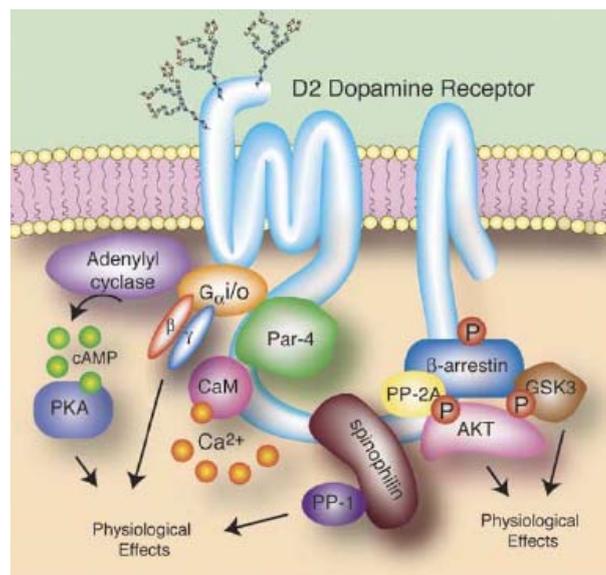
Figure I.4. D2 receptor-mediated signaling pathways

D2 receptor mediates signaling transduction mainly via G protein and arrestin2-PP2A-AKT complex. The activation of $G_{\alpha i/o}$ results in inhibition of adenylate cyclase and activation of protein kinase A. Additionally, in response to D2 receptor activation, arrestin2-PP2A-AKT signaling complex can be formed, which leads to inactivation of AKT. This figure adapted from Bibb (2005).

There are other pathways which are modulated by G protein-independent signaling mechanisms. Beaulieu and colleagues reveal that D2 receptor can mediate protein kinase B (Akt) signaling pathway independently of coupled G protein. Between D2 receptor and Akt, β-arrestin 2 is served as the biochemical connection. The activation of D2 receptor induces the formation of a signaling complex involving β-arrestin 2, Akt and protein phosphatase-2A (PP2A) that mediates the effects of dopaminergic transmission (Beaulieu et al., 2004; 2005). In addition, D2 receptor also can bind to spinophilin to link to protein phosphatase-1 (Smith et al., 1999).

Two isoforms of D2 receptor, D2S and D2L, show distinct signaling effect in presynapse and postsynapse respectively. The D2S receptor specifically regulates the state of phosphorylation and activity of tyrosine hydroxylase (TH) in presynaptic terminals, whereas the D2L receptors are mainly involved in the regulation of DARPP32 (dopamine and cAMP-regulated phosphoprotein with molecular weight 32 kDa) phosphorylation in postsynaptic neurons (Lindgren et al., 2003). Interestingly, analysis of postmortem tissues from schizophrenia patients revealed a decrease of DARPP32 in layer II of prefrontal cortex in the patients (Albert et al., 2002).

I.2.3. Dopamine D2 receptor deficient mice and overexpressing mice

There are several lines of D2 receptor knockout mice have been generated (Baik et al., 1995; Kelly et al., 1997; Jung et al., 1999). D2 receptor knockout mice exhibit pituitary lactotroph hyperplasia and chronic hyperprolactinemia (Kelly et al., 1997), significantly lower level of locomotor activity and Parkinsonian-like locomotor impairment (Baik et al., 1995; Kelly et al., 1998). D2 receptor knockout mice fail to show amphetamine-induced disruption of prepulse inhibition of startle (PPI) indicating D2 receptor plays an essential role in amphetamine-induced sensorimotor gating deficits (Ralph et al., 1999). Reward-related responses to a number of abuse drugs are abnormal in D2 receptor knockout mice, such as ethanol (Phillips et al., 1998), morphine (Elmer et al., 2002), opiate (Dockstader et al., 2001) and cocaine (Chausmer and Katz, 2001; Chausmer et al., 2002). It is known that short isoform of

D2 receptor functions as an autoreceptor in presynaptic terminals (Benoit-Marand et al., 2001), thus, the ability of dopamine to inhibit the firing of neurons and dopamine release is lost in D2 receptor knockout mice (Mercuri et al., 1997). However, despite the loss of this function, neither synaptic dopamine level nor tissue dopamine content is significantly changed in D2 receptor knockout mice (Rouge-Pont et al., 2002).

It is indicated that increased activity of D2 receptors in the striatum has been linked to the pathophysiology of schizophrenia (Wong et al., 1986; Laruelle et al., 1998). Kellendonk and colleagues generate transgenic mice which selectively overexpress D2 receptors in the striatum to investigate the direct consequence of increased D2 receptor function in the striatum (Kellendonk et al., 2006). Overall behavior of the mutant mice is normal with unaltered locomotor activity, sensorimotor gating and generalized anxiety. However, the mice show deficits in prefrontal-dependent processes, such as working memory and behavioral flexibility, and conditional associative learning (Drew et al., 2007; Bach et al., 2008).

I.3. Trafficking of G protein-coupled receptors (GPCRs)

G protein-coupled receptor is one of the largest cell surface receptor families which mediate intracellular signaling in response to numerous hormones and neurotransmitters through coupling to heterotrimeric G-proteins (Dohlman et al., 1991).

The magnitude of receptor signaling is precisely dictated by the level of receptor available at the plasma membrane, which is primarily determined by the balance of three processes: 1) secretory pathway by which newly synthesized receptors are transported from endoplasmic reticulum through Golgi-apparatus to cell surface by secretory vesicles; 2) endocytosis which allows the internalization of receptors upon stimulation by their ligands; 3) recycling pathway which recycles internalized receptors back to the cell surface (Fig.I.5).
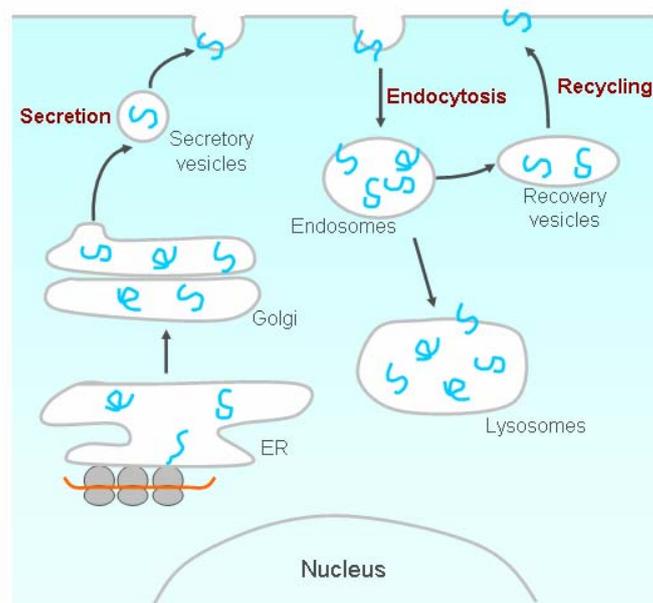
Figure I.5. Trafficking of G protein-coupled receptors (GPCRs)

GRCR-mediated signaling is dictated by the level of GPCRs at the plasma membrane, which is regulated by three processes, secretory pathway, endocytosis and recycling pathway. See text for details.

I.3.1 Secretory pathway of GPCRs

GPCRs start their life at the endoplasmic reticulum (ER), where they are synthesized, folded and assembled (Lee et al., 2004). Once the nascent polypeptide chains emerged into the ER lumen, they undergo a series of modifications including signal peptide cleavage, glycosylation, folding, and disulfide-bond formation with the help of a number of chaperone proteins, including BiP, GPRP94, calnexin, calretivulin, protein disulfide isomerase, and peptidyl praline *cis-trans* isomerase. Quality control mechanisms of the ER do not only prevent midfolded proteins from exiting the ER, but also ensure that abnormal proteins are degraded. Misfolded or misassembled proteins are exported from the ER into the cytosol, where they are ubiquitinated and subject to proteasomal degradation.

Properly folded proteins are recruited and packaged in ER-derived coat protein complex II (COPII)-coated vesicles and transported serially to the ER-Golgi intermediate compartment (ERGIC), Golgi-apparatus and trans-Golgi network (TGN).

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During the transport, receptors undergo post-translational modifications, such as glycosylation, to achieve mature status. The Golgi complex is a major site of N-linked oligosaccharide modification within secretory pathway. A highly organized set of glycosyl transferase, glycosidases, and nucleotide- or lipid-linked glycosyl donors and transporters cooperates to produce these modifications. Matured receptors then move from the TGN to their final destination at the plasma membrane by information contained within the cytosolic domains of the proteins (Enns, 2001).

I.3.2 Endocytosis of GPCRs

I.3.2.1 Categories of endocytosis

Endocytosis allows cells to internalize macromolecules and particles into transport vesicles derived from the plasma membrane (Conner and Schmid, 2003). It controls entry into the cell and has a crucial role in development, the immune response, neurotransmission, intracellular communication, signal transduction and cellular and organismal homeostasis. GPCRs-mediated signaling is extensively regulated by multiple processes, and endocytosis is a major mechanism of receptor signal attenuation. Endocytosis occurs by multiple mechanisms that fall into four main categories: clathrin-mediated endocytosis, caveolar endocytosis, pinocytosis and phagocytosis (Fig.I.6).
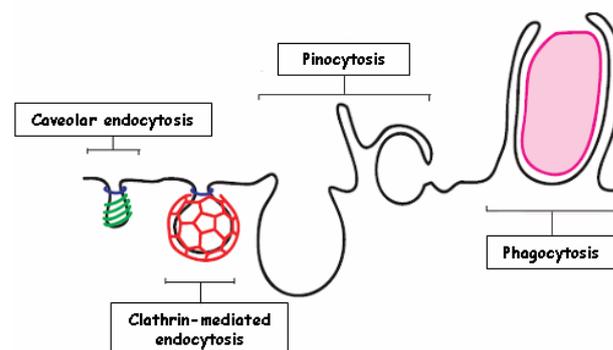
Figure I.6. Endocytosis categories

There are four main types of endocytosis: caveolar endocytosis, clathrin-mediated endocytosis, pinocytosis and phagocytosis. Endocytic pathways differ with regard to the size of endocytic vesicles, the nature of cargoes (ligands, receptors and lipids) and the mechanism of vesicle formation. This figure adapted from Nichols (2003).

Clathrin-dependent endocytosis is the best characterized pathway. Most membrane proteins undergo endocytosis via this pathway. Three mechanistically defined stages, each of which corresponds to a morphologically distinct intermediate, are recognized during the formation of clathrin-coated vesicles: (1) assembly of clathrin into a polygonal lattice and formation of coated pits, (2) invagination of coated pits, and (3) pinching-off of the coated vesicles (Mousavi et al., 2004). Many proteins are involved in the clathrin-mediated endocytosis. The most abundant proteins found in coated pits are clathrin and the heterotetrameric protein AP-2 (adaptor protein-2). Several accessory proteins are implicated in the formation of clathrin-coated vesicles, such as AP180 (Ford et al., 2001), Epsin (Chen et al., 1998), Eps15 (Benmerah et al., 1999). The clathrin-coated pits invaginate in a process involving proteins such as endophilin (Gad et al., 2000), dynamin (van der Bliek et al., 1993) and Rab5 (Bucci et al., 1995). The deeply invaginated coated pits pinch off from the plasma membrane in a dynamin-dependent manner and to form clathrin-coated vesicles.

Endocytosis mediated by caveolae and glycolipid rafts is another important endocytosis pathway. Caveolae are cholesterol- and sphingolipid-rich smooth invaginations of the plasma membrane that partition into raft fractions and whose expression is associated with caveolin-1. Caveolae are therefore a subdomain of the biochemically defined glycolipid raft (Anderson, 1998; Kurzchalia and Parton, 1999). Caveolae and raft pathways mediate the internalization of sphingolipids and sphingolipid binding toxins, GPI-anchored proteins, the autocrine motility factor (AMF), endothelin, growth hormone, and interleukin-2 (IL2) receptors, viruses (including SV40), and bacteria (Nichols and Lippincott-Schwartz, 2001; Duncan et al., 2002; Johannes and Lamaze, 2002; Pelkmans and Helenius, 2002; Conner and Schmid, 2003).

Clathrin-dependent endocytosis and caveolar/raft-mediated endocytosis are two major pathways by which membrane-bound receptors are removed from cell surface. It was believed that certain protein employs one endocytosis mechanism, either clathrin-dependent or clathrin-independent. Recently, Sigismund and his colleagues

challenge this principle. They found that epidermal growth factor receptor (EGFR) was internalized via clathrin-dependent pathway by stimulation with low doses of ligand. However, high doses of ligand led to receptor internalization via caveolar/raft-dependent pathway (Sigismund et al., 2008). Thus, proteins can uitilze different endocytic pathways under different conditions.

Phagocytosis in mammals is conducted primarily by specialized cells, including macrophages, monocytes and neutrophils, which clear large pathogens like bacteria or yeast, or large debris such as the remnants of dead cells and arterial deposits of fat (Aderem and Underhill, 1999).

Pinocytosis accompanies the membrane ruffling that is induced in many cell types upon stimulation by growth factors or other signals. Unlike phagocytosis, these protrusions do not "zipper up" along a ligand-coated particle, but collapse onto and fuse with plasma membrane to generate large endocytic vesicles (Conner and Schmid, 2003).

I.3.2.2. Fate of endocytosed proteins

Following their activation at the plasma membrane, most GPCRs undergo a cascade of desensitization events that culminates in endocytosis. After endocytosis, receptors can be sorted differentially between recycling endosomes and lysosomes (Tsao and von Zastrow, 2000). Receptors that are recycled to the plasma membrane quickly resensitize to agonist stimulation, whereas receptors that are degraded are irreversibly desensitized because of a decreased number of functional receptors on cell surface. The sorting of individual GPCRs between recycling and degradative fates is thus a critical regulatory mechanism and is therefore tightly regulated. Early endosomes, also referred as the sorting endosomes, have traditionally been considered as the initial sorting stations, where cargos are destined for recycling or degradation. Lakadamyali et al show that there two distinct populations of early endosomes: a dynamic population, which is highly mobile on microtubules and matures rapidly

toward late endosomes for protein degradation, and a static population, which matures much more slowly and is enriched in recycling cargos (Lakadamyali et al., 2005).
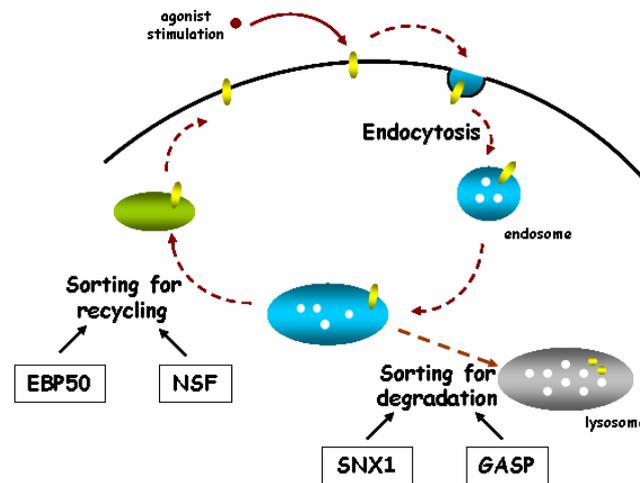
Figure I.7. The sorting of endocytosed proteins

Following agonist stimulation, cell surface receptors undergo endocytosis to endosomes. Afterwards, receptors can be sorted differentially between recycling endosomes and lysosomes. Some receptors are recycled back to the plasma membrane and quickly resensitize to agonist stimulation, whereas other receptors are targeted to lysosomes for degradation. So far it is known that EBP50 and NSF are responsible for the recycling sorting, while SNX1 and GASP contribute to the lysosomes sorting.

So far, it has been identified several proteins contribute to the sorting of endocytosed receptors. EBP50 and NSF are responsible for recycling sorting, in contrast, SNX1 and GASP contribute to the lysosomes sorting (Fig.I.7). Cao and colleagues could show that ezrin-radixin-moesin binding phosphoprotein 50 (EBP50) sort beta2 adrenergic receptor for recycling (Cao et al., 1999). Several studies reveal that another factor, N-ethylmaleimide-sensitive factor (NSF), is responsible to recycle beta2 adrenergic receptor and AMPA receptor back to plasma membrane (Cong et al., 2001; Song et al., 1998; Noel et al., 1999). Whereas, sorting nexin 1 (SNX1) contributes to target receptors to lysosomes, like epidermal growth factor receptor (EGFR) and protease-activated receptor-1 (PAR1) (Kurten et al., 1996; Wang et al., 2002). Another lysosome sorting protein is G-protein coupled receptor-associated sorting protein (GASP), which preferentially sort GPCRs to the lysosomes for degradation after

endocytosis (Simonin et al., 2004; Whistler et al., 2002; Martini et al., 2007; Thompson et al., 2007).

Interestingly, Sigismund and colleagues report that clathrin-mediated endocytosis and caveolae/raft-mediated endocytosis exert distinct effect on receptor signaling: clathrin-mediated endocytosis is essential to recycle endocytosed EGFR to maintain sustained EGFR signaling; whereas EGFR internalization through caveolae and raft pathway are efficiently degraded (Sigismund et al., 2008).

I.3.2.3. Internalization of dopamine D2 receptor

D2 receptor internalization is thought to occur through classic clathrin-mediated endocytosis (Paspalas et al., 2006). Paspalas et al reported that the internalization of D2 receptor is clathrin-dependent in primate prefrontal cortex. They have captured *in situ* the insertion of D2 receptor in clathrin-coated membrane pits, resulting in receptor sorting in primary endosomes.

D2 receptor internalization is precisely regulated, and impaired internalization leads to receptor supersensity, which has been implicated in psychiatric disorders, such as schizophrenia and drug addiction (Carlsson, 2001; Nestler, 2001). Abundant studies have focused on the regulatory effect of phosphorylation on D2 receptor internalization. The desensitization of activated GPCRs is mediated by the phosphorylation of serine and threonine residues within the intracellular domains of receptors (Ferguson, 2001). Receptor phosphorylation serves to uncouple receptors from G-protein activation and promote arrestin binding and internalization (Krupnick and Benovic, 1998). Protein kinase C, instead of protein kinase A, can phosphorylate D2 receptor within the third cytoplasmic domain and promote receptor internalization through a β-arrestin- and dynamin-dependent way (Namkung and Sibley, 2004). Co-expression of G protein-coupled receptor kinase 2 (GRK2) or 5 (GRK5) is required for D2 receptor internalization, which is regulated by dynamin and rab5A (Ito et al., 1999; Iwata et al., 1999). GRK6 deficiency in mice leads to dopaminergic

supersensitivity due to disrupted D2 receptor internalization (Gainetdinov et al., 2003). Kabbani and colleagues reported that neuronal calcium sensor NCS-1 attenuated agonist-induced receptor internalization via a mechanism that involves a reduction in D2 receptor phosphorylation (Kabbani et al., 2002). Apart from phosphorylation modulation, recently it has been found that BLOC-1 protein dysbindin, encoded by schizophrenia susceptibility gene, can modulate D2 receptor internalization, whereas has no effect on D1 receptor (Iizuka et al., 2007).

Regarding to the fate of endocytosed D2 receptor, it was revealed that D2 receptor could interact with GASP and targeted for degradation as a consequence, thereby D2 receptor responses in brain fail to resensitize after agonist treatment (Bartlett et al., 2005).

I.4 Indication for association between NCAM and dopamine D2 receptor

Schizophrenia is a chronic psychiatric disorder which affects about 1% of the world population and usually first appears in early adulthood. Schizophrenia is characterized by the simultaneous presentation of two types of symptoms: "positive" symptoms that include delusions, hallucination, and bizarre thoughts, and "negative" symptoms that include social withdrawal with affective flattening, poor motivation, and apathy. Although antipsychotic medications have alleviated the symptoms in schizophrenia patients, the pathogenesis of the illness remain poorly understood.

Of the many contemporary theories of schizophrenia, the most enduring has been the "dopamine" hypothesis besides "serotonin" and "glutamate" hypothesis (Snyder, 1976; Carlsson et al., 2001). Increased activity of D2 receptor signaling is believed to play an important role in the pathogenesis of schizophrenia. Major antipsychotic drugs exert their functions by blocking D2 receptor and the dopamine-releasing drugs worsen symptoms (Seeman et al., 1975; Creese et al., 1976). Schizophrenia patients show increased baseline occupancy of D2 receptor by dopamine and the number of

D2 receptor elevates in the striatum of schizophrenia brains (Wong et al., 1986; Abi-Dargham et al., 2000; Seeman and Kapur, 2000).

Increasing evidence indicate that NCAM is also related to psychiatric disorders (Atz et al., 2007; Sullivan et al., 2007; for a recent review see, Brennaman and Maness, 2008). NCAM180 deficiency in mice leads to impairment in prepulse inhibition of startle (PPI), which is one characteristic of schizophrenic patients (Wood et al., 1998). Transgenic mice expressing extracellular region of NCAM (NCAM-EC) show higher basal locomotor activity and enhanced responses to amphetamine, an indirect dopamine agonist, and deficit in prepulse inhibition (Pillai-Nair et al., 2005). In addition, schizophrenic patients show reduced PSA-NCAM level in hippocampus (Barbeau et al., 1995) and increased NCAM fragment in cerebrospinal fluid or in hippocampus and cortex (Poltorak et al., 1996; van Kammen, et al, 1998; Vawter et al., 1998).

Since both D2 receptor and NCAM are associated with schizophrenia, it seems likely that there is a functional relationship between D2 receptor and NCAM. Previous phage display analysis discovered one peptide which showed binding to NCAM180 intracellular domain by screening a random 12mer peptide library. Interestingly, the sequence of this peptide shares similarity to a sequence stretch within the third intracellular domain of D2 receptor, indicating NCAM and D2 receptor are potential binding partner and the NCAM/D2R interaction might exert certain function in psychiatric disorders.

II. AIMS OF THE STUDY

From phage display analysis, dopamine D2 receptor was indicated as a putative binding partner of NCAM intracellular domain. The aims of my study are:

First, to identify the interaction between NCAM and D2 receptor, and characterize which regions within NCAM and D2 receptor are responsible for this interaction. To realize this aim, coimmunoprecipation, biochemical crosslinking and GST pull down assay would be performed.

Second, to determine the functional consequence of the NCAM/D2R interaction. To address this question, functional *in vivo* approach in intact mice and *in vitro* assays in cells will be employed in my study.

III. MATERIALS

III.1. Chemicals

All chemicals were obtained from the following companies in p.a. quality: Bio-Rad (Hercules, CA, USA), Carl Roth (Karlsruhe, Germany), Invitrogen (Karlsruhe, Germany), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) and Sigma-Aldrich (Deisenhofen, Germany). Restriction enzymes were obtained from New England Biolabs (Frankfurt am Main, Germany). DNA purification kits were purchased from Life Technologies (Karlsruhe, Germany), Pharmacia Biotech (Freiburg, Germany), Macherey & Nagel and Qiagen (Hilden, Germany). Plasmids and molecular cloning reagents were obtained from Clontech (Heidelberg, Germany), Invitrogen (Groningen, Netherlands), Pharmacia Biotech, Promega (Mannheim, Germany), Qiagen (Hilden, Germany) and Stratagene (La Jolla, California, USA). Oligonucleotides were ordered from Metabion (Munich, Germany). Cell culture material was ordered from Nunc (Roskilde, Denmark) or Life Technologies. Glutathione-agarose was purchased from Sigma-Aldrich. Ni-NTA agarose was obtained from Qiagen.

III.2. Solutions and buffers

Bi-distilled water was used for solutions preparation.

III.2.1 For coomassie blue staining

Staining solution	1% coomassie blue
	45% methanol
	10% acetic acid
Destaining solution	45% methanol
	10% acetic acid

III.2.2 For DNA agarose gel electrophoresis

TAE buffer (50x)   2 M Tris-acetate, pH8.0

100 mM EDTA

DNA sample buffer (5x)   0.025% (w/v) orange G

20% (v/v) glycerol in TAE buffer

Ethidiumbromide solution   10 μg/ml ethidiumbromide in TAE buffer

III.2.3 For immunocytochemistry

Antibody buffer   0.5% (w/v) lambda-carrageenan in PBS

Blocking buffer   5% (v/v) normal goat/donkey serum in PBS

0.2% (v/v) TritonX-100

0.02% (w/v) sodium azide

III.2.4 For Lysis buffers (co-IP and cross-linking)

Lysis buffer P+   1x PBS, pH 7.4

1 mM $MgCl_2$

1 mM $MnCl_2$

1 mM EDTA

1 mM NaF

0.5 mM Na_3VO_4

0.5 mM H_2O_2

1 μM okadic acid

1x complete[TM] EDTA-free protease inhibitor mixture

Lysis buffer P-	1x PBS, pH 7.4
	protein kinase C inhibitor peptide
	1x complete^{TM} EDTA-free protease inhibitor mixture
RIPA buffer	50 mM Tris, pH 7.5
	150 mM NaCl
	1 mM $Na_4P_2O_7$
	1 mM NaF
	1 mM EDTA
	1 mM PMSF
	2 mM Na_3VO_4
	1% NP-40

III.2.5 For recombinant proteins expression

For His-tagged proteins

IPTG stock solution	238 mg/ml in ddH$_2$O results in a 1M stock solution
XGAL stock solution	40 mg/ml in dimethyl formamide
Lysis buffer	50 mM NaH$_2$PO$_4$, pH 8.0
	300 mM NaCl
	10 mM Imidazole
Wash buffter 1	50 mM NaH$_2$PO$_4$, pH 8.0
	600 mM NaCl
	10 mM Imidazole

Wash buffter 2   50 mM NaH_2PO_4, pH 8.0

300 mM NaCl

20 mM Imidazole

Wash buffter 3   50 mM NaH_2PO_4, pH 8.0

300 mM NaCl

40 mM Imidazole

Wash buffter 4   50 mM NaH_2PO_4, pH 8.0

300 mM NaCl

60 mM Imidazole

Elution buffter   50 mM NaH_2PO_4, pH 8.0

300 mM NaCl

250 mM Imidazole

For GST-fused proteins

Lysis buffer   1x PBS, pH7.4

1% Triton X-100

1x complete[TM] EDTA-free protease inhibitor mixture

Wash buffter   1x PBS, pH7.4

1% Triton X-100

Elution buffter   20 mM reduced glutathione

50 mM Tris-HCl, pH 8.0

III.2.6 For SDS polyacrylamide gel electrophoresis

Sample buffer (2x)	125 mM Tris-HCl, pH 6.8
	4% SDS
	20% glycerol
	10% β-mercaptoethanol
	0.00625% bromphenol blue

Running buffer	25 mM Tris
	192 mM glycin
	0.1% (w/v) SDS

Stacking gel 4%	3.68 ml H_2O
	0.625 ml of 1 M Tris-HCl, pH 6.8
	0.05 ml of 10% SDS
	0.665 ml of 30% Acrylamide-Bis 37:1
	25 μl of 10% APS
	5 μl TEMED

Separating gel 10%	3.45 ml H_2O
	4.65 ml of 1 M Tris-HCl, pH 8.8
	0.125 ml of 10% SDS
	4.17 ml of 30% Acrylamide-Bis 37:1
	62.5 μl of 10% APS
	6.25 μl TEMED

III.2.7 For silver staining

Fixation solution	50% (v/v) methanol
	5% (v/v) acetic acid
	45% (v/v) H_2O

Sensitivity solution	0.02% sodium thiosulfate
Staining solution	0.1% silver nitrate
Developing solution	0.04% formaldehyde
	2% sodium carbonate
Stop solution	1% acetic acid

III.2.8 For subfraction isolation

Homogenization buffer	0.25 M sucrose
(for Golgi-apparatus)	25 mM KCl
	5 mM $MgSO_4$
	1 mM PMSF
	25 mM HEPES, pH 7.3
	1x complete[TM] EDTA-free protease inhibitor mixture
Homogenization buffer	0.32 M sucrose
(for synaptosomes)	1 mM $MgCl_2$
	1 mM $CaCl_2$
	1 mM $NaHCO_3$
	5 mM Tris-HCl, pH 7.5
	1x complete[TM] EDTA-free protease inhibitor mixture
Sucrose gradient solution	0.25 M/0.8 M/1.15 M/1.3 M/2.0 M sucrose
(for endosomes)	1 mM $MgCl_2$
	1 mM $CaCl_2$
	5 mM Tris-HCl, pH 7.5

Sucrose gradient solution	0.25 M/1.1 M/1.84 M
(*for Golgi-apparatus*)	25 mM KCl
	5 mM $MgSO_4$
	25 mM HEPES, pH 7.3

Sucrose gradient solution	0.8 M/1.0 M/1.2 M sucrose
(*for synaptosomes*)	1 mM $MgCl_2$
	1 mM $CaCl_2$
	5 mM Tris-HCl, pH 7.5

III.2.9 For Western blot analysis

Blocking buffer	5% (w/v) fatty-free milk powder in PBS

Blotting buffer	25 mM Tris
	192 mM glycin
	20% methanol
	0.001% (w/v) SDS

Stripping buffer	0.5 M NaCl
	0.5 M acetic acid

III.3. Bacterial media

(Media were autoclaved and antibiotics were supplemented prior to use)

Ampicillin stock solution	100 mg/ml in H_2O

Kanamycin stock solution	25 mg/ml in H_2O

LB medium	10 g/l bacto-tryptone, pH 7.4
	10 g/l NaCl
	5 g/l yeast extract
LB/Amp medium	100 mg/l ampicillin in LB medium
LB/Amp plate	20 g/l agar in LB medium
	100 mg/l ampicillin
LB/Kan plate	20 g/l agar in LB medium
	25 mg/l kanamycin

III.4. Cell culture medium

III.4.1 For hippocampal cell culture

Dissection buffer	Hank's balanced salt solution (HBSS)
	4 mM $NaHCO_3$
	10 mM HEPES
	6 mg/ml D-glucose
	5 µg/ml Gentamycin
	3 mg/ml BSA
	12 mM $MgSO_4$
Digestion buffer	135 mM NaCl
	5 mM KCl
	7 mM Na_2HPO_4
	4 mM $NaHCO_3$
	25 mM HEPES, pH 7.4

culture medium for the first two days	Neurobasal^{TM}-A medium (Gibco)

culture medium for the first two days	Neurobasal^{TM}-A medium (Gibco)
	2 mM L-glutamine
	1x B-27 supplement
	12.5 ng/ml b-FGF
	10% horse serum
culture medium for the next days	Neurobasal ^{TM}-A medium (Gibco)
	2 mM L-glutamine
	1x B-27 supplement
	12.5 ng/ml b-FGF
	5 µM Ara C

III.4.2 For HEK 293 cells stably expressing myc-D2L

HEK 293 cell medium	high glucose Dulbecco modified Eagle's medium (DMEM)
	1 mM L-glutamine
	1 mM sodium pyruvate
	50 U/ml penicilline/streptomycine
	10% (v/v) fetal calf serum (FCS)
	2 µg/ml puromycin

III.5. Bacterial strains and cell lines

Escherichia coli **DH5α**	Invitrogen
Escherichia coli **M15 [pREP4]**	Qiagen
Escherichia coli **BL21 (DE3)**	Novagen
HEK293 cells	Human embryonic kidney cells

III.6. Molecular weight standards

III.6.1 1kb DNA ladder (Invitrogen)

14 bands within the range from 100-12000 bp

III.6.2 BenchMark^{TM} prestained protein Ladder (Invitrogen)

Band No.	Apparent molecular weight (kDa)
1	181.8
2	115.5
3	82.2
4	64.2*
5	48.8
6	37.1
7	25.9
8	19.4
9	14.8
10	6

* orientation band (pink in color)

III.6.3 Precision plus protein^{TM} dual color standards (BioRad)

Band No.	Apparent molecular weight (kDa)
1	250
2	150
3	100
4	75*
5	50
6	37
7	25*
8	20
9	15
10	10

* orientation band (pink in color)

III.7. Antibodies

III.7.1 Primary antibodies

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anti-Actin	rabbit polyclonal antibody (Sigma- Aldrich)
	WB: 1:1000
anti-AP2	mouse monoclonal antibody (BD Biosciences)
	WB: 1:1000
anti-clathrin	mouse monoclonal antibody against the clathrin heavy chain (BD Biosciences)
	WB: 1:2000
anti-D1R	rabbit polyclonal antibody against the fourth extracellular domain of dopamine D1 receptor (Millipore)
	WB: 1:1000
anti-D2R	mouse monoclonal antibody which against amino acids 1-50 of dopamine D2 receptor (Santa cruz)
	WB: 1:200
	ICH: 1:25
anti-DARPP32	rabbit monoclonal antibody against a synthetic peptide corresponding to residues surrounding Glu160 of human DARPP32 (Cell signaling technology)
	WB: 1:1000
anti-DARPP32 (pThr34)	rabbit polyclonal antibody against phospho-DARPP32 (pThr34) (AbD Serotec)
	WB: 1:1000
anti-GAPDH	mouse monoclonal antibody (Millipore)
	WB: 1:5000

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anti-GM130	mouse monoclonal antibody against rat GM130 869-982 (BD biosciences) WB: 1:1000
anti-GST	goat polyclonal antibody (GE healthcare) WB: 1:4000
anti-His	rabbit polyclonal antibody against a 6xHis synthetic peptide (Cell signaling technology) WB: 1:1000
anti-Lamp	rabbit polyclonal antibody against a synthetic peptide conjugated to KLH derived from C-terminus of human Lamp2b (Abcam) WB: 1:1000
anti-myc	mouse monoclonal antibody against amino acids 408-439 within the C- terminal domain of c-myc of human origin (Santa cruz) WB: 1:200 ICH: 1:50
anti-NCAM (5B8)	mouse monoclonal antibody against the C-terminus of the intracellular domain of NCAM 140 and 180 WB: 1:1000
anti-NCAM (D3)	mouse monoclonal antibody which recognizes an epitope on NCAM 180 within the intracellular domain encoded by exon 18 WB: 1:600
anti-NCAM (rabbit)	rabbit polyclonal antibody against the extracellular domain of NCAM WB: 1:1000 ICH: 1:200
anti-PDI	mouse monoclonal antibody against bovine PDI 109-214 (BD biosciences) WB: 1:1000

anti-TH	rabbit polyclonal antibody against tyrosine hydroxylase (Chemicon) WB: 1:1000
anti-TH (pSer40)	rabbit polyclonal antibody against phospho-tyrosine hydroxylase (pSer40) (AbD Serotec) WB: 1:1000

III.7.2 Secondary antibodies

All horseradish peroxidase-coupled (HRP) secondary antibodies were purchased from the Jackson Laboratory (Dianova, Hamburg, Germany) and were used in a dilution of 1:10,000-1:20,000 in 5% milk in PBS for immunoblotting.

For immunocytochemistry, Cy2, Cy3 and Cy5-coupled secondary antibodies were obtained from Jackson Immunoresearch Laboratories (Dianova, Hamburg, Germany) and were used in a dilution of 1:200-1:400.

III.8. Synthesized peptide

The peptides were synthesized by Dr. Christian Schafer (Copenhagen, Denmark).

III.8.1 NCAM peptide

Peptide	Sequence
NCAM-1	H-DITCYFLNKCGLLMCIAVNLC-OH
NCAM-2	H-NLCGKAGPGAKGKDMEEG-OH

III.8.2 D2R peptide

H-SPIPPSHHQLTLPDPSHHGLHSNPD-OH

III.9. Plasmids

pcDNA3 *(Invitrogen)*	mammalian expression vector, Ampicillin resistance
pcDNA3-NCAM140 (from Prof. Dr. Maness, University of South Carolina)	mammalian expression vector of full length NCAM140
pcDNA3-NCAM180 (from Prof. Dr. Maness, University of South Carolina)	mammalian expression vector of full length NCAM180
pGEM-T Easy *(Promega)*	vector for subcloning of PCR amplified DNA fragments via T/A cloning. Ampicillin resistance
pGEX-4T-2 *(Amersham Pharmacia Biotech)*	prokaryotic expression vector for recombinant expression of proteins carrying a GST at the 5' end of the multiple cloning sites for purification. Ampicillin resistance
pQE30 *(Qiagen)*	prokaryotic expression vector for recombinant expression of proteins carrying polyhistidine domain (6xHis) at the 5' end of the multiple cloning sites for purification. Ampicillin and Kanamycin resistance
pQE30-exon18	prokaryotic expression vector for His-tagged exon18 domain of NCAM180
pQE30-NCAM140 ICD	prokaryotic expression vector for His-tagged NCAM140 ICD protein
pQE30-NCAM180 ICD	prokaryotic expression vector for His-tagged NCAM180 ICD protein

III.10. Oligonucleotides

For cloning

Name	Sequence 5'-3'	Amino acids
IC3-D2R-BamHI-up	<u>GGATCC</u>AAAATCTACATCGTCCTCC	211-374
IC3-D2R- SalI-dn	<u>GTCGAC</u>TCACTGAGTGGCTTTCTTCTCCT	211-374
mD2R-S311C-fw	CTCACTCTCCCTGATCCAT**G**CCACCACGG C CTACATAGC	311
mD2R-S311C-dn	GCTATGTAGGCCGTGGTGG**C**ATGGATCA GG GAGAGTGAG	311

For the sequencing of full-length of D2 receptor

Name	Sequence 5'-3'
D2L-1	CACCACCAACTACCTGATAG
D2L-2	CAGACCAGA ATGAGTGTATC
D2L-3	AGGAGCTGGAAATGGAGATG
D2L-4	TCTTCATCACGCACATCCTG
D2S-1	TCACAGTGAATCCTGCTG

III.11. Mouse model

NCAM deficient mice (NCAM-/-) (Cremer et al., 1994) were generated by breeding heterozygous mutant mice to keep on a mixed C57BL/6J background. Mice were kept under standard conditions with food and water *ad libitum* and a light:dark cycle of 12:12 hr. Animals were sacrificed in a CO_2 chamber before the removal of brains. All animal experiments were approved by the University and State of Hamburg animal care committees and were conformed to NIH guidelines.

IV. METHODS

IV.1. Molecular biological methods

IV.1.1. Polymerase chain reaction (PCR)

Vectors for the rat full length D2R cDNA in pcDNA3 and rat full length D2S cDNA in pCD-SR were kindly provided by Prof. Dr. David R. Sibley (NINDS, National Institutes of Health, Bethesda, Maryland, USA). The third intracellular domain of D2R was amplified by PCR (primers: IC3-D2R BamHI-up 5'-GGA TCC AAA ATC TAC ATC GTC CTC C-3' and IC3-D2R SalI-dn 5'-GTC GAC TCA CTG AGT GGC TTT CTT CTC CT-3'). BamHI restriction site was induced at the 5' end and SalI restriction site at 3' end.

DNA template (10 ng/µl)	1 µl
Primer A (5 pmol/µl)	5 µl
Primer B (5 pmol/µl)	5 µl
10x PfuUltra reaction buffer	5 µl
MgCl$_2$ (50 mM)	2 µl
dNTP (10 mM)	5 µl
PfuUltra DNA polymerase	1 µl
ddH$_2$O	26 µl

PCR programme

cycles	temperature	time
1	95°C	3 min
28	95°C	30 sec
	58°C	30 sec
	72°C	1 min
1	72°C	10 min

IV.1.2. Horizontal agarose gel electrophoresis of DNA

1-2% (w/v) agarose gels were prepared in 1x TAE buffer depending on the size of DNA fragments. TAE buffer was filled in the electrophoresis chambers (BioRad). Load DNA samples to the gel and run the gel at constant voltage (10v/cm gel length) until the orange-G dye reached the end of the gel. The gel was dyed in an ethidiumbromide staining solution for 20 min. Bands were visualized using the E.A.S.Y. UV-light documentation system (Herolab, Wiesloh, Germany).

IV.1.3. Extraction of DNA from agarose gel

DNA fragments were isolated and purified from agarose gel by QIAquick® Gel Extraction kit (Qiagen).

IV.1.4. T/A cloning

Since PfuUltra DNA polymerase doesn't give an additional dATP to the 3'-end of PCR product as Tag polymerase does, dATP was add to the 3'-end of PCR fragment afterwards for the following T/A cloning. Reaction was incubated at 70°C for 30 min.

purified PCR fragment	6.7 μl
dATP (2 mM)	1 μl
10x Taq reaction buffer	1 μl
MgCl$_2$ (50 mM)	0.3 μl
Tag polymerase	1 μl

PCR fragment with 3'-adenosine overhang was cloned into pGEM®-T Easy vector.

PCR fragment with 3'-adenosine overhang	3 μl
pGEM®-T Easy vector	1 μl
2x ligation buffer	5 μl
Ligase	1 μl

The ligation mixture was incubated at 4°C for overnight. Then the whole mixture was transformed into competent *E. coli* cells.

IV.1.5. Transformation of bacteria

Aliquots of competent DH5α bacteria were thawed on ice for 10 min. 50-100 ng of plasmid DNA were supplemented to 100 μl of bacteria suspension and incubated for 30 min on ice. After a heat shock at 42°C for 1 min and consecutive incubation on ice for 2 min, 900 μl LB medium were added to the bacteria followed by incubation at 37°C for 60 min with constant shaking. The cells were collected by centrifugation at 4,000 g for 2 min at RT. Cells were resuspended in 100 μl LB medium and plated on LB plates containing appropriate antibiotics. The plates were incubated at 37°C overnight to allow single bacteria colonies to grow.

IV.1.6. Plasmid isolation from Escherichia coli culture

The preparation of plasmid DNA was based on alkaline lysis of bacteria, denaturation of protein by chaotropic salts and the isolation of DNA from contaminants using a glass fiber matrix. For small scale plasmid isolation, 3 ml LB containing appropriate antibiotics were inoculated with a single colony and incubated for overnight at 37°C with constant shaking. Cells were harvested by centrifugation at 12,000 rpm for 1 min at RT. Plasmids were isolated according to the manufacturer's protocol (Amersham Pharmacia Mini preparation kit). To prepare large amount of plasmid DNA, 500 ml bacteria culture was taken to isolate plasmids using the Maxiprep kit (Qiagen) with the same principle.

IV.1.7. Determination of DNA concentration

DNA concentration was determined spectroscopically by Amersham-Pharmacia spectrometer, which measures the absorbance at 260nm, 280nm and 320nm. Absorbance at 260nm was between 0.1 and 0.6 for reliable determinations by appropriate dilution of DNA. The ratio of the DNA absorbance at 260nm and 280nm was used to monitor the purity of DNA. A ratio of A_{260}/A_{280} between 1.8 and 2.0

indicated sufficient purity of DNA for further experiments.

IV.1.8. Ligation

pGEM®-T Easy vector containing IC3-D2R insert was digested with BamHI and SalI to release the IC3-D2R, which was then cloned in frame into BamHI/SalI site of the pGEX-4T-2. The ratio of insert/vector molarity is 3:1 to achieve high ligation efficiency.

Ligation reaction mixture

Vector	100 ng
Insert	as required
10x buffer for T4 DNA ligase	1 µl
T4 DNA ligase	1 µl
add ddH$_2$O up to 10 µl	

Ligation mixture was incubated at 16°C for overnight. 5 µl of ligation mixture was transformed into competent *E. coli* DH5α for appropriate antibiotics selection. Positive clones were picked up and further analyzed by restriction enzyme digestion and sequence analysis.

IV.1.9. DNA sequencing

DNA sequencing was performed by the sequencing facility of the ZMNH (step-by-step protocols for DNA sequencing with sequenase-version 2.0, 5th ed., USB, 1990).

IV.1.10. Site directed mutagenesis

Mutation of IC3-D2R (S311C), which is associated with schizophrenia (Itokawa et al., 1993), was generated by using QuickChange® II XL-Site Directed Mutagenesis Kit (Stratagene, Amsterdam, Netherlands). To create the IC3-D2R (S311C) mutant, the sense primer 5'-CTC ACT CTC CCT GAT CCA T**GC** CAC CAC GGC CTA CAT AGC-3' and the antisense primer 5'-GCT ATG TAG GCC GTG GTG G**C**A TGG ATC

AGG GAG AGT GAG-3' were used (sequence differences to wild-type D2R are given in bold letters).

IV.2. Biochemical methods

IV.2.1. Production of recombinant proteins in Escherichia coli using the pQE-system or pGEX-system

The expression of recombinant proteins in *E. coli* was achieved by cloning the cDNA of the desired protein in frame with an ATG start codon and the purification tag of the corresponding expression plasmid.

IV.2.1.1. Recombinant expression of proteins

The pQE expression vector encoding the intracellular domains (ICDs) of NCAM 180 and NCAM140 and CHL1 with N-terminal 6xHis purification tag, or pGEX-4T-2 expression vector encoding the third intracellular loop of D2R with N-terminal GST tag were transformed into *E. coli* M15 or BL21. After overnight growth of transformed cells in LB plates containing the appropriate antibiotics, single colony was inoculated into 20 ml LB medium with antibiotics and incubated overnight at 37°C with constant shaking. The non-induced overnight culture was transferred into a 1 liter expression culture (1:50) and incubated at 37°C under vigorous shaking until the culture reached an optical density (OD_{600}) of 0.6. Protein expression from large scale preparations was induced by 1 mM isopropyl-D-thiogalactopyranoside (IPTG). The protein expression was controlled by collecting small aliquots of the culture after IPTG induction every hour. After 4 h of growth at 37°C, cells were harvested at 4,000 g for 20 min at 4°C.

IV.2.1.2. Bacteria lysis and French press

Harvested cells were resuspended thoroughly in lysis buffer. Subsequently, cell suspension was transferred to a pre-cooled French-Pressure-20K chamber (Spectronic Instruments/SLM Aminco, 10000 psi, 5 min) and lysed by compression. French press

procedure was performed twice to lyse cells completely.

IV.2.1.3. Purification and concentration of proteins

After centrifugation (10,000 g, 20 min, 4°C), cleared cell lysates were taken. His-tagged proteins were purified in soluble form under native conditions using Ni-NTA beads (Qiagen, Hilden, Germany) according to the handbook "The QIAexpressionistTM". GST fused proteins were purified with glutathione agarose resin (Sigma-Aldrich). Following dialysis against PBS, proteins were concentrated using Vivaspin (Vivascience AG, Hannover, Germany) and stored at -80°C.

IV.2.1.4. Determination of protein concentration

Protein concentration was determined by the BCAssay kit (Bicinchoninic Acid assay, KMF Laborchemie Handels). 10 μl of samples or BSA standards (from 100 μg/ml to 2000 μg/ml) were placed on a 96-well plate and incubated with 200 μl of mixture of reagent A and reagent B (1:50) for 30 min at 37°C. Then, the absorbance was measured at 560nm by spectrophotometer and the protein concentration was calculated from the BSA standard curve.

IV.2.2. SDS-polyacrylamide gel electrophoresis and Western blot

IV.2.2.1. SDS-polyacrylamide gel electrophoresis

Proteins were separated by use of discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean III system (BioRad, Munich, Germany). The SDS-polyacrylamide gel consists of a stacking gel (4% or 5% acrylamide) and a separating gel (10% or 12% acrylamide). After complete polymerization of the gel, the electrophoresis chamber was set up according to the manufacturer's protocol. Protein samples were heated at 95°C for 5 min in SDS sample buffer, then loaded to the gel. Run the gel at constant voltage of 60V for 20 min and then 100V until the bromphenol blue line migrated to the bottom of the gel. Gels were either stained with coomassie blue or silver, or subjected to Western blot.

IV.2.2.2. Coomassie blue staining of SDS polyacrylamide gels

SDS-polyacrylamide gels were dyed in coomassie blue staining solution for 1 hr at RT with constant shaking, then destained in destaining solution until the background almost disappeared. This staining is sensitive enough to detect 0.1 μg of protein in a single band.

IV.2.2.3. Silver staining of SDS polyacrylamide gels

SDS-polyacrylamide gels were fixed in fixation solution for 30 min at RT with constant agitation. Gels were intensively rinsed with water and washed with water for another one hour. Then, gels were quickly sensitized with sensitizing solution for 1 min and washed twice with water. Afterwards, gels were stained in chilled 0.1% AgNO$_3$ for 30 min at 4°C. Finally gels were briefly developed in developing solution and the reaction was stopped with 1% acetic acid.

IV.2.2.4. Western blot analysis

Proteins were transferred from SDS-polyacrylamide gel onto a nitrocellulose membrane (Protran, Nitrocellulose BA 85, Schleicher & Schüll, Dassel, Germany) using a Mini Transblot apparatus (BioRad) as described in the manufacturer's protocol. The blotting "sandwich" was assembled according to the manufacturer's recommendations. Proteins were transferred electrophoretically in blotting buffer at constant voltage 80V for 150 min at 4°C.

After electrophoretic transfer, the nitrocellulose membrane was removed from the blotting sandwich. The side of membrane presenting the proteins was placed right-side up in a glass jar. Then the membrane was washed once with PBS and blocked in 5% fatty-free milk in PBS for 1 h at RT. Afterwards, the primary antibody was diluted in milk solution and incubated for overnight at 4°C on a shaking platform. The primary antibody was removed and the membrane was washed 8 times with PBST (PBS+0.1% tween-20), 5 min each. The appropriate secondary antibody coupled with HRP was applied in milk solution for 1 h at RT, followed by washing with PBST for 8 times, 5 min each.

Immunoreactivity was visualized by enhanced chemiluminescence detection system (ECL) or ECL with extended duration (Pierce Biotechnology, Rockford, IL, USA) for detection of weak signals. The membrane was covered with detection solution (1:1 mixture of solutions I and II) for 2 min, and placed between two plastic foils and exposed to X-ray film (Kodak Biomax-ML, Sigma-Aldrich, Steinheim, Germany) for various time periods followed by development and fixation of film. Bands intensities were densitometrically quantified using the image software TINA 2.09 (University of Manchester, UK).

IV.2.3. Protein-protein binding assay

IV.2.3.1. Coimmunoprecipitation assay

Preparation of brain homogenate and cell lysate

Brains from C57BL/6J mice were homogenized in lysis buffer P+ with a Dounce homogenizer (Weaton, Telfon pestle, 2 ml or 10 ml) on ice. 100 μl of brain homogenate (1-2 mg of proteins) was taken for further lysis in 1ml lysis buffer P+ containing 1% NP-40 for 1 h at 4°C on a rotator, and then subjected to coimmunoprecipitation.

Stably myc-D2R expressed HEK 293 cells, which was kindly provided by Prof. Dr. Kim A. Neve (Oregon Health & Science University, Portland, Oregon, USA), were transfected with NCAM180 or NCAM140 by FuGENE 6 transfection reagent (Roche Diagnostics, GmbH). Two days after transfection, cells were washed with PBS and lysed in lysis buffer P+ containing 1% NP-40 for 30 min on ice. Cells were further disrupted by repeated aspiration through 25 gauge needle. Pellet cellular debris by centrifugation at 700 g for 10 min at 4°C and supernatant was taken for coimmunoprecipitation.

Coimmunoprecipitation with protein A/G plus agarose

Brain homogenate, synaptosomes or cell lysate were pre-cleared by incubation with protein A/G plus agarose beads (Santa Cruz Biotechnology) for 3 h at 4°C on a

rotating wheel. Mouse D2R antibody or c-myc antibody were added to pre-cleared samples and incubated at 4°C overnight on a rotator. Then, pre-washed protein A/G plus agarose beads were added and incubated at 4°C for 8 h under constant agitation. Beads were washed with lysis buffer P+ containing 1% NP-40 for 4 times, followed 2 times with lysis P+ buffer. Precipitated proteins were eluted from beads with 2xSDS sample buffer by heating at 95°C for 5 min. Samples were separated by SDS-PAGE and subjected to Western blot analysis. Membranes were detected with NCAM antibody 5B8.

IV.2.3.2. Biochemical crosslinking with Sulfo-SBED

Biochemical crosslinking was performed under two different conditions, lysis P+ or lysis P- condition. Since the crosslinker Sulfo-SBED (Pierce Science, Bonn, Germany) is light sensitive, the procedure was performed in darkness until the photoactivation by UV light.

The trifunctional crosslinker was coupled to NCAM180-ICD, NCAM140-ICD or CHL1-ICD for 30 min at RT on a rotating wheel. Unbound cross-linker was removed by overnight dialysis against PBS at 4°C. Then brain homogenate from 2- to 3-month-old C57BL/6J was added to the ICDs-crosslinker complex and incubated for 1 h at RT, followed by photoactivation under UV light (365 nm). To isolate the potential binding partners-ICDs-crosslinker complex, two steps of isolation was performed. First, Ni-NTA beads were used to precipitate His-tagged ICDs of NCAM or CHL1. Then, magnetic streptavidin DYNA-beads (Dynal diagnostics, Hamburg, Germany) were applied to precipitate biotin-labeled proteins. After washing 4 times with PBS, precipitated proteins were eluted from beads with 2xSDS sample buffer by heating at 95°C for 5 min. Samples were separated by SDS-PAGE and subjected to Western blot analysis. Western blot was performed by using NeutrAvidin-HRP to detect biotin labeled proteins and using D2R antibody to detect if D2R was binding partner.

IV.2.3.3. GST pull down assay

Pull down assay

GST pull down assay were performed to check the direct interaction between IC3-D2R and NCAM intracellular domain. All procedures were performed on ice or at 4°C.

GST-tagged IC3-D2R and His-tagged NCAM-ICD were incubated in PBS containing 1% BSA at 4°C for overnight on a rotator. Then, pre-washed glutathione agarose beads were added and incubated at 4°C for 8 h under constant agitation to pull down GST-tagged proteins. Beads were washed with PBS containing 1% NP-40 for 4 times, followed 2 times with PBS. Precipitated proteins were eluted from beads with 2xSDS sample buffer by heating at 95°C for 5 min. Samples were separated by SDS-PAGE and subjected to immunoblotting. Membranes were detected with NCAM antibody 5B8, then stripped and incubated with GST antibody to check GST input.

Competition pull down assay

To narrow down the binding sites between D2R and NCAM, competition pull down assay were performed by using peptides as competitors.

The same procedure as described before except that D2R peptide and NCAM180-ICD or NCAM peptides and GST-IC3-D2R were pre-incubated in PBS+1% BSA at 4°C overnight on a rotator. Then, GST-IC3-D2R or NCAM180-ICD were added and further incubated at 4°C for overnight.

IV.2.4. Isolation of subcellular fractions by discontinuous gradient centrifugation

IV.2.4.1. Isolation of synaptosomes, plasma membrane and endosomes from total brain homogenate

All procedures were performed on ice or at 4°C.

Whole brains were isolated from adult NCAM-/- mice and control littermates and transferred into ice-cold homogenization buffer. Brains were homogenized in a

Dounce homogenizer (Weaton, Telfon pestle, 10 ml). Crude brain homogenate was centrifuged at 1,000 g for 10 min. Take supernatant and centrifuge at 17,000 g for 20 min.

Synaptosomes isolation

17,000 g pellet was resuspended in homogenization buffer and loaded to the discontinuous sucrose grandient, which consisted of 0.8 M, 1.0 M and 1.2 M sucrose from the top to the bottom. After centrifugation at 100,000 g for 1 h, the interphase materials between 1.0 M and 1.2 M sucrose, which contains synaptosomes, were collected and diluted with homogenization buffer. Synaptosomes were pelleted by centrifugation at 17,000 g for 20 min.

Plasma membrane isolation

17,000 g pellet was washed once with homogenization buffer, then lysed by hypoosmotic shock in 9 volumes icd-cold H_2O plus protease inhibitors, followed by rapidly adjusted to 5 mM Tris by 1 M Tris-HCl (pH 7.5). Mix constantly in cold room for 30 min to ensure complete lysis. Lysate was centrifuged at 25,000 g for 20 min to yield a pellet, lysed membrane fraction. Pellet was resuspended in homogenization buffer and loaded to the top of the discontinuous sucrose gradient, which consisted of 0.8 M, 1.0 M and 1.2 M sucrose from the top to the bottom. After centrifugation at 150,000 g for 2 h, the interphase materials between 1.0 M and 1.2 M sucrose, which contains membrane fractions, were collected and diluted with homogenization buffer. Membrane fractions were pelleted by centrifugation at 150, 000 g for 30 min.

Endosomes isolation

17,000 g supernatant was collected and centrifuged at 100,000 g for 1 h. Pellet was resuspended in 2 M sucrose gradient solution, and other sucrose gradient was loaded to the top containing 0.25 M, 0.8 M, 1.15 M, 1.3 M of sucrose. After centrifugation at 100,000 g for 2 h, the interphase fractions between 0.8 M and 1.15 M sucrose, which

contains endosomes, were collected and precipitated by methanol-chloroform. Briefly, 4 vol of methanol was added to endosomes fraction and mix, followed by adding 1 vol of chloroform and 3 vol of water. After centrifugation at 9,000 g for 3 min, the liquid interface containing proteins was collected. 3 vol of methanol was added to precipitate proteins by centrifugation at 9,000 g for 2 min.

IV.2.4.2. Isolation of Golgi apparatus (Wong et al., 1998)

Whole brains isolated from adult NCAM-/- mice and control littermates were homogenized in homogenization buffer by a Dounce homogenizer (Weaton, Telfon pestle, 10 ml). Brain homogenate was centrifuged at 10,000 g for 10 min to remove unbroken cells and nuclei. Supernatant was taken and centrifuged at 100,000 g for 1 h to get crude membrane pellet. Membrane pellet was resuspended in minimal volume of homogenization buffer containing 0.25 M sucrose and adjusted to 1.25 M sucrose by adding 2.3 M sucrose. Afterwards, samples were loaded to the top of 1.84 M sucrose, followed by 1.1 M sucrose and 0.25 M sucrose on the top. After centrifugation at 120,000 g for 3 h, Golgi-enriched fraction from the 1.1 M/0.25 M sucrose interphase was collected and diluted by 0.25 M sucrose homogenization buffer. Golgi fraction was pelleted by centrifugation at 100,000 g for 1 h.

IV.2.4.3. Isolation of Endoplasmic reticulum

Endoplasmic reticulum (ER) was isolated by a discontinuous OptiPrep density gradient using the Endoplasmic reticulum isolation kit (ER0100, Sigma-Aldrich). Fractions were collected at the interphases of the OptiPrep gradient and assayed for ER protein PDI and the lysosomal protein Lamp2b. The fraction at the interphase between 15% and 20% of the OptiPrep gradient was taken as endoplasmic reticulum, which was enriched in PDI.

IV.2.4.4. Isolation of Lysosomes

Lysosomes were prepared by a discontinuous OptiPrep gradient using the lysosome isolation kit (LYSISO1, Sigma-Aldrich). Fractions were collected at the interphases of

the OptiPrep gradient and assayed for the lysosomal protein Lamp2b and ER protein PDI. The fraction at the interphase between 8% and 12% of the OptiPrep gradient was taken as lysosomes, which was enriched in Lamp2b and negative for PDI.

IV.3. Cell biological methods

IV.3.1. Cell culture

IV.3.1.1. Maintenance of HEK 293 cells

HEK 293 cells were cultured with 15 ml of appropriate medium in 75 cm^2 flasks or 2 ml in 6-well plate at 37°C, 5% CO_2 and 90% relative humidity. Cells were passaged when they grow confluently. Medium was removed and cells were shortly washed with HBSS. Then cells were detached by Trypsin-EDTA and resuspended in fresh culture medium. Cells were passaged 1:6 for maintenance.

IV.3.1.2. Transfection of HEK 293 cells

Fugene 6 (Roche) was chosen for transient transfection of HEK 293 cells. One day before transfection, cells were seeded in 6-well plates or 10 cm dishes. When they grow to 50-80% confluency, cells were transfected with 1 μg of DNA per well. 3 μl Fugene 6 were used in each transfection as described in the manufacturer's transfection protocol. Two days after transfection, cells were used for biochemical analysis.

IV.3.1.3. Primary hippocampal cell culture

1) Dissection

Postnatal day 1-3 mice were decapitated. Brains were removed from the skull and cut along the midline. hippocampi were prepared and split into 1 mm thick pieces.

2) Digestion

Hippocampi were washed three times with dissection solution and treated with trypsin and DNAseI in digestion buffer for 5 min at RT. After removal of the digestion solution, hippocampi were washed twice with dissection solution and the digestion

reaction was stopped by adding trypsin inhibitor for 5 min at RT.

3) Dissociation

Hippocampi were dissociated in dissection solution containing DNAseI by glass pasteur pipettes which have successively smaller diameters. After homogenous suspension was obtained, dissection solution was added to cell suspension.

4) Removal of cell debris and plating of cells

Cell suspension was centrifuged (960 rpm, 15 min, 4°C) to pellet cells. Cells were washed once with dissection solution and counted in a Neubauer cell chamber. Cells were plated on 0.01% PLL coated coverslips at the density of 1,000 cells/mm^2. Two days later, AraC was added to culture medium to prevent the growth of astrocytes. Culture medium was changed every 2 days.

IV.3.2. Cell surface biotinylation

All procedures were performed on ice or at 4°C.

Cells were washed twice with ice-cold PBS containing 2 mM MgCl$_2$ and 0.5 mM CaCl$_2$ (PBS^{2+}). Then cell surface proteins were biotinylated with 0.5 mg/ml sulfo-NHS-SS-biotin (Pierce Science, Bonn, Germany) in PBS^{2+} for 10 min, which is membrane impermeable. Extra biotin was quenched by 20 mM glycine in PBS^{2+} for 5 min. Cells were washed twice with PBS^{2+} and lysed in RIPA buffer for 30 min. Cells were further disrupted by repeated aspiration through a 25 gauge needle. Cellular debirs were pelleted by centrifugation at 700 g for 10 min at 4°C and supernatant was taken for further immunoprecipitation. Magnetic streptavidin beads were added to cell lysate and incubated for overnight to precipitate biotinylated surface proteins under constant agitation. Beads were washed four times with RIPA buffer and twice with PBS. Precipitated proteins were eluted from beads with 2xSDS sample buffer in the absence of mercaptoethanol by heating at 95°C for 5 min. Samples were separated by SDS-PAGE and subjected to Western blot analysis.

IV.3.3. Immunocytochemistry

The subcellular localization of NCAM and D2 receptor in cultured hippocampal

neurons was examined using immunofluorescence confocal microscopy. 3 weeks after culture, cells were washed in PBS and fixed with 4% ice-cold formaldehyde and 4% sucrose in PBS for 15 min at RT. After quickly rinsed with PBS for twice, cells were blocked in PBS containing 5% non-immune goat serum (NGS) and 0.2% triton X-100 for 30 min at RT. After removal of the blocking solution, cells were incubated with mouse D2R antibody and rabbit polyclonal NCAM antibody at 4°C overnight in a humidified chamber. Unbound primary antibodies were washed away with PBS for 3 times, 5 min each. Then secondary antibodies coupled with Cy2 or Cy3 were added to cells and incubated at RT for 1 h in the dark. Cells were then washed three times with PBS. Afterwards, DAPI was added to label nuclei for 2 min at RT in the dark, followed by twice wash with PBS. Coverslips were mounted on glass slides with Fluoromount G. Glass slides were stored in darkness at 4°C after they were dry.

IV.3.4. *Antibody feeding immunocytochemistry* (Bartlett et al., 2005; Zhang et al., 2007)

To study D2R internalization processes, stably myc-D2R expressed HEK 293 cells were transfected with NCAM180 by FuGENE 6 transfection reagent. 24 hours later, live cells were fed with myc antibody (1:50) at 37°C for 10 min in incubator, then stimulated with 10 μM dopamine for 10 min or 60 min. Cells were fixed with 2% PFA for 5 min at RT, then block with 5% NGS for 30 min at RT. Cy3-conjugated secondary antibody (1:400) was applied for 30 min at RT to label cell surface D2R. Cells were washed with PBS and fixed again in 2% PFA for 5 min at RT, followed by permeation with 0.2% Triton X-100 for 5 min and blocking with 5% NGS for 30 min at RT. Then, Cy5-conjugated secondary antibody (1:200) was applied for 30 min at RT to label internalized D2R. After washing with PBS, NCAM polyclonal antibody (1:400) was added for 60 min at RT, followed by Cy2-conjugated secondary antibody. In the end, DAPI was added to label nuclei for 2 min at RT in the dark. Coverslips were mounted on glass slides with Fluoromount G. Glass slides were stored in darkness at 4°C after they were dry.

Confocal images were acquired using a Leica confocal microscope. For quantification

of internalization, the confocal settings for image acquisition were kept the same for all cells. Image stacks were flattened into a single image using a maximum projection and analyzed with Image-J. Surface and internal D2R fluorescence intensities were measured as the integrated pixel intensities in the red and blue channels, respectively. Total D2R fluorescence was determined as the sum of the surface (red) and the internal (blue) fluorescence intensities. For each cell, the internalization index was defined as the ratio of the internalized fluorescence intensity to the total fluorescence intensity.

IV.3.5. Degradation Assay

Stably myc-D2R expressed HEK 293 cells were transfected with NCAM180 by FuGENE 6 transfection reagent. Two days later, cells were stimulated with 10 μM dopamine at 37°C for the indicated time point in the presence of 10 μg/ml protein synthesis inhibitor cycloheximide (Tocris). Cells were then lysed with RIPA buffer and subjected to Western blot analysis.

IV.4. Radioimmunoassay

Whole brains isolated from adult NCAM-/- mice and control littermates were homogenized in 0.01 N HCl containing 1 mM EDTA and 4 mM $Na_2S_2O_5$ on ice. After centrifugation at 10,000 rpm for 20 min at 4°C, supernatant was taken and subjected to dopamine determination by radioimmunoassay according to the manufacturer's protocol (Labor diagnostika nord, Nordhorn, Germany) and protein concentration measurement by BCA assay. Dopamine content was normalized to protein concentration.

IV.5. Measurement of locomotor activity

IV.5.1. Drug administration

The D1R-specific antagonist SKF83566 (Tocris) or D2R-specific antagonist raclopride (Sigma-Aldrich) were dissolved in saline and injected intraperitoneally (i.p.)

before behavioral testing. To investigate the effect of dopamine receptor agonists in dopamine-depleted mice, a combination of reserpine (5 mg/kg, i.p., 20 h before the test, Sigma-Aldrich) and methyl-DL-tyrosine (250 mg/kg, i.p., 1 h before the test, Sigma-Aldrich) were applied to deplete dopamine in mice (Gainetdinov et al., 2003), followed by administration of the D1R/D2R agonist apomorphine (1 mg/kg, s.c., Sigma-Aldrich). Reserpine, methyl-DL-tyrosine and apomorphine were dissolved in distilled water with drops of glacial acetic acid, hydrochloric acid or 0.1% ascorbic acid, respectively. All injections were given in a volume of 0.1 ml/30 g of body weight.

IV.5.2. Measurement of locomotor activity

Locomotion was evaluated in the open field. The open field consisted of a wooden box (50 x 50 x 40 cm) laminated with rough, matted, light-gray resin and illuminated by a white bulb (100 Lux). After drug administration, mice were genly introduced into a cylinder placed at one corner of the box for 5 sec. As the cylinder was lifted, mice could move freely in the arena for a duration of 30 min. Locomotor activity was measured at 5 min intervals and cumulative counts were taken for data analysis with the software EthoVision (Noldus, Wageningen, The Netherlands).

IV.6. Statistical analysis

Values in graphs were presented as mean ± SEM. Data were analyzed by independent samples t test. The threshold value for acceptance of differences between group mean values was 5%.

V. RESULTS

V.1. NCAM and D2 receptor partially col-ocalize in hippocampal neurons.

To obtain indications for the NCAM/D2R interaction to occur *in vivo*, I first examined the subcellular localization of NCAM and D2 receptor in hippocampal neurons. Primary cultured hippocampal neurons were co-stained with NCAM and D2 receptor after fixation and permeablization. Fig.V.1 shows a representative hippocampal neuron with long processes. NCAM partially co-localizes with D2 receptor at the cell body, also along neurites, indicating these two proteins have chance to interact with each other.

Figure V.1. Co-localization of NCAM and D2 receptor in primary hippocampal neuron.

3-week-old cultured hippocampal neurons were immunostained with NCAM rabbit polyclonal antibody (green) and D2 receptor mouse monoclonal antibody (red). Nuclear was stained with DAPI. Images of a representative neuron are shown here. Note that NCAM co-localized with D2 receptor at the cell soma and along neurites. Scale bar: 20 μm.

V.2. Interaction between NCAM and D2 receptor

V.2.1. Co-immunoprecipation assay

Immunoprecipitation experiments were performed to examine whether there is interaction between NCAM and D2 receptor in physiological context. First, brain homogenate was taken for immunoprecipitation. Brain homogenate was incubated with mouse monoclonal D2 receptor antibody for immunoprecipitation, followed by

incubation with protein A/G agarose beads to precipitate potential binding partners/D2 receptor complexes. After SDS-PAGE, Western blot analysis was performed using NCAM antibody 5B8 for detection of co-precipitated NCAM. As shown in Fig.V.2A, immunoprecipitation of D2 receptor from brain homogenate led to a co-immunoprecipitation of endogenous 180 isoform of NCAM. In particular, NCAM180, but no NCAM140, shows co-precipitation with D2 receptor.
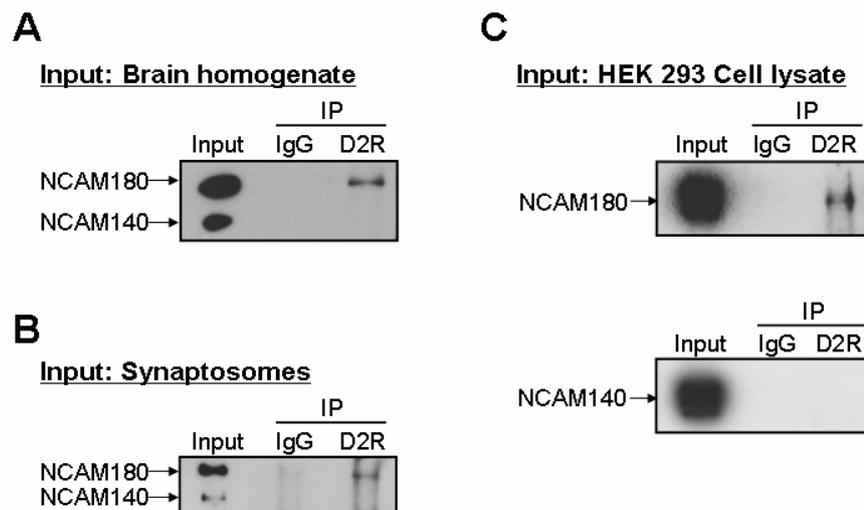
Figure V.2. NCAM180 interactes with D2 receptor *in vivo*.

Co-immunoprecipitation of NCAM180 and D2R in protein extracts prepared from brain homogenate **(A)**, synaptosomes fraction **(B)**, or HEK293 cell lysate **(C)** transfected with D2R and NCAM180 (C upper panel) or NCAM140 (C lower panel) by using anti-D2R antibody. IgG was used as a control in all immunoprecipitation experiments. Immunoprecipitates were subjected to SDS-PAGE and Western blot by using NCAM antibody 5B8 for detection of putative coimmunoprecipitates. NCAM180, rather than NCAM140, was co-precipitated with D2 receptor in brain homogenate, synaptosomes and cell lysate.

Since both NCAM and D2 receptor are present and function in synapses, we examined whether NCAM and D2 receptor could form complex in synaptosome fractions. It turned out that NCAM180, but not NCAM140, was co-precipitated with D2 receptor in synaptosomes (Fig.V.2B). Co-precipitation of NCAM180 and D2 receptor was also confirmed by using transfected cell lysates. HEK293 cells stably expressing D2R were transiently transfected with NCAM180 or NCAM140. Two days after transfection, transfected cells were lysed for immunoprecipitation with

mouse D2 receptor antibody. 5B8 antibody was used for detection of co-precipitated NCAM. FigV.2C shows co-immunoprecipitated NCAM180 with D2R in NCAM180 transfected cells (C upper panel), but no such co-precipitation was observed in NCAM140 transfected cells. In all co-immunoprecipitation experiments, mouse non-specific IgG was used as control and there was no NCAM immunoactive band in the control. It's interesting that only NCAM180, but no NCAM140, could be co-immunoprecipitated with D2 receptor in all experiments. Since NCAM140 is localized to migratory growth cones and axon shafts of developing neurons, whereas NCAM180 is enriched in postsynaptic densities of mature neurons (Persohn et al., 1989; Schuster et al., 2001), co-immunoprecipitation result indicated that the interaction between NCAM and D2 receptor occured in postsynaptic terminus.

V.2.2. Biochemical crosslinking

To further characterize the interaction between NCAM and D2 receptor, biochemical crosslinking experiment was performed. Since phage display analysis indicated the intracellular domain of NCAM (NCAM ICD) might mediate the NCANM/D2R interaction, NCAM-ICD was applied in crosslinking assay. The trifunctional crosslinker Sulfo-SBED containing a biotin moiety was coupled to the ICDs of NCAM180 and NCAM140, or CHL1 as a control, followed by incubation with brain homogenate from 2- to 3-month old C57/BL mice. After UV-crosslinking, the samples were separated by SDS-PAGE under reducing conditions, which leads to the transfer of the biotin moiety from the crosslinker to the molecules that had bound to the ICD-cross-linker conjugates, and analyzed by Western blot. Neutravidin-HRP was used for detection of biotin-labeled proteins and mouse D2 receptor antibody for D2 receptor detection. In line with results from co-immunoprecipitation, crosslinking experiment revealed that NCAM180-ICD could interacte with D2 receptor, whereas no binding was observed in NCAM140-ICD and CHL1-ICD controls (Fig.V.3A).

Furthermore, since phosphorylation plays an important role for protein-protein interactions, we performed the crosslinking experiment under condition that favors

either phosphorylation with phosphatase inhibitors or dephosphorylation with protein kinase C inhibitor. As shown in Fig.V.3B, under phosphorylation conditions more D2 receptors were detectable in comparison to dephosphorylation conditions, which indicates the interaction between D2 receptor and NCAM180-ICD was enhanced by phosphorylation.
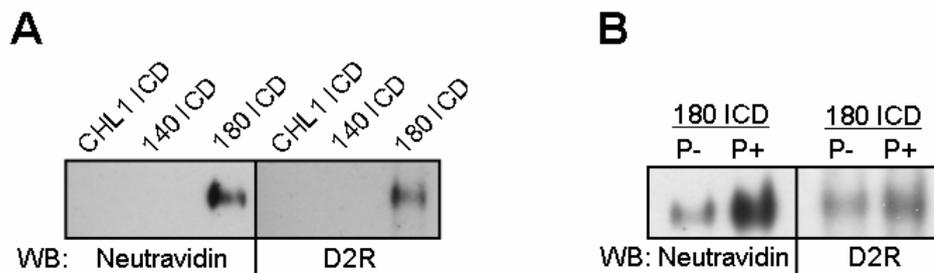
Figure V.3. Binding of D2 receptor to NCAM180 ICD by biochemical cross-linking.

The trifunctional crosslinker Sulfo-SBED containing a biotin moiety was coupled to ICDs of NCAM180 and NCAM140, or CHL1 as a control, followed by incubation with brain homogenate. After UV crosslinking and denatured under reducing conditions, the biotin was transferred to crosslinked partner molecules. Potential binding partners were isolated and separated by SDS-PAGE under reducing conditions. Western blot was performed by using anti-D2 receptor antibody and using neutravidin-HRP to detect the biotin-labeled proteins. It's shown that D2 receptor binds to NCAM180-ICD **(A)**, furthermore, under phosphorylation condition, more D2 receptor binds to NCAM180-ICD (increase by 60%) when compared with under dephosphorylation lysis condition **(B)**.

V.2.3 Pull down assay

V.2.3.1 Direct interaction between NCAM and D2 receptor

To confirm the interaction data shown above and examine whether NCAM/D2R interact directly, GST pull down assay was performed by using recombinant GST tagged IC3-D2R (GST-IC3-D2R) and His tagged NCAM-ICD since it's indicated that NCAM/D2R interaction could be mediated by NCAM-ICD and IC3-D2R by phage display and crosslinking assays. Increasing amounts of NCAM180-ICD or NCAM140-ICD was incubated with GST or GST-IC3-D2R at a constant concentration overnight, followed by pull down with glutathione beads. Pulled down proteins were subjected to SDS-PAGE and Western blot analysis with NCAM

antibody 5B8 and GST antibody to check GST proteins input. Fig.V.4 shows that NCAM180-ICD as well as NCAM140-ICD can interact with GST-IC3-D2R in a concentration dependent manner *in vitro*. No such pull down of NCAM-ICD was observed when incubation with GST control.
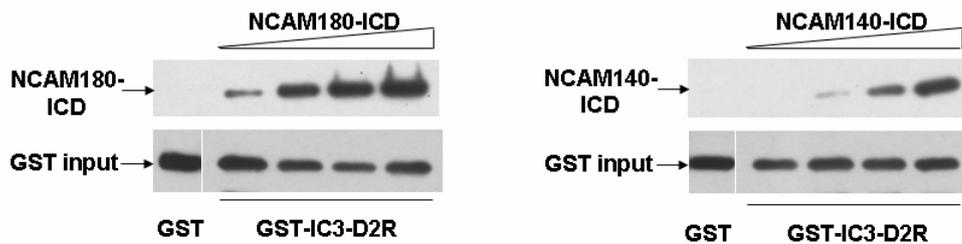
Figure V.4. NCAM ICD directly interactes with the third intracellular loop of D2 receptor.

GST or GST-IC3-D2R was incubated with increasing amount of recombinant NCAM180-ICD or NCAM140-ICD, followed by pull down with glutathione beads. The pulled down proteins were subjected to SDS-PAGE and analyzed by Western blot analysis. Both NCAM180-ICD and NCAM180-ICD were pulled down with GST-IC3-D2R in a concentration dependent manner, but not with GST control. GST input showed that the amount of GST-IC3-D2R was similar or even lower than GST control.

Further analysis was performed to clarify the binding sites on NCAM and D2R that mediated their interaction by pull down assays. Since both NCAM180-ICD and NCAM140-ICD can interact with D2R in this *in vitro* assay, it is unlikely that NCAM180 specific "exon 18" determines the interaction of NCAM to D2R. Recombinant His-tagged NCAM "exon 18" was incubated with GST or GST-IC3-D2R overnight followed by pull down with glutathione beads. Pulled down complex was analyzed by Western blot with anti-Penta His antibody. Fig.V.5B showed no detectable band of "exon 18" by His antibody, indicating exon 18 encoding protein of NCAM180 indeed did not interact with IC3-D2R. We proceeded to examine whether the C-terminal segment of NCAM-ICD could mediate NCAM/D2R interaction. The same pull down assay as "exon 18" was performed except incubation IC3-D2R with N-terminally truncated NCAM140-ICD (NCAM140-ICD △N, lacking amino acids 730-772; Fig. V.5A) instead of "exon 18".

It's revealed that NCAM140-ICD △N did not interact with IC3-D2R either (Fig.V.5B). Then, the binding region was narrowed down to the N-terminus of NCAM-ICD. To further identify the binding site, two peptides matching the N-terminal sequences of NCAM-ICD (peptide1: 730-750; peptide2: 748-765) were applied for competition in the pull down assays, in which NCAM peptides were pre-incubated with IC3-D2R, followed by addition of NCAM-ICD and pull down by glutathione beads. NCAM peptide2 showed strong competition with the binding of NCAM-ICD to IC3-D2R, and dramatically less NCAM-ICD was pulled down together with IC3-D2R in the presence of NCAM peptide2. However, no such competition was observed with NCAM peptide1. This result indicated peptide2 region was the binding site for NCAM-ICD to D2 receptor, and the membrane proximal part of NCAM-ICD carries the binding site for NCAM-ICD to D2R (Fig. V.5C).
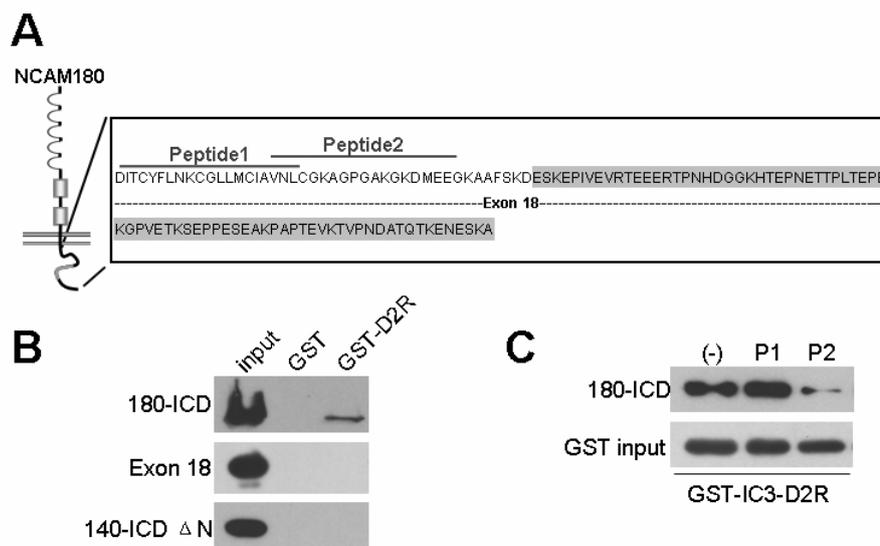
Figure V.5. NCAM-ICD interactes with D2 receptor via N-terminus of intracellular domain.

(A) Schematic structure of NCAM180-ICD. Gray highlights N-terminal truncated fragment of NCAM140-ICD (140-ICD △N). Dashed line indicates exon18 encoded sequence of NCAM. **(B)** Pull down was performed with recombinant His-tagged NCAM180-ICD, "exon18" and NCAM140-ICD △N with GST-IC3-D2R or GST control. Precipitated proteins were detected by anti-Penta His antibody. NCAM180-ICD, but not "exon18" and NCAM140-ICD △N, was pulled down with GST-IC3-D2R. **(C)** Pull down with NCAM180-ICD and GST-IC3-D2R in the presence of NCAM peptide1 and 2. NCAM peptide2, instead of peptide1, showed strong inhibition to the NCAM/D2R interaction. (-) designates no application with NCAM peptides.

In terms of the binding domain on D2 receptor, phage display analysis strongly suggested that amino acids 296-320 within IC3-D2R might mediate NCAM/D2R interaction. To confirm phage display data, D2R peptide (296-320 aa) derived from IC3-D2R which shared similarity with phage peptide was applied for competition in the GST pull down assay, in which increasing amounts of D2R peptide were pre-incubated with constant concentration of NCAM180-ICD, followed by addition of IC3-D2R and pull down by glutathione beads. As shown in Figure V.6, dramatically less NCAM-ICD was pulled down together with IC3-D2R in the presence of D2R peptide in a concentration dependent manner, indicating NCAM/D2R binding site located within 296-320 of IC3-D2R (Fig.V.6).

Taken all these *in vitro* interaction data together, these observations indicate that NCAM180 interacte with IC3-D2R at amino acids 296-320 via an N-terminal segement (amino acids 748-765) of NCAM180-ICD.
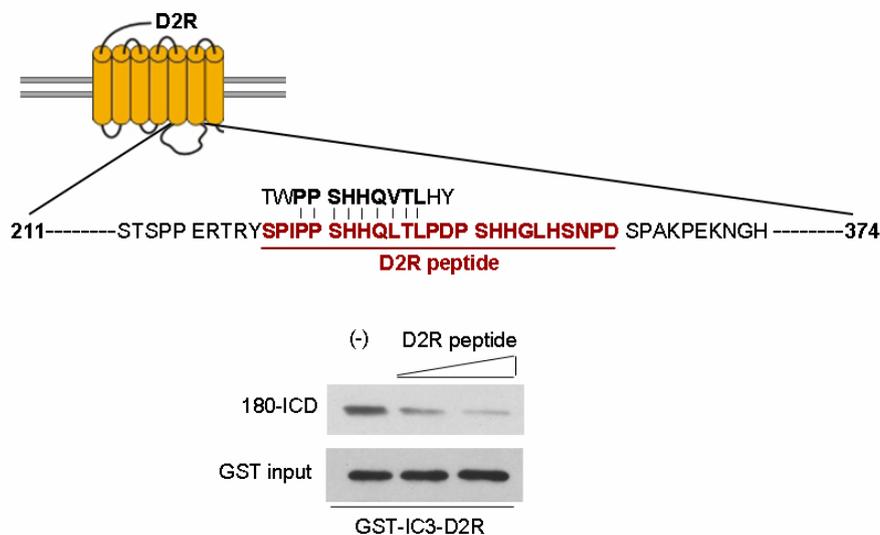
Figure V.6. NCAM-ICD interactes with the third intracellular loop of D2 receptor at amino acids 296-320 region.

Upper image presents schematic structure of D2 receptor. Red line highlights D2R peptide (amino acids 296-320) which holds similarity with phage peptide ("|" show identical amino acid). Pull down was carried out with NCAM180-ICD and GST-IC3-D2R in the presence of increasing concentrations of D2R peptide as a competitor. D2R peptide showed remarkably inhibition to the NCAM-D2R interaction in a concentration-dependent manner. (-) designates no application with D2R peptide.

V.2.3.2 NCAM/D2R interaction is disrupted by the mutation of D2 receptor (S311C).

The polymorphism of serine to cysteine at 311 residue of D2 receptor, which locates within the binding region for NCAM, has been implicated as a risk factor in schizophrenia (Itokawa et al., 1993). To investigate the effect of this mutation on the NCAM/D2R interaction, mutated GST-IC3-D2R (S311C) was generated by site-directed mutagenesis and recombinantly expressed in *E. coli*. Pull down assay was performed by incubation NCAM180-ICD with wild-type IC3-D2R or mutated IC3-D2R (S311C). Western blot analysis with pulled down proteins revealed the amount of NCAM180-ICD pulled down together with IC3-D2R was reduced when serine was mutated to cysteine at 311aa of D2 receptor (35% less in mutated D2R) (Fig.V.7), indicating mutated form of D2 receptor has lower affinity to NCAM180-ICD. Thus, NCAM/D2R interaction was disrupted by the mutation of D2 receptor (S311C).
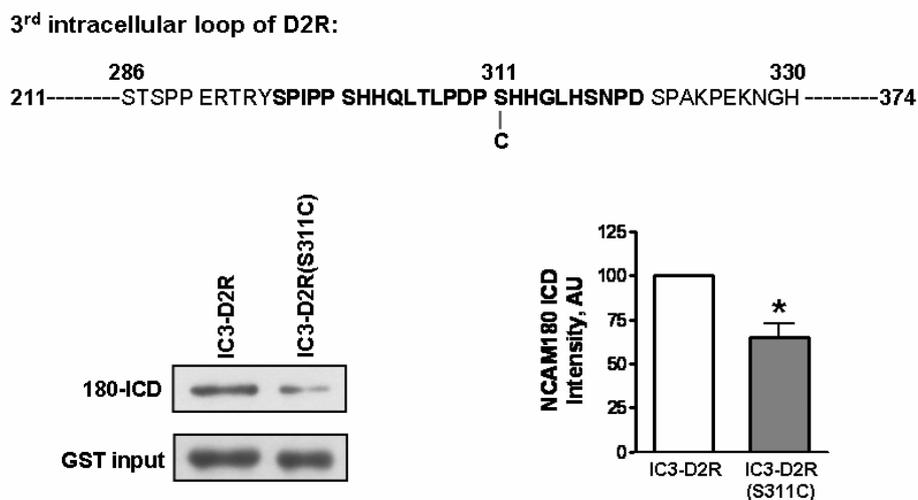
Figure V.7. NCAM/D2R interaction is disrupted by mutation of D2 receptor (S311C).

Bold letters highlights D2R peptide (amino acids 296-320), which carries the binding sequence of D2R to NCAM. GST-tagged wild type IC3-D2R and mutated IC3-D2R (S311C) were incubated with NCAM180-ICD, followed by pull down with glutathione beads. Pulled down proteins were analyzed by Western blot with NCAM antibody 5B8 and GST antibody. Optical density was quantified and set to 100% in wild-type GST-IC3-D2R. The amount of precipitated NCAM180-ICD was normalized to GST-IC3-D2R or GST-IC2-D2R (S311C) input. Compared with wild-type IC3-D2R, less NCAM180-ICD was pulled down with mutated IC3-D2R (S311C). Mean values ± SEM are shown, *$p<0.05$, *n=4.*

V.3. NCAM deficiency leads to abnormal subcellular distribution of D2 receptor

V.3.1 Total D2 receptor expression is not changed in adult NCAM deficient mouse brains.

To address the functional consequence of NCAM/D2R interaction, we first checked the subcellular distribution of D2 receptor in NCAM deficient mouse brains. First of all, dopamine receptor protein level was analyzed in total brain homogenate from adult NCAM deficient mice and wild-type littermates by Western blot analysis. Actin was served as a loading control. NCAM deficient mice show no difference in total protein level of D2 receptor as well as D1 receptor in brains in comparison with wild-type mice (Fig.V.8), indicating NCAM deficiency in mice did not change the total dopamine receptor expression in adult brains.
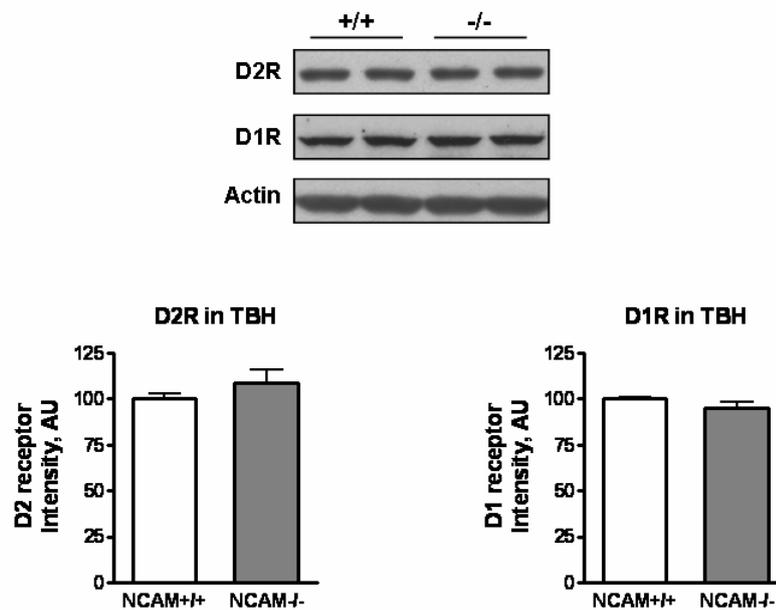
Figure V.8.   Unaltered total expression of dopamine receptors in NCAM deficient mouse brains.

Brain homogenate from 2- to 3-month old NCAM wild-type (NCAM+/+) and NCAM deficient (NCAM-/-) mice were probed by Western blot with antibodies against D1 receptor (D1R), D2 receptor (D2R) and actin as a loading control. Optical density was quantified and set to 100% in NCAM+/+ mice. In total brains, there is no difference of D2 receptor as well as D1 receptor protein expression between two genotypes. TBH: total brain homogenate. Mean values ± SEM are shown, *n=5*.

Both NCAM and D2 receptor are enriched in synapses, where they exert their biological functions, thus it's interesting to examine whether synaptic distribution of D2 receptor is altered in the absence of NCAM. Fractions enriched in synaptosomes were prepared by sucrose gradient from adult mouse brains and subjected to Western blot with antibodies against D2 receptor. No difference of D2 receptor levels was observed in synaptosomal fractions in NCAM deficient mice as comparison to wild-type mice (Fig.V.9), indicating that the total expression of D2 receptor and the synaptosomal distribution of D2 receptor is not altered in adult NCAM deficient mice.
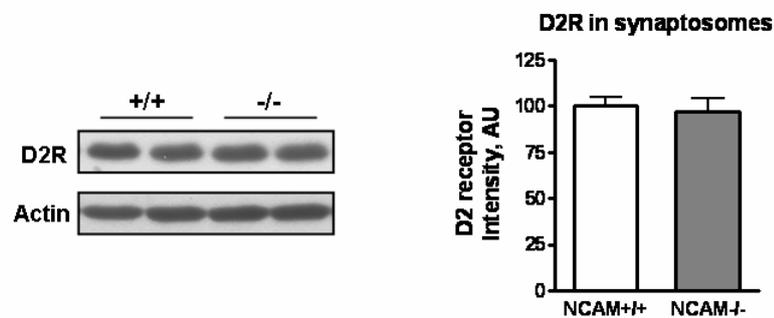
Figure V.9. Unaltered synaptosomal distribution of D2 receptor in NCAM deficient mouse brains.

Synaptosomal fraction from 2- to 3-month old NCAM+/+ and NCAM-/- mouse brains were prepared by sucrose gradient, and subjected to Western blot analysis with antibodies against D2 receptor (D2R). Actin was served as loading control. Optical density was quantified and set to 100% in NCAM+/+ mice. NCAM-/- mice have the same amount of D2 receptor in synaptosomal fraction as compared with NCAN+/+ mice. Mean values ± SEM are shown, *n*=5-6.

V.3.2 Increased D2 receptors on the cell surface with augmented receptor-mediated signaling in NCAM deficient mouse brains.

Since the total D2 receptor level was not dysregulated in NCAM deficient mice, we proceeded to examine the subcellular distribution of D2 receptor in more details. The magnitude of receptor signaling is precisely dictated by the levels of receptor available at the plasma membrane, thus plasma membranes from synaptosomal fractions of mouse brains were prepared and the cell surface localization of dopamine receptor was measured by Western blot analysis. Glyceraldehyde 3 phosphate

dehydrogenase (GAPDH) was served as loading control.

As shown in Figure V.9, NCAM deficiency in mice led to dramatic increase of D2 receptor intensity (two-fold increase) in plasma membrane fraction in comparison to wild-type mice, quantification of the levels revealed a two-fold increase of D2 receptors. In contrast, no such dysregulation was observed in the levels of D1 receptor in plasma membrane, which exhibited the comparable amount in NCAM deficient mice as NCAM wild-type mice (Fig.V.9). This result indicated that the trafficking of D2 receptor, but not D1 receptor might be dysregulated in NCAM deficient mice.
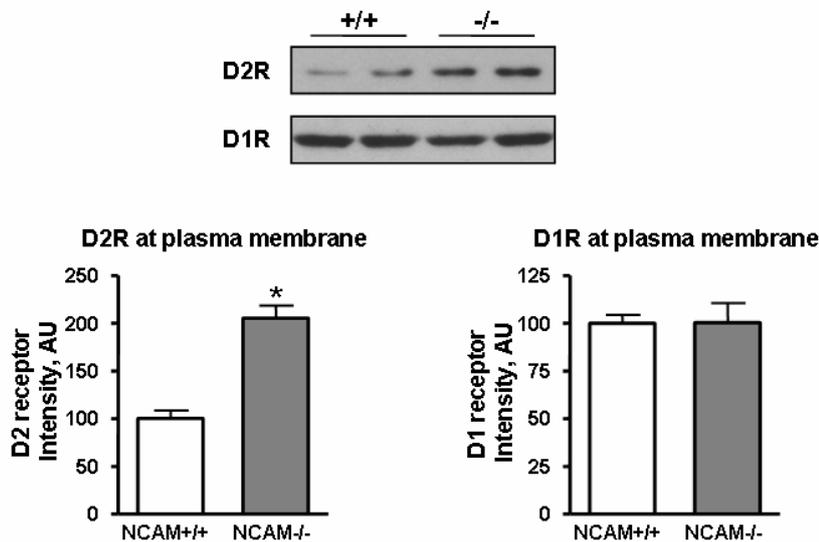
Figure V.10. Increased D2 receptor at plasma membrane from NCAM deficient mouse brains.

Plasma membrane fractions were isolated from 2- to 3-month old NCAM+/+ and NCAM-/- mouse brains by sucrose gradient and probed by Western blot with antibodies against D1 receptor (D1R) and D2 receptor (D2R). Optical density was quantified and set to 100% in NCAM+/+ mice. NCAM-/- mice showed two-fold increase of D2 receptor in plasma membrane compared with NCAM+/+ mice, whereas D1R remained unaltered in NCAM-/- mice. Mean values ± SEM are shown, *$p<0.05$, n=5-6.

Three are two isoforms of D2 receptor, long isoform (D2L) and short isoform (D2S) (Dal Toso et al., 1989; Giros et al., 1989). D2L has additional 29 amino acids in the third intracellular loop and is present mainly postsynaptically, whereas, D2S is located in presynaptic boutons and functions as autoreceptor (Khan et al., 1998). Lindgren found that the activation of different isoforms of D2 receptor led to different signal

effect: the activation of D2L can inhibit the phosphorylation of DARPP32 at Thr34 residue, whereas, D2S inhibit the phosphorylation of tyrosine hydroxylase (TH) at Ser40 residue after activation (Lindgren et al., 2003). To figure out which isoform is dysregulated in plasma membrane fraction from NCAM deficient mice, and further examine the effect of NCAM deficiency in D2R-mediated signaling transduction, the levels of phosphorylated tyrosine hydroxylase (Ser40) and phosphorylated DARPP32 (Thr34) were analyzed by Western blot using specific antibodies which exclusively recognize the specific phosphorylated forms of tyrosine hydroxylase and DARPP32. Phosphorylated protein levels were normalized to total protein expression. GAPDH was served as loading control.
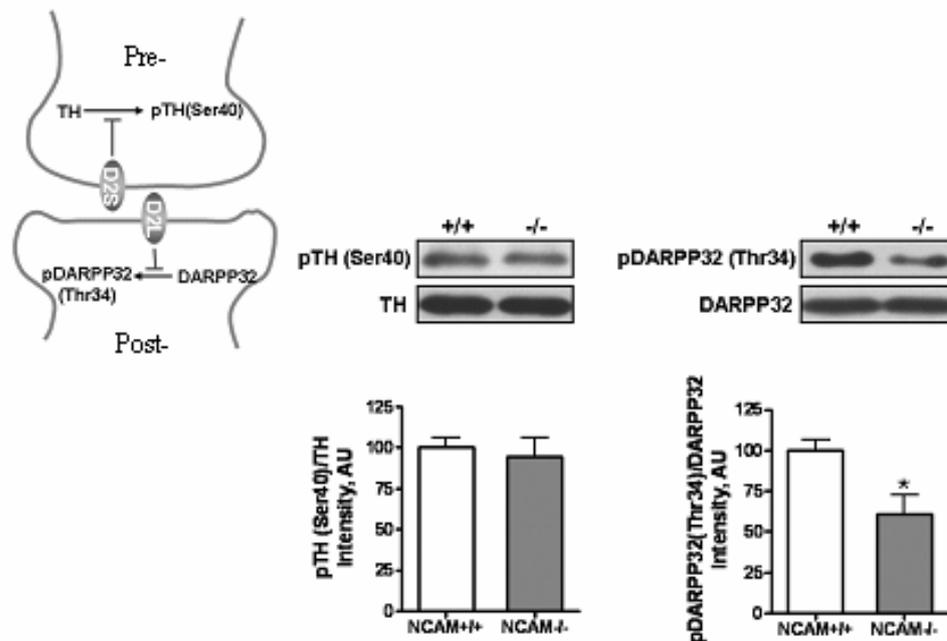
Figure V.11. Reduction of phospho-DARPP32 (Thr34) in NCAM deficient mouse brains.

Schematic representation of signaling pathways mediated by presynaptic short isoform of D2R (D2S) and postsynaptic long isoform of D2R (D2L). Brain homogenates from 2- to 3-month old NCAM+/+ and NCAM-/- mice were subjected to Western blot with antibodies against phospho-TH (Ser40), total TH, phospho-DARPP32 (Thr34) and total DARPP32. The levels of phosphorylated TH or DARPP32 was normalized to total TH or DARPP32, respectively. GAPDH was served as loading control in all blots (data not shown). Optical density was quantified and set to 100% in NCAM+/+ mice. Phosphorylated DARPP32 (Thr34) was significantly reduced in NCAM-/- mouse brains, whereas no difference was observed in phosphorylated TH (Ser40) between both genotypes. Mean values ± SEM are shown, *$p<0.05$, $n=6$.

As shown in Figure V.11, total TH and DARPP32 expression were not altered in NCAM deficient mice. However, NCAM deficiency resulted in the remarkable reduction of phospho-DARPP32 (Thr34) (~ 40% less compared with wild type mice), in contrast, no effect on phospho-TH (Ser40) was observed (Fig.V.11). These observations indicate that D2L, not D2S, is upregulated on the cell surface in NCAM deficient mice, which led to excessive D2 receptor signaling, particularly in postsynaptic terminus.

V.3.3 Unaltered dopamine content in NCAM deficient mouse brains.

Augmented D2 receptor signaling could be explained either by secondary event due to dysregulated dopamine level or by primarily altered receptor strength to the same level of dopamine. To address this question, dopamine contents in NCAM deficient mice and wild-type littermates were measured by radioimmunoassy. It's revealed that dopamine content was not affected in NCAM deficient mice (Fig.V.12), which show comparable dopamine level in brains as wild-type mice. This result indicated that enhanced D2 receptor signaling in NCAM deficiency did not result from dopamine content which remained unaltered (Fig.V.12).
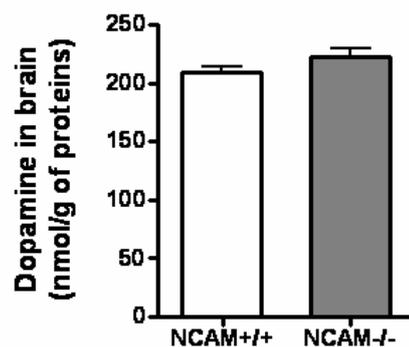
Figure V.12, Unaltered dopamine content in NCAM deficient mouse brains.

Brain homogenate from 2- to 3-month old NCAM+/+ and NCAM-/- mice were shortly centrifuged at 10,000 g to remove debris and nuclei, and supernatant was subjected to dopamine determination by radioimmunoassay and protein concentration measurement by bicinchoninic acid assay. Dopamine levels were normalized to total protein contents. No difference of dopamine levels was observed between NCAM+/+ and NCAM-/- mouse brains. Mean values ± SEM are shown, n=5-6.

V.3.4 Reduction of D2 receptor localization in endocytic compartments in NCAM deficient mice.

Since the dopamine level is not changed in NCAM deficient mice, attention was drawn back to D2 receptor itself to search for the mechanisms by which more D2 receptors were present on the cell surface. It's known that the amount of cell surface protein is mainly determined by three processes: 1) secretory pathway: neo-synthesized proteins in rough endoplasmic reticulum (ER) are transported from Golgi apparatus to cell surface via secretory vesicles; 2) endocytosis: plasma membrane proteins are internalized from cell surface to endocytic compartment, thus the amount of surface proteins decrease and proteins-mediated signaling will be attenuated; 3) recycling: some endocytosed proteins can be recycled back to cell surface via recycling vesicles and resensitize to agonist stimulation.

After endocytosis, receptors can be sorted differentially between recycling endosomes, which allow receptors to go back to cell surface, and lysosomes which degrade receptors and decrease number of receptors. It's reported that internalized D2 receptors were targeted to late endosomes and lysosomes for degradation, thereby D2 receptor responses fail to resensitize after agonist treatment (Bartlett et al., 2005). Since D2 receptors don't have recycling pathway, in my present study, I focused on the other two processes: secretion and endocytosis.

First, fractions enriched in Golgi apparatus and endoplasmic reticulum, two important components of secretory pathway, were prepared from adult mouse brains to examine the amount of D2 receptor by Western blot analysis. Difference was found neither in endoplasmic reticulum nor in Golgi apparatus between NCAM deficient mice and wild-type mice (Fig.V.13), indicating secretion pathway for D2 receptor in not altered in NCAM deficiency. Golgi matrix protein (GM130) and protein disulfide isomerase (PDI) were served as loading control for Golgi apparatus and endoplasmic reticulum, respectively.
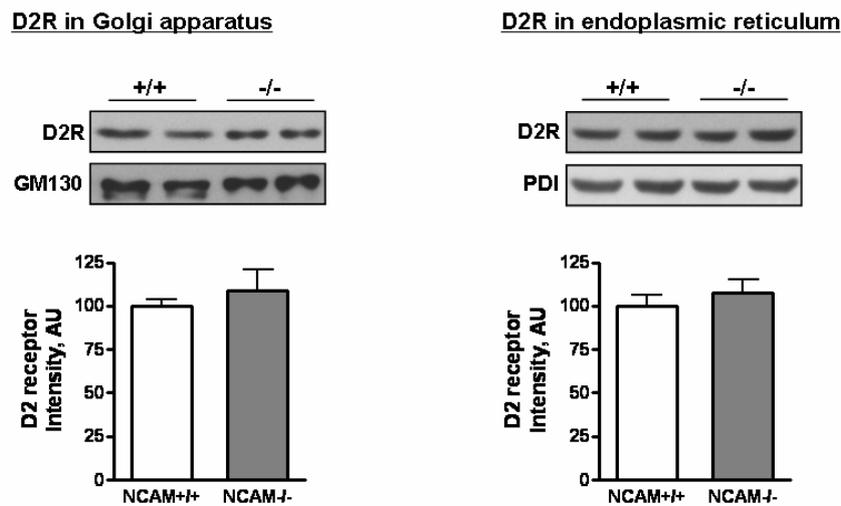
Figure V.13. Unaltered D2 receptor expression in Golgi apparatus and endoplasmic reticulum from NCAM deficient mouse brains.

Golgi apparatus and endoplasmic reticulum fractions were isolated from 2- to 3-month old NCAM+/+ and NCAM-/- mouse brains and probed by Western blot with antibodies against D2 receptor, Golgi matrix protein (GM130) as Golgi marker and protein disulfide isomerase (PDI) as endoplasmic reticulum marker. D2 receptor expression in Golgi apparatus and endoplasmic reticulum were normalized to GM130 and PDI, respectively. Optical density was quantified and set to 100% in NCAM+/+. No difference of D2 receptor expression was observed in Golgi apparatus and endoplasmic reticulum between NCAM+/+ and NCAM-/- mice. Mean values ± SEM are shown, n=3-5.

We proceeded to examine the endocytosis pathway of D2 receptor in NCAM deficient mice. Fractions enriched in endosomes and lysosomes were prepared and analyzed by Western blot with D2 receptor antibody and lysosome-associated membrane protein (Lamp2) antibody as lysosome marker. Significant reduction of D2 receptor was found in both endosomes and lysosomes fractions from NCAM deficient mouse brains (Fig.V.14). Consistent with double increase in membrane fraction, quantification showed double decrease of D2 receptor in in endosomes and lysosomes from NCAM deficient mice brains when compared with wild-type mice. Thus, we conclude that NCAM deficiency leads to abnormal subcellular D2 receptor distribution, more receptors on the cell surface and fewer receptors in the endosomes and lysosomes, indicating NCAM might play a role in D2 receptor internalization .

D2R in endosomes

D2R in lysosomes
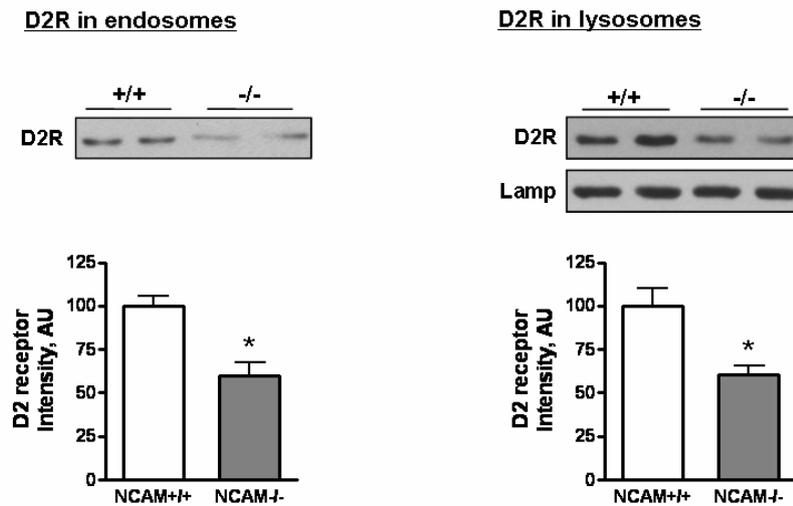
Figure V.14. Reduction of D2 receptor in endosomes and lysosomes from NCAM deficient mouse brains.

Endosomes and lysosomes fractions were isolated from 2- to 3-month old NCAM+/+ and NCAM-/- mouse brains and probed by Western blot with antibodies against D2 receptor (D2R) or lysosome associated membrane protein (Lamp) as a lysosome marker. The level of D2 receptor was normalized to loading control Lamp in lysosomes. Optical density was quantified and set to 100% in NCAM+/+ mice. Significant reduction of D2 receptor in endosomes fractions was observed in NCAM-/- mice brains when compared with NCAM+/+ mice (~50% less in NCAM-/- mice). The same reduction was observed in lysosomes fractions (~50% less in NCAM-/- mice). Mean values ± SEM are shown, *$p<0.05$, n=5.

V.4. NCAM promotes D2 receptor internalization *in vitro*

V.4.1 NCAM/D2R interaction is regulated by dopamine stimulation.

Altered subcellular distribution of D2 receptor in NCAM deficient mouse brains suggested that NCAM might be involved in the internalization process of D2 receptor. Agonist-stimulated receptor internalization is a common feature of G protein-coupled receptors in the regulation of receptor responsiveness to its ligand (Böhm et al., 1997). To investigate whether the formation of the NCAM/D2R complex depends on dopamine stimulation, D2R-expressing HEK293 cells were transiently transfected with NCAM180. Two days after transfection, cells were stimulated with dopamine for 10 min, 60 min or left untreated. Then cells were lysed and cell lysate was taken for immunoprecipitation with D2 receptor antibody. Co-precipitated NCAM180 was

visulized by NCAM180 specific antibody D3. The total NCAM180 was also checked by Western blot analysis using D3 antibody and the amount of GAPDH determined by Western blot was served as a loading control.minutes of dopamine stimulation.
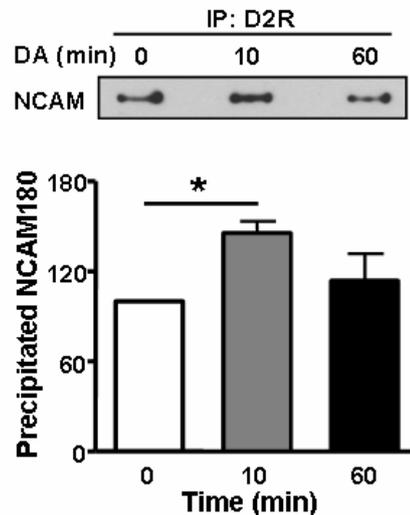
Figure V.15. Dopamine stimulation enhances NCAM/D2R association.

D2R expressed HEK293 cells were transiently transfected with NCAM180. Two days after transfection, cells were stimulated with 10 μM dopamine for 10 min, 60 min or left untreated. Then cells were lysed for immunoprecipitation with D2 receptor antibody. NCAM180 specific antibody D3 was used for detection of co-precipitated NCAM180. Co-precipitated NCAM180 was normalized to total NCAM180 (data not shown). Optical density was quantified and set to 100% in non-dopamine stimulated group. After stimulation with dopamine for 10 min, a significant increase in the amount of NCAM precipitates with D2 receptor was observed. 60 min after dopamine treatment, the amount of precipitated NCAM was comparable to that obtained under non-stimulated condition. Mean values ± SEM are shown, *$p<0.05$, $n=3$.

There was no change of NCAM180 total expression after dopamine stimulation (data not shown). However, Western blot analysis of immunoprecipitates showed a significant increase in the levels of NCAM that co-immunoprecipitated with D2 receptor after 10 min dopamine stimulation. Sixty minutes after dopamine treatment, the levels of precipitated NCAM were comparable to that are obtained under non-stimulated condition (Fig.V.15). This result indicates that the NCAM/D2R interaction is enhanced upon dopamine stimulation, and suggests that NCAM modulates the D2R function after dopamine stimulation.

V.4.2 NCAM reduces the cell surface localization of D2 receptor upon dopamine stimulation.

To further document the role of NCAM on D2 receptor internalization, the surface expression of D2 receptor was determined by cell surface biotinylation approach. In brief, cell surface proteins were labeled with biotin by Sulfo-NHS-LC-biotin. Since it is membrane impermeable, Sulfo-NHS-LC-biotin exclusively binds to membrane-bound proteins and can be used to distinguish between cell surface proteins and intracellular proteins. Cell surface proteins labeled with biotin were isolated by streptavidin beads and analyzed by Western blot. Total protein expression level in cells was determined by Western blot on whole cell lysate. Surface D2 receptors were normalized to total D2 receptor.
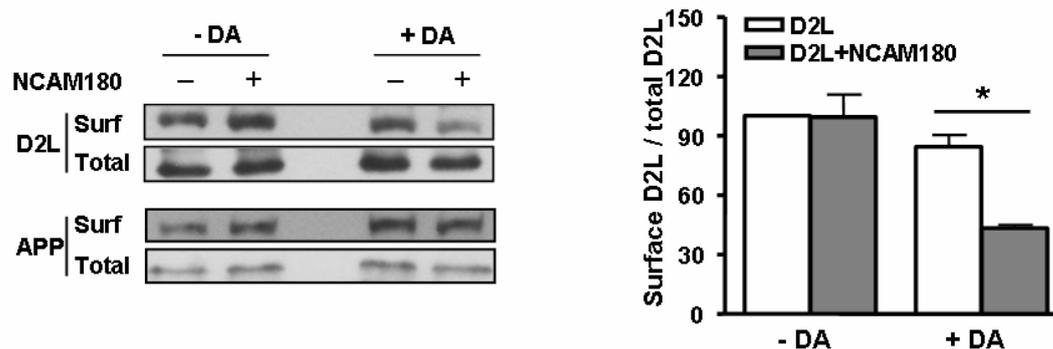
Figure V.16. NCAM reduces cell surface localization of D2 receptor upon dopamine stimulation.

Myc-D2R expressed HEK293 cells were transiently transfected with NCAM180. 48 hours after transfection, cells were stimulated with 10 μM dopamine (DA). Afterwards, cell surface biotinylation was performed with Sulfo-NHS-LC-biotin. Biotin-labeled surface proteins (surf) and total proteins (total) were analyzed by Western blot analysis. Surface D2 receptor was normalized to total D2 receptor. Optical density was quantified and set to 100% in mock transfected group without dopamine stimulation. Under non-stimulated condition, the amount of cell surface D2 receptor in NCAM transfected cells was comparable to mock transfected group. However, after dopamine stimulation, the presence of D2 receptor on cell surface was significantly reduced when NCAM was co-expressed. In contrast, another transmembrane protein, amyloid precursor protein (APP), showed constant level on cell surface. Mean values ± SEM are shown, *$p<0.05$, $n=3$.

Under non-stimulated condition, the level of cell surface D2 receptor in NCAM transfected cells was comparable to mock transfected group. Whereas, after dopamine stimulation, the presence of D2 receptor on cell surface was significantly reduced when NCAM was co-expressed (50% less than control group), which indicated NCAM promoted the internalization of D2 receptor upon dopamine stimulation. Another transmembrane protein, amyloid precursor protein (APP), which was endogenously expressed by HEK293 cells, showed a constant level on cell surface independently on NCAM and dopamine stimulation (Fig.V.16). Thus, NCAM could reduce cell surface D2 receptor upon dopamine stimulation.

V.4.3 NCAM promotes D2 receptor internalization.

To determine whether the decreased expression of D2 receptor on cell surface in NCAM expressing cells was due to enhanced internalization, antibody feeding immunocytochemistry was performed. In brief, myc-D2R expressing HEK293 cells were transiently transfected with NCAM180. Two days later, cell surface D2R were labeled with myc antibody in live cells. Afterwards, cells were stimulated with dopamine to induce D2R internalization. After fixation, remaining surface receptors and internalized receptors were stained under nonpermeabilized or permeabilized conditions respectively in the same cell to distinguish remaining surface and internalized receptors. Fluorescence intensities were quantified, and internalization index was defined as the ratio of internalized fluorescence intensity to total fluorescence intensity.

Figure V.17 shows representative images of antibody feeding immunocytochemistry experiment: there were two NCAM expressing cells (green color) which exhibited more internalized D2Rs (blue color) compared with other NCAM negative cells. Quantification of fluorescence intensity revealed that the internalization of D2 receptor was significantly higher in NCAM expressing cells both under basal condition and dopamine-stimulated condition (Fig.V.17), indicating that NCAM expression promoted the internalization of D2 receptor in HEK293 cells.
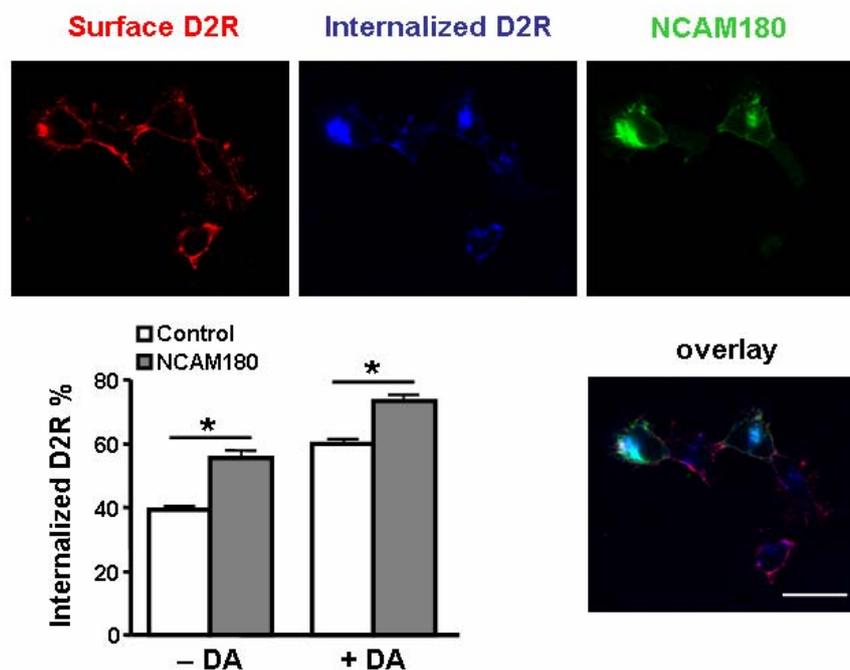
Figure V.17. NCAM promotes D2 receptor internalization.

Myc-D2R expressing HEK293 cells were transfected with NCAM180. Two days later, cells were fed myc antibody to label cell surface myc-tagged D2 receptors, followed by induction of internalization with 10 μM dopamine. After fixation, cells were processed to differential staining of remaining surface (before permeablization) and internalized (after permeablization) receptors. Representative images showed remaining surface D2 receptors (red), internalized D2 receptors (blue) and NCAM positive cells (green). Internalized D2R index, defined as the ratio of internalized to total fluorescence intensities, was presented as mean ± SEM for 45-55 cells under each condition. NCAM180 promotes D2 receptor internalization in HEK cells. *$p<0.05$. This experiment was performed two times and showed the identical results. Scale bar: 25 μm.

V.4.4 NCAM accelerates D2 receptor degradation upon dopamine stimulation.

Most internalized D2 receptors go to lysosomes for degradation and do not recycle back to the cell surface (Bartlett et al., 2005). We proceeded to examine the effect of NCAM on D2 receptor degradative fate after endocytosis upon dopamine stimulation. HEK293-D2R cells transfected with NCAM were treated with dopamine to stimulate D2 receptor internalization and degradation in the presence of cycloheximide which blocked the neo-protein synthesis. After different period, cells were harvested and the amount of D2 receptor was analyzed by Western blot on total cell lysate. GAPDH was

served as loading control.

As shown in Figure V.18, D2 receptor degraded overtime after dopamine stimulation. More interestingly, the rate of D2 receptor degradation was remarkably accelerated in NCAM-expressing cells (T/2 ~90 min and 50 min in control and NCAM-transfected cells, respectively, Fig.V.18). Three hours after dopamine stimulation, D2 receptors were hardly detectable in NCAM-transfected cells, in contrast, there were still D2 receptors in mock-transfected control cells.
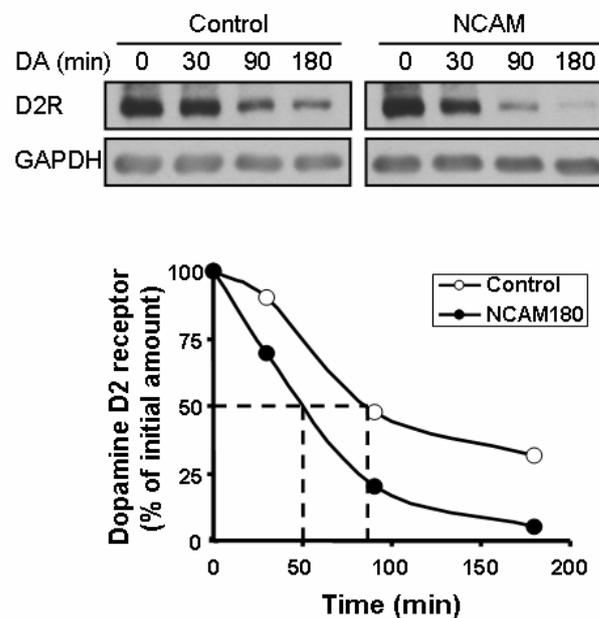
Figure V.18. NCAM promotes D2 receptor degradation upon dopamine stimulation.

Myc-D2R expressing HEK293 cells were transiently transfected with NCAM180. Two days later, cells were stimulated with 10 μM dopamine (DA) stimulation for 30 min, 90 min or 180 min, in the presence of 10 μg/ml of protein synthesis blocker cycloheximide. Cell lysate were subjected to SDS-PAGE and Western blot. GAPDH was served as loading control. Optical density was quantified and set to 100% in non-DA stimulated groups. Results shown here are representative of three independent experiments. Dashed lines designate 50% degradation.

Taken all these *in vitro* data all together, it's indicated that NCAM reduces cell surface D2 receptor and promotes the internalization and subsequent degradation of D2 receptor.

V.5 Dopamine-related locomotor behavior is altered in NCAM deficient mice

V.5.1 The behavioral response to dopamine receptor activation is enhanced in dopamine-depleted NCAM-/- mice

Since D2R internalization is dysregulated in the absence of NCAM, which in turn leads to increased amount of D2Rs at the cell surface with augmented D2R-mediated signaling, we investigated whether dopamine-related locomotor activity was also influenced in NCAM-/- mice. Although NCAM deficiency did not cause alterations of total dopamine levels in brain tissue (Fig.V.12), it is possible that the release of dopamine is altered in NCAM-/- mice. To assess direct sensitivities of dopamine receptors in NCAM-/- mice, endogenous dopamine was ablated by treatment with reserpine which depletes intracellular storage of monoamines and thus dopamine, and with methyl-DL-tyrosine to inhibit dopamine synthesis. Dopamine-depleted mice were then challenged with the non-selective dopamine receptor agonist apomorphine to assess locomotor responses to dopamine receptor activation. We investigated the locomotor activity in open fields. Mices were placed in open field boxes immediately after drug injection and then monitored for locomotor activity for 30 min at 5 min intervals. Movement time courses and total distance moved were counted.

As shown in Figure V.19, after dopamine depletion, locomotor activity was dramatically reduced both in NCAM+/+ and NCAM-/- mice, indicating endogeneous dopamine was successfully ablated by pharmacological treatment. Administration of dopamine receptor agonist, apomorphine, could partially restore the locomotor activity in dopamine-depleted NCAM+/+ and NCAM-/- mice. Furthermore, in comparison to dopamine-depleted NCAM+/+ mice, dopamine-depleted NCAM-/- mice showed a markedly enhanced response to apomorphine (Fig.V.19), demonstrating that dopamine receptor responsiveness is enhanced in NCAM-/- mice.
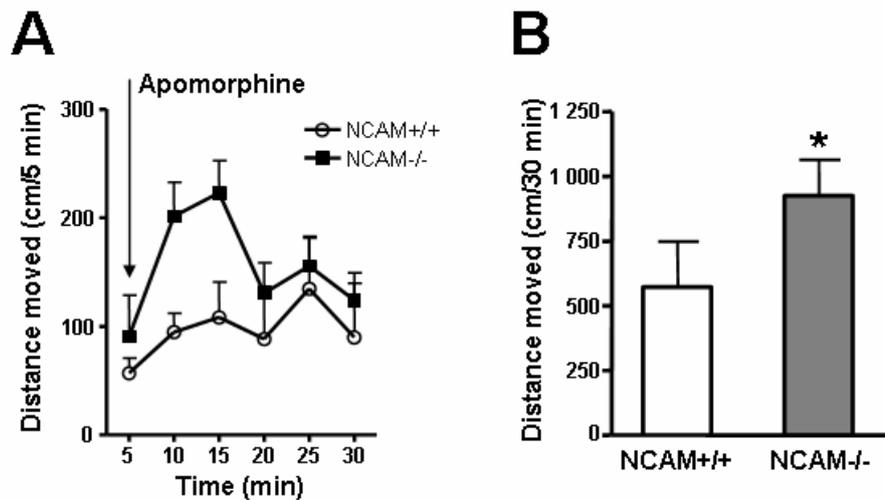
Figure V19. Behavioral responses to dopaminergic agonists are altered in dopamine-depleted NCAM-/- mice.

A combination of reserpine (5 mg/kg, i.p.) and methyl-DL-tyrosine (250 mg/kg, i.p.) was applied to deplete dopamine in NCAM+/+ and NCAM-/- mice. After injection of the dopamine receptor agonist apomorphine (1 mg/kg, s.c.), dopamine-depleted NCAM+/+ and NCAM-/- mice were placed into the open field and locomotor activity was immediately monitored for 30 min at 5 min intervals. **(A)** Time course of the effect of apomorphine on the locomotor activity of dopamine-depleted NCAM+/+ and NCAM-/- mice. Distance moved was counted at 5 min intervals. Mean ± SEM values are shown. *n*=5. **(B)** Total distance moved was measured in dopamine-depleted NCAM+/+ or NCAM-/- mice for a period of 30 min after injection with apomorphine. Means ± SEM are shown. *, p < 0.05. *n*=5.

V.5.2 Hyperactivity of locomotion in NCAM deficient mice resulted from dysregulated D2 receptor signaling

Since the dopaminergic response was enhanced in dopamine-depleted NCAM-/- mice, we further investigated which class of dopamine receptor was responsible for this hyperactivity of locomotion in NCAM-/- mice by using dopamine receptors antagonists. To test the locomotor responses to dopamine receptor antagonists, the D1R-specific antagonist SKF83566 or the D2R-specific antagonist raclopride were injected into NCAM-/- mice and NCAM+/+ littermates. Immediately after drug administration, mice were placed in open field boxes and then monitored for locomotor activity.
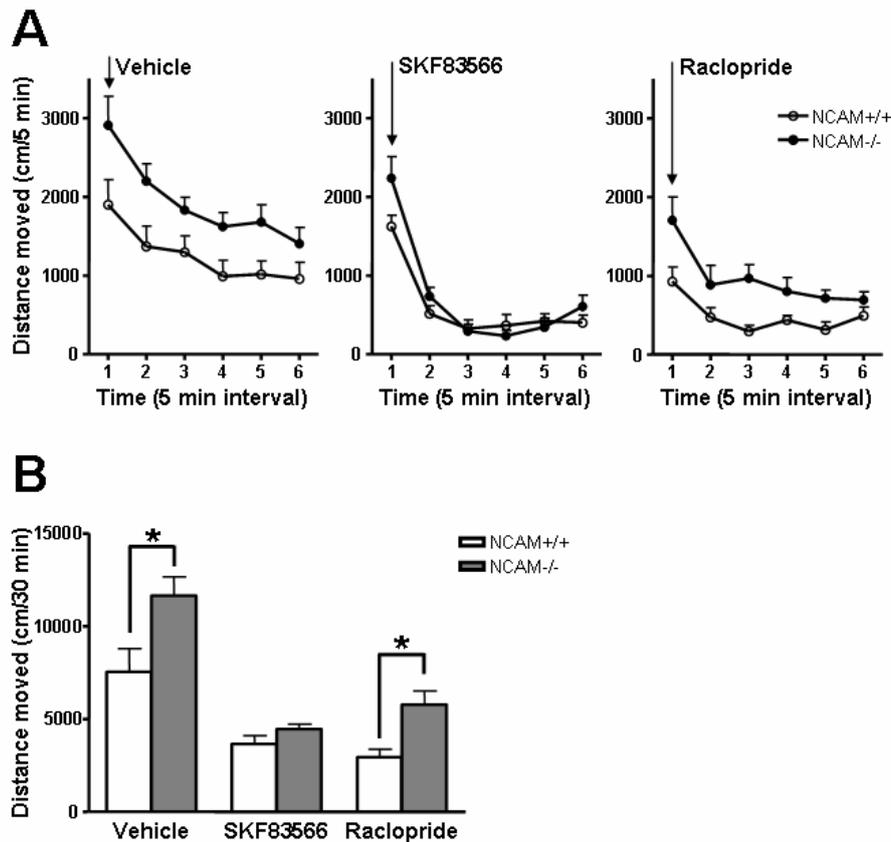
Figure V.20. Behavioral responses to dopaminergic antagonists are changed in NCAM-/- mice.

NCAM+/+ and NCAM-/- mice were placed into the open field after injection with D1 receptor antagonist SKF83566, D2 receptor antagonist raclopride or vehicle control (0.1 mg/kg of body weight, i.p.), and locomotor activity was monitored for 30 min at 5 min intervals. **(A)** Time courses of the effect of dopamine receptor antagonists on the locomotor activity of NCAM+/+ and NCAM-/- mice. Mean ± SEM values are shown. **(B)** Total distance moved was measured in NCAM+/+ or NCAM-/- mice for a period of 30 min after injection with dopamine receptor antagonists. Means ± SEM are shown. NCAM-/- mice showed higher activity of locomotion. SKF83566 injection resulted in comparably inhibitory effect in both genotypes. In contrast, the locomotor activity was still significant higher in NCAM-/- mice after administration of the same relative low dose of raclopride. *, $p < 0.05$. n=7-9.

In line with the observation from the dopamine-depletion experiment, higher locomotor activity was found in vehicle-treated NCAM-/- mice when compared with NCAM+/+ mice. After treatment with dopamine receptor antagonists, both D1R antagonist SKF83566 and D2R antagonist raclopride reduced locomotor activity in NCAM-/- mice as well as NCAM+/+ mice. No significant difference of locomotor activity was observed between genotypes after SKF83566 treatment, and SKF83566

led to comparable and low activity levels of locomotion in both genotypes (Fig.V.20). However, following administration of raclopride, locomotor activity was significantly higher in NCAM-/- mice when compared to NCAM+/+ mice (Fig.V.20), indicating enhanced activity of D2R-triggered signaling in NCAM-/- mice. These observations on locomotor activity strongly suggest that dysregulated D2R levels contribute to the hyperactivity of locomotion observed in NCAM-/- mice.

VI. DISCUSSION

Dopaminergic signaling through D2 receptor is crucial for the development and function of the nervous system. Impairment in the function of D2 receptor has been implicated in several neural disorders, such as schizophrenia and Parkinson's disease (Iversen and Iversen, 2007). Thus, to elucidate the regulatory mechanisms of D2 receptor signaling is extremely important and believed to provide beneficial insights to clinical therapeutics of neuropsychiatric disorders.

In the present study, we identified neural cell adhesion molecule (NCAM) as a novel binding protein of D2 receptor, which can regulate D2 receptor internalization/degradation and attenuate D2 receptor-mediated signaling upon ligand stimulation. The NCAM/D2R interaction was mediated by the N-terminus of NCAM intracellular domain and the third intracellular loop of D2 receptor. Furthermore, by *in vivo* functional analysis, we show that NCAM deficiency in mice leads to hyperactivity of dopamine-related behaviors, which probably results from enhanced D2 receptor responsiveness.

VI.1. Characterization of the NCAM180/D2R interaction

D2 receptor is the member of G protein-coupled receptor superfamily which contains seven transmembrane helices, three extracellular loops and three intracellular loops. Among these domains, the third intracellular loop of D2 receptor is the longest and extremely important for the function of D2 receptor. The majority of binding partners interact with D2 receptor within the third intracellular loop. For example, signaling molecules bind to the third intracellular domain of D2 receptor, such as G-protein (Obadiah et al., 1999), calmodulin (Bofill-Cardona et al., 2000), prostate apoptosis response 4 (Par-4) (Park et al., 2005) and protein phosphatase-1-binding protein spinophilin (Smith et al., 1999). In addition, it's discovered that several cytoskeleton and trafficking-associated proteins also interact with D2 receptor in this loop, like protein 4.1N (Binda et al., 2002), filamin-A (Lin et al., 2001) and arrestin (Macey et

al., 2004; Lan et al., 2008). Lee et al reported that the third cytoplasmic loop of D2 receptor directly interacted with dopamine transporter (DAT) to facilitate its cell surface localization (Lee et al., 2007). Our study identified a novel binding partner of D2 receptor, neural cell adhesion molecule (NCAM). To our knowledge, this is the first report describing the interaction between adhesion molecule and D2 receptor.

Phage display analysis indicated that D2 receptor might be a putative binding partner to NCAM180 intracellular domain (NCAM-ICD). It was confirmed by biochemical crosslinking analysis that D2 receptor specifically interacted with NCAM180-ICD, but not with NCAM140-ICD. The same results were observed by co-immunoprecipitation, which demonstrated that only NCAM180 could form complex with D2 receptor, whereas NCAM140 did not show co-precipitation with D2 receptor. As is known, NCAM180 is expressed in postsynaptic region, whereas NCAM140 is considered as presynaptically localized isoform of NCAM (Pollerberg et al., 1987). Since NCAM180, but not NCAM140 interacts with D2 receptor, it indicates that NCAM/D2R interaction occurs in postsynaptical terminus, and also implicates that it is the long isoform of D2 receptor that interacts with NCAM180 since long isoform of D2 receptor is postsynaptic form of D2 receptor, while short isoform of D2 receptor is presynaptic receptor (Khan et al., 1998).

Physiological interaction between NCAM and D2 receptor was determined by co-immunoprecipitation. From the phage display analysis, it is expected that there is direct interaction between the third intracellular domain of D2 receptor (IC3-D2R) and NCAM180-ICD. To address the direct interaction, GST pull down assays were performed by using recombinant NCAM-ICD and GST tagged IC3-D2R. Both NCAM180-ICD and NCAM140-ICD can directly interact with D2 receptor *in vitro* in pull down assays. Although co-immunoprecipitation only showed the physiological binding of D2 receptor to NCAM180, not to NCAM140, pull down assay reveals that both NCAM180 and NCAM140 have the ability to interact with D2 receptor *in vitro*. Furthermore, it is clarified that NCAM/D2R interaction was mediated by N-terminal intracellular domain of NCAM and the third intracellular loop of D2 receptor at

amino acids 296-320 by pull down assays.

The binding region of D2 receptor to NCAM is located in the third intracellular domain, which contains several phosphorylation sites. Increasing evidences show that phosphorylation is essential for certain process of D2 receptor, like internalization. Protein kinase C (PKC) can phosphorylate D2 receptor within the third intracellular loop and promote receptor internalization through a β-arrestin- and dynamin-dependent way (Namkung and Sibley, 2004). Besides, G protein-coupled receptor kinase (GRK) and neuronal calcium sensor (NCS-1) are also shown to modulate D2 receptor internalization via phosphorylation (Ito et al., 1999; Iwata et al., 1999; Kabbani et al., 2002). In the present study, we found that the interaction between NCAM and D2 receptor was enhanced under phosphorylation condition, indicating that phosphorylation plays a crucial role in the NCAM180/D2R interaction and might modulate certain function of NCAM and D2 receptor.

A positive relationship between schizophrenia and the serine-311-cysteine polymorphism of D2 receptor gene has been implicated (Itokawa et al., 1993). Since 311serine is within the binding region for NCAM, does this mutation have any effect on the NCAM/D2R interaction? Pull down assays revealed that the mutation of D2 receptor at 311serine did disrupt the NCAM/D2R interaction. The mutated D2 receptor has lower binding affinity to NCAM compared with unmutated form. Together with the observations that NCAM plays a regulatory role in D2 receptor internalization, we speculate that D2 receptor (S311C) internalization might be also impaired, which results in enhanced D2 receptor signaling and probably the onset of schizophrenia. This needs to be further investigated.

VI.2. NCAM promotes D2 receptor internalization

To investigate the functional effect of the NCAM180/D2R interaction, D2 receptor expression was analyzed in NCAM deficient mouse brains. NCAM deficient mice show unaltered D2 receptors both in total brain homogenate and synaptosomes

fraction. However, NCAM deficiency leads to altered subcellular distribution of D2 receptor. NCAM deficient mice present more D2 receptors on the cell surface with excessive D2 receptor signaling. Augmented D2 receptor signaling could be explained either by secondary event due to dysregulated dopamine level or by primarily altered receptor strength to the same level of dopamine. Since the dopamine content is not altered in the absence of NCAM, what is the mechanism underlying the increase of D2 receptor on the cell surface? Analysis with subcellular fractions demonstrate that there are dramatically reduced D2 receptors in endosomes and lysosomes in NCAM deficient mice, but no difference is observed in Golgi apparatus and endoplasmic reticulum fractions, indicating unaltered secretory pathway but dysregulated endocytosis process. When NCAM is absent, D2 receptor internalization is disrupted, as a consequence, D2 receptor in endosomes and lysosomes is decreased and the level of cell surface D2 receptor is increased, which in turn leads to augmented D2 receptor signal transduction as demonstrated by reduced phosphorylation of DARPP32 at Thr34. In particular, the abnormal D2 receptor-mediated signaling is only observed in phospho-DARPP32 (Thr34) in NCAM deficient mice, but no difference in phospho-TH (Ser40). The reduction of phospho-DARPP32 (Thr34) and unalteration of phospho-TH (Ser40) indicates that the long isoform of D2 receptor in postsynaptic region is dysregulated and presynaptic short isoform of D2 receptor remains unchanged. This is consistent with interaction analysis, which implicates NCAM180/D2R interaction occurs in postsynaptic region.

To document the role of NCAM in D2 receptor internalization, *in vitro* functional analysis was carried out. G-protein coupled receptors undergo endocytosis after ligand stimulation (Sartania et al., 2007; Martini et al., 2007; Böhme et al., 2008). Quantitative immunoprecipitation revealed NCAM/D2R interaction was enhanced upon dopamine stimulation. These results indirectly support the hypothesis that NCAM is contributed to D2 receptor internalization. To further demonstrate this issue, D2 receptor internalization was analyzed *in vitro* by using cell surface biotinylation approach and antibody feeding immunocytochemistry. We found that much less D2

receptors stayed on the cell surface and more D2 receptors were internalized and degraded in the presence of NCAM180 in HEK293 cells. Taken all these together, we conclude that NCAM promotes D2 receptor internalization and degradation and reduces the cell surface localization of D2 receptor.

So far, the molecular mechanism by which NCAM modulates D2 receptor internalization remains to be determined. It's shown that NCAM can direct interact with adaptor protein AP2 and recruit it to the plasma membrane to promote the endocytosis of synaptic vesicles in presynaptic boutons (submitted paper by Shetty et al.). Since NCAM specifically modulate D2 receptor internalization and exert no effect on D1 receptor which shows comparable level on cell surface in NCAM deficient mice, we propose that in postsynaptic spines, in contrast to presynaptic boutons, NCAM doesn't contribute to the general recruitment of AP2 and has more specialized regulatory function in proteins internalization. Otherwise, more general impairment of postsynaptic receptor internalization would be expected. In stead, we propose that NCAM might function as a "bridge" to link its binding proteins, such as D2 receptor, to endocytic machinery to facilitate its internalization. Further studies will undoubtedly be needed to demonstrate the specific mechanism of the regulation of D2 receptor endocytosis by NCAM.

G protein-coupled receptors undergo constitutive and agonist-stimulated internalization (Rankin et al., 2006). Cell surface biotinylation data indicate that NCAM is likely to be only involved in agonist-induced D2 receptor internalization since no effect was observed under non-stimulated condition. However, antibody feeding internalization assay shows that NCAM exerts its function under both non-stimulated and dopamine-stimulated conditions. An explanation for this discrepancy of the results from the two different approaches may be that the application of antibody to live cells in the antibody feeding assay leads to the clustering of receptors (Diestel et al., 2007), which are likely triggering NCAM and D2R internalization even in the absence of dopamine and thus mimic agonist stimulation. Thus, we assume that NCAM mainly functions in agonist-induced D2

receptor internalization. However, we do not exclude the possibility that NCAM might be also involved in constitutive internalization of D2R.

VI.3. NCAM modulates dopamine-related behavior

We speculate dopamine-related behavior in NCAM deficient mice is altered based on the founding that D2 receptor internalization and D2 receptor-mediated signaling are dysregulated in the absence of NCAM. As shown by open field test, locomotion activity is much higher in NCAM deficient mice when compared with wildtype mice. Dopamine receptors are classified into D1-like (D1, D5) and D2-like (D2, D3, D4) based on their physiological and pharmacological properties (Missale et al., 1998). An important question arising from the present study is which subtype of dopamine receptor is regulated by NCAM. Considering our biochemistry data which demonstrated dysregulated D2 receptor but unaltered D1 receptor in NCAM deficiency, it's indicated that elevated D2 receptor on cell surface might contribute to the abnormal locomotor activity in NCAM deficient mce. Locomotor responses to selective antagonists of dopamine receptors proves that D2 receptor, but not D1 receptor, is modulated by NCAM, since attenuated inhibitory response to D2 receptor antagonist is observed in NCAM deficient mice, whereas no significant difference is seen with D1 receptor antagonist in comparison with wild type mice. Our observations also support the requirement for the cooperative action of D1 and D2 receptors in the control of locomotion (White et al., 1988; Kobayashi et al., 2004) since remarkably reduction of locomotion activity is observed when either dopamine receptor is blocked.

Taken all data together, we propose the following working model: upon dopamine stimulation, NCAM forms complex with D2 receptor at plasma membrane via their intracellular domains, to exert its modulatory effect on D2 receptor internalization, as a consequence, modulates D2 receptor-mediated signaling and dopamine-related behavior (Fig VI). Thus, NCAM plays a critical role in the strength of dopaminergic signaling and transmission.

In conclusion, we have identified a novel molecular mechanism by which D2 receptor internalization and signaling can be modulated by NCAM via direct *in cis* interaction in postsynaptic spines. NCAM deficiency increase the level of cell surface D2 receptor and enhance the strength of D2 signaling, which in turn leads to dopaminergic hyperactivity. These observations provide a new alternative pathway by which dopaminergic system is modulated. Investigation of the function and mechanism of this regulatory pathway in dopaminergic transmission should provide new interesting insights into understanding and potentially clinically therapy of neuropsychiatric disorders.
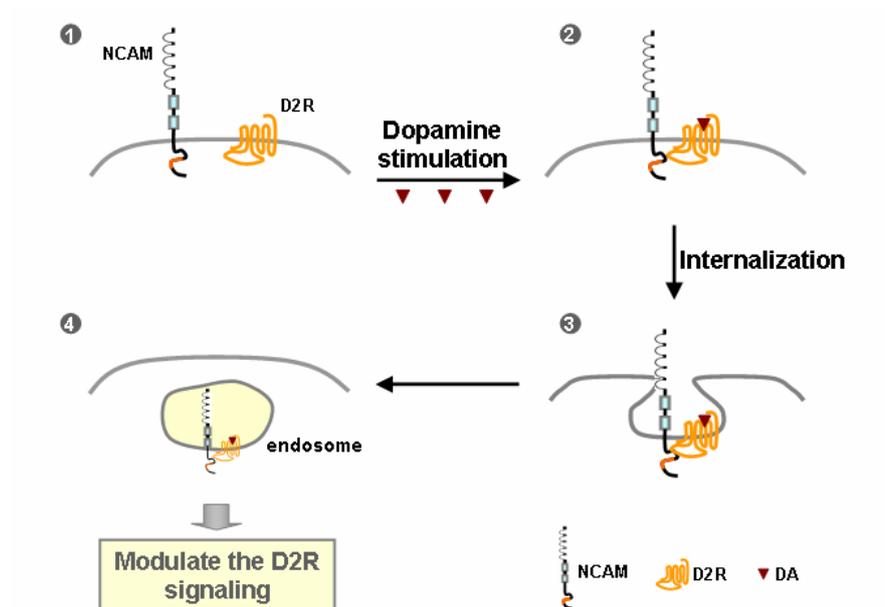
Figure VI. Proposed model illustrating the regulation of the D2 receptor signaling by NCAM180.

(1) NCAM and D2 receptor are present at plasma membrane; (2) Upon dopamine stimulation, NCAM forms complex together with D2 receptor; (3) NCAM facilitates the D2 receptor internalization into endocytic compartments; (4) NCAM reduces the levels of D2 receptor on the cell surface, leading to attenuation of D2 receptor-mediated signaling transduction.

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VIII. ABBREVIATIONS

AA	Arachidonic acid
Ab	Antibody
AC	Adenyl cyclase
Amp	Ampicillin
AP-2	Aaptor protein-2
APS	Ammonium peroxosulfate
bp	Base pair
BSA	Bovine serum albumine
CA	Cornus ammoni
CAM	Cell adhesion molecule
CHL1	Close homologue of L1
CNS	Central nervous system
D2L	Dopamine D2 receptor long isoform
D2R	Dopamine D2 receptor
D2S	Dopamine D2 receptor short isoform
DA	Dopamine
DAG	Diacylglycerol
DARPP32	Dopamine and cAMP-regulated phosphoprotein with molecular weight 32 kDa
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EBP50	Ezrin-radixin-moesin binding phosphoprotein 50
ECL	Enhanced chemiluminescence
E. Coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

FAK	Focal adhesion kinase
FCS	Fetal calf serum
FGFR	Fibroblast growth factor receptor
FNIII	Fibronectin type III domain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GASP	G-protein coupled receptor-associated sorting protein
GPCRs	G-protein coupled receptors
GPI	Glycophosphatidyl inositol
GST	Glutathione S transferase
HEPES	2-(4-(2-hydroxyethyl)-piperazine-ethane sulfonic acid
His	Histidine tag
hr	Hour
HRP	Horseradish peroxidase
ICD	Intracellular domain
Ig	Immunoglobulin
IPTG	Isopropyl-D-thiogalactopyranoside
LB	Luria bertani
LTD	Long term depression
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
min	minute
mRNA	Messenger ribonucleic acid
NCAM	Neural cell adhesion molecule
NSF	N-ethylmaleimide-sensitive factor
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase

PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase Cγ
PNS	Peripheral nervous system
PSA	Polysialic acid
PSD	Postsynaptic density
rpm	Rounds per minute
RPTP	Receptor protein tyrosine phosphatrase
RT	Room temperature
sec	Second
SDS	Sodium dodecyl sulfate
SNX1	Sorting nexin 1
Sulfo-SBED	Sulfosuccinimidyl-2-[6-(biotinamido)-2-(p-azidobenzamido)-Hexanoamido]ethyl-1,3-di-thiopropionate
TEMED	N,N,N',N',-tetramethylethylene diamine
TH	Tyrosine hydroxylase
v/v	Volume per volume
w/v	Weight per volume
x-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside

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As a native English speaker hereby I confirm that the PhD thesis by Meifang Xiao titled as "Neural cell adhesion molecule NCAM modulates Dopamine-related behavior by regulating dopamine D2 receptor internalization in mice (*Mus musculus* L., 1758)" is written in correct grammar and appropriate style.