THE ROLE OF SRC TYROSINE KINASE IN SYNAPTIC PLASTICITY AND NEUROLOGICAL DISORDERS

By

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Abstract

The non-receptor protein tyrosine kinase Src is expressed throughout the central nervous system and is involved in diverse biological functions like cell growth, differentiation, and postsynaptic signalling. Despite the well-documented functions of Src in hippocampal synaptic plasticity, roles in social behaviours, motor function, cognition, and synaptic signalling in other brain regions remain largely untested. This work investigates the neurocellular and behavioural effects of in-vivo inhibition and disinhibition of the Src tyrosine kinase pathway in mice. To this end, we employed a cell permeant Src inhibitory peptide, mutant mice harbouring a loss-of-function point mutation in Src, and a mutant mouse with deficits in the inhibitory C-terminal Src kinase (Csk). Alterations in Src signalling were associated with profound changes in NMDA receptor signalling, synaptic plasticity, motor function, cued fear conditioning, and a variety of social behaviours, underscoring the ubiquity and importance of Src signalling in the mammalian central nervous system.

Blocking the interaction of the Src tyrosine kinase with the NMDAR complex impaired auditory conditioned fear memory and social recognition. Inhibition of Src-NMDAR interactions also attenuated NR2B phosphorylation and decreased NR2B surface expression in the amygdala. Furthermore, at the lateral to basolateral nucleus pathway (LA-BLA), inhibition of Src impaired long-term potentiation. Mice harbouring a Src point mutation (Src^{thl/lhl}) mice) exhibited behavioural abnormalities and growth retardation. We also observed differences in behaviour phenotypes analogous to mouse models of Williams-Beuren syndrome (WBS) and humans with
WBS. WBS is neurodevelopmental disorder characterized by distinctive facial features, hypersociability, mild to moderate mental retardation, and a unique cognitive disability (Meyer-Lindenberg et al., 2006). Sociability and social vocalization were increased in three different social affiliation tasks in $Src^{(thl/thl)}$ mice. Mutant mice exhibited hyperactivity in the open field and spent significantly less time in the centre of the open field. Also, motor function was impaired in three different motor performance tasks. The $Src^{(thl/thl)}$ mice showed an enhanced startle response to loud stimuli, impaired cued fear conditioning, and deficient visiospatial memory in the Morris water maze. Furthermore, $Src^{(thl/thl)}$ mice were not able to learn a visual object recognition task. These results underscore the importance of Src in an array of behavioural, motor, and cognitive functions in mice.

The C-terminal Src kinase (Csk) acts to suppress Src activity, so Csk(+/−) mice were employed to examine the behavioural impact of enhanced Src signalling. Decreased Csk expression led to enhanced long-term and short-term social olfactory recognition and social transmission of food preference. We also found elevated NR2B phosphorylation in the olfactory bulb and amygdala, two brain regions critical for the behavioural expression of anxiety and social recognition in mice.

Deficiencies in the Src tyrosine kinase pathway were associated with impaired synaptic plasticity in the amygdala and behaviour disturbances that are relevant to WBS. Conversely, up-regulation of the Src tyrosine kinase pathway by reducing Csk expression increased social olfactory cognition. A more detailed understanding of the Src pathway could facilitate the development of new treatments for diseases characterized by aberrant social behaviours.
Dedication

I dedicate this work to the loving memory of my father Dr. Akbar Sinai.
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### 3 « THE ROLE OF SRC IN SYNAPTIC PLASTICITY AND LEARNING/MEMORY »

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- Src inhibition reduces NR2B surface expression and synaptic plasticity in the amygdala

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- Association of Src mutation in mice with phenotypes related to Williams Beuren Syndrome

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- Impaired olfactory social memory in 129P2 inbred mouse strain can be rescued by reducing Csk expression in the brain

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Chapter 1
Introduction

1 « Src family kinases »

1.1 « History »

Peyton Rous first described a virus that induced tumours in chickens (Rous, 1911). In 1950, it was shown that tumours can arise after infection with the Rous sarcoma virus (Thomas and Brugge, 1997, Rubin, 1955). In the 1970s, Brugge identified v-Src as the transforming protein of the oncogenic Rous sarcoma virus in serum from tumour-bearing rabbits (Brugge et al., 1977). In addition, it was demonstrated that the viral Src gene (v-Src) has a highly conserved and ubiquitously expressed cellular homologue, c-Src (Stehelin et al., 1976). Although Src is ubiquitously expressed, it is expressed at high levels in neurons (Cotton and Brugge, 1983), platelets (Golden et al., 1986), and osteoclasts (Horne et al., 1992). In addition to the ubiquitously expressed isoform of Src, there is also a neuron specific isoform that result from differential splicing of extra exons into the SH3 domain (Brugge et al., 1985). A great deal of information about the cellular functions of the Src has come from studies of Src(-/-) knockout mice. Despite being involved in a variety of cellular processes, including proliferation, differentiation, and migration, the only readily observable phenotype of Src(-/-) mice is the development of osteopetrosis, a defect in bone re-absorption that leads to an overgrowth of bone (Soriano et al., 1991). In 1992, Seth Grant et.al has shown that mutations in the src gene did not interfere with either the induction or the maintenance of LTP (Grant et al., 1992).
More recently, Src(-/-) mice have also been found to have a defect in lactation, implicating Src in mammary gland development as well (Watkin et al., 2008). These findings suggest that although Src is expressed in most cells and is involved in many cellular processes, it is not necessary for the majority of them. The endogenous Src was the first known protein to possess protein tyrosine kinase activity (Bishop et al., 1983), and is now one of the most intensively studied kinases. At present, eight members belonging to the Src family kinases (SFKs) are known: Fyn, Yes, Lyn, Yrk, Hck, Blk, Fgr, and Lck. The SFKs mediate a broad spectrum of physiological responses, including cell cycle control, proliferation, differentiation, migration, and survival (Thomas and Brugge, 1997). Five of these SFKs, Src, Fyn, Yes, Lck, and Lyn, are highly expressed in the mammalian central nervous system (CNS), where they regulate neuronal differentiation and neurite outgrowth (Kuo et al., 1997a, Kuo et al., 1997b, Hoffman-Kim et al., 2002). The NMDA receptor was the first ion channel shown to be modulated by Src (Wang and Salter, 1994). Electrophysiological and pharmacological studies indicated that tyrosine phosphorylation and dephosphorylation of NMDA receptor subunits could enhance and suppress NMDAR-mediated synaptic currents (Wang and Salter, 1994).

1.2 Structure

Mutational studies and structural modeling based on crystallography data have revealed the structure of Src. In vertebrates, Src family proteins have similar structures (Brown and Cooper, 1996) and range in molecular mass from 52 to 62 kDa. All Src proteins contain six distinct functional domains: Src homology domain 4 (SH4), a unique domain, SH3 domain, SH2 domain, a catalytic domain (SH1), and a C-terminal regulatory region. The SH4 domain at the amino (N) terminus of the protein contains from 15 to 17 amino acid residues with contains myristolation and palmitoylation motifs important for anchoring Src to the plasma membrane.
The unique domain (U) spans amino acid residues 18-84 and is distinct in each Src family member. This region may be responsible for interactions with other protein tyrosine kinases (PTKs) and PKC. The SH3 domain located between amino acid residues 85-140 plays an important role in interactions with protein substrates. This region is also responsible for intramolecular interactions controlling catalytic kinase activity (Pawson, 1995). The SH3 domain of Src binds to proline-rich regions in substrates. All known SH3 ligands carry a PXXP sequence (where X is any amino acid). The SH2 domain (amino acid residues 141-260) controls a range of proteins interactions. It recognizes a short amino acid sequence carrying phosphotyrosine. In the Src tyrosine kinase family, SH2 is more conserved than the SH3 domain and can bind to specific proteins phosphorylated by tyrosine. The catalytic domain at amino acid residues 265-516 is found in all proteins of the Src family. It confers tyrosine kinase activity and contains the activation loop that includes the key activator residue tyrosine (Y) 416. Phosphorylation of Y416 stimulates complete activation of Src and provides a binding site for the SH2 domains of other cellular proteins. The C-terminal regulatory region at amino acid residues 517 to 536 plays a significant role in regulation of Src kinase activity. All kinases of the Src family have a C-terminal regulatory subregion of 15-19 conserved amino acid residues that include the key regulator residue Y527. When Y527 is phosphorylated, the C-terminus can bind to the SH2 domain, leading to a closed protein conformation. The close conformation shows 98% lower kinase activity than the p-Y416 active form (Salter and Kalia, 2004) (Figure 1).
Figure 1-1 Domain structure and regulation of activity in SFKs

The Src family kinases contain 6 distinct functional domains: Src homology 4 (SH4) domain, unique domain (U), SH3 domain, SH2 domain, linker region, catalytic domain (SH1 domain, N- and C-lobes), and regulatory domain (right). Intramolecular interactions and internal tyrosine phosphorylation and dephosphorylation regulate protein structure and kinase activity.

Phosphorylation of Y527 (by Csk) promotes the association of the SH2 domain with Y527. This in turn promotes dephosphorylation of Y416, resulting in the closed or inactive state (Y416/p-
Y527. Protein phosphatases, such as PTP, can dephosphorylate Y527. Autophosphorylation at Y416 in the catalytic domain prevents the SH2–C-terminus interactions, resulting in the open and active form p-Y416/Y527 (Figure adapted from Salter and Kalia 2004).

1.3 Regulation of Src kinases

Kinase activity is tightly controlled through phosphorylation at two regulatory sites, Tyr 416 and Tyr 527. Autophosphorylation of the Src family kinases on Tyr 416, located within the activation loop, increases activity by stabilizing the kinase domain in an open conformation that promotes kinase-substrate interactions and catalysis. In contrast, the phosphorylation of Tyr 527 in the C-terminal tail of the Src family kinases by Csk (C-terminal Src Kinase) (Nada et al., 1991) results in the intramolecular binding of the C-terminal tail to the Src Homology 2 (SH2) domain and the concomitant docking of the Src Homology 3 (SH3) domain onto the SH2-kinase linker. Together, these intramolecular interactions stabilize the kinase domain in an inactive close conformation. The SFKs are usually present in an inactive state. Full inactivation of the Src family kinases requires both dephosphorylation of the activation loop and phosphorylation of the C-terminal tail (Figure 1). SFK becomes activated through dephosphorylation of the C-terminal tyrosine or through binding to another protein that displaces the intramolecular interaction.

1.4 Csk a negative regulator of Src

In 1980, Csk (C-terminal Src kinase) was identified as a negative regulator of Src that promoted Src deactivation by phosphorylating Y527, resulting in the closed conformation (Figure 1) (Okada et al., 1991). Deletion of the Csk gene leads to early embryonic lethality with severe
neutral tube defects. In contrast, Csk gain-of-function can down-regulate SFK-mediated cell signalling (Chow et al., 1993). Also, Csk-Src double knockout mice show more normal neural development, which again suggests that Csk is a negative regulator of Src (Thomas et al., 1995). The genomic sequences of Src and Csk genes show 40% identity. Indeed, the sequence of Csk includes a SH3 domain, a SH2 domain, and a catalytic domain similar to Src. In contrast to Src, however, Csk lacks the N-terminal lipid anchoring domain, the activation loop, and a C-terminal tyrosine-containing Y527 (Figure2), resulting in a distinct regulatory mechanism. Some evidence suggests that the SH2 and/or SH3 domains of Csk are essential for SFK regulation (Sabe et al., 1994, Cloutier et al., 1995) and Csk is only functional when attached to the membrane (Chow et al., 1993, Cloutier et al., 1995, Howell and Cooper, 1994). It is known that Csk is recruited to lipid rafts in the membrane by a phosphoprotein, Cbp, that can tightly bind to the SH2 domain of Csk upon phosphorylation to block SFK signalling (Kawabuchi et al., 2000, Brdicka et al., 2000). In general, proteins that bind to the SH2 domain of Csk, such as Cbp, Paxillin, and FAK, appear to act as important regulators of Csk activity (Sabe et al., 1994).

![Image: Molecular structures of Src and CSK]

Figure 1-2 Molecular structures of Src and CSK
1.5 The NMDA receptors

The NMDA receptors (NMDARs) are heteromeric complexes composed of NR1 (ζ in mouse) and NR2 (ε in mouse) subunits (Monyer et al., 1992). In some cases, an NR3 subunit can assemble with NR1 but the role of this subunit in the central nervous system is still unclear (Cull-Candy et al., 2001). The NR1 subunit is essential for pore formation and for the binding of several regulatory ligands, whereas NR2 subunits (NR2A-D) bind glutamate and determine the kinetic characteristics of specific NMDAR multimers (Monyer et al., 1992). Unlike the NR1 subunit, NR2A and NR2B are tyrosine phosphorylated by Src family kinases (Lau and Huganir, 1995). Electrophysiological studies demonstrated that NMDA receptor activation required two independent glutamate and two independent glycine binding sites (Clements and Westbrook, 1991), suggesting that functional NMDA receptors are tetramers composed of two NR1 and two NR2 subunits (Mayer and Armstrong, 2004). In contrast to most other ionotropic glutamate receptors that only permeable to sodium and potassium, NMDA receptors are also permeable to calcium (Mayer and Westbrook, 1987).

1.5.1 The role of the NMDA receptor and Src in synaptic plasticity

The most commonly studied form of synaptic plasticity in the CNS is NMDAR-dependent long term potentiation at glutamatergic synapses. It is widely believed that the NMDA receptor is important for learning and memory by acting as a coincidence detector of presynaptic and postsynaptic activity that initiates changes in synaptic strength, leading to the formation of new functional neuronal circuits. Coincident pre-synaptic glutamate release from axonal action
potential firing and post-synaptic action potentials (likely back-propagating from the cell body) are essential for full NMDA receptor activation. Post-synaptic depolarization relieves the channel blockade mediated by extracellular Mg$^{2+}$, allowing influx of ions, including calcium into the postsynaptic neuron. The calcium ion triggers a multitude of biochemical signalling cascades catalyzed by enzymes (kinases, lipases, and proteases) located in the postsynaptic membrane.

In essence, the post synaptic calcium signals conveys information about the depolarization state of the post synaptic neuron (Salter and Kalia, 2004). During low frequency synaptic transmission, glutamate binds to two different receptors that are usually co-localized on individual dendritic spines (the small outgrowths from the dendritic shaft that are the postsynaptic site of synaptic contacts). The first is the AMPA receptor, which has a channel permeable to Na$^+$ and K$^+$. These monovalent cations are responsible for generating postsynaptic responses when the cell is close to its resting membrane potential. The second is the NMDA receptor, which is a ligand and voltage-dependent channel because of the Mg$^{2+}$ blockade at hyperpolarization membrane potentials. Under low-frequency stimulation, there is little postsynaptic depolarization so NMDA receptor activity is minimal. In response to higher frequency stimulation, however, depolarization from temporal summation and short-term plasticity (facilitation of glutamate release) causes Mg$^{2+}$ to dissociate from its binding site within the NMDA receptor pore, allowing Ca$^{2+}$ as well as Na$^+$ influx into the dendritic spine (Salter and Kalia, 2004). The ensuing rise in intracellular Ca$^{2+}$ is the critical trigger for long-term potentiation (LTP) following high-frequency stimulation. If the Ca$^{2+}$ does not reach the threshold for LTP, it can generate either long term depression (LTD) or a short term potentiation
(STP, sometime called post-tetanic potentiation) that decays to baseline over 5 to 20 minutes (Nicoll and Malenka, 1999).

The Src family tyrosine kinases are essential for induction of LTP in the CA1 region of the hippocampus, the structure where the mechanisms of LTP have been most thoroughly investigated (Lu et al., 1998). The dynamic regulation of NMDARs by SFKs and protein tyrosine phosphatases (PTPs) modulates the threshold for NMDAR-dependent synaptic plasticity. Under basal conditions, NMDAR activity is suppressed by partial blockade of the channel by Mg\(^{2+}\) and by the activity of the STEP phosphotyrosine phosphatase. High-frequency (tetanic) stimulation increases NMDAR-mediated currents (by relief of Mg\(^{2+}\) inhibition) and activates the cell adhesion kinase β (CAKβ) and Src, which overcome the suppression by STEP, and by sensitizing the NMDARs to raised Na+. Upregulated NMDARs allow greatly increased entry of Ca\(^{2+}\), which binds to calmodulin (CaM) and activates CaMKII (calcium/calmodulin-dependent protein kinase) and PKC, the key initial triggers for LTP. Expression of LTP is ultimately mediated by insertion of additional AMPARs into the postsynaptic membrane or by enhanced AMPAR channel conductance (Huang et al., 2001), resulting in larger excitatory post-synaptic potentials (EPSPs).

1.5.2  The exocytosis of NMDA receptors

Functional NMDA receptors are likely formed from co-assembly of two NR1 subunits and two NR2 subunits (Ulbrich and Isacoff, 2007). The NR1 subunit is not trafficked to the cell surface when expressed alone due to the presence of an endoplasmic reticulum (ER) retention signal (Scott et al., 2001). Such ER retention motifs are also present in the C-terminal of NR2 subunits. Co-expression and association of NR1 and NR2 subunits masks these ER retention motifs in the
third trans-membrane domain of the protein, leading to cell surface expression (Horak et al., 2008). Thus, it is necessary for NR1 and NR2 subunits to co-assemble in the ER to form functional receptors before they can be trafficked to the plasma membrane.

The NR1/NR2A and NR1/NR2B are the major receptor subtypes expressed in the brain. The NR2B-containing NMDA receptors are more abundant in the early stages of neuronal development and are present at both synaptic and extra-synaptic sites (Cull-Candy et al., 2001), while NR2A-containing receptors are predominant in the adult brain (Moon et al., 1994). The ER, the Golgi apparatus, and the trans-Golgi network are responsible for trafficking of NMDAR to the neuronal plasma membrane. NR2B may also bypass trans-Golgi and be directly transported to Golgi outpost in dendrites (Jeyifous et al., 2009). Multimeric NMDA receptors exit from the ER after assembly and are transferred to the Golgi network where they are packed in mobile transport units. These receptors move along the microtubules and are eventually recruited to the synapses by intracellular signals (Washbourne et al., 2002). During this process, NMDA receptors are transported along the microtubules more rapidly than AMPA receptors (Washbourne et al., 2002). Intracellular NMDA receptors are associated with several other proteins to form an exocytosis complex before they are targeted to the membrane.

1.5.3 The endocytosis of NMDA receptor

Dynamic regulation of receptor endocytosis is critical for both the expression of synaptic plasticity (LTP) through AMPA receptor insertion and for regulating LTP threshold by altering the postsynaptic NMDA receptor population. Both NR2A and NR2B subunits have been shown to interact with the adaptor protein-2 (AP-2), followed by an attachment to clathrin-coated vesicles (Roche et al., 2001). The internalization of NMDARs is regulated by the clathrin-coated
vesicle pathway. This pathway is regulated by interactions between a tyrosine-based internalization motifs located in NR2 and the clathrin adaptor protein 2 (AP2), which delivers NMDARs into clathrin-coated vesicles. The NR2A and NR2B subunits contain different internalization motifs (Lavezzari et al., 2004), the LL motif in NR2A and the YEKL motif in NR2B (Lavezzari et al., 2004, Roche et al., 2001). In both HEK cells and neurons, activity-dependent phosphorylation of the NR2B YEKL motif at Y1472 by Src suppresses clathrin-mediated endocytosis of NMDARs (Zhang et al., 2008). Similarly, phosphorylation of Y1472 is also required for proper localization of NR2B-containing receptors at synapses in the amygdala of NR2B Y1472F knock-in mice. These mice showed reduced surface expression of NR2B and impaired NMDAR-mediated signalling through calcium/calmodulin-dependent kinase II (CaMKII). The NR2B knock-in mice also exhibited reduced LTP at amygdala synapses and impaired cued fear conditioning, implicating phosphorylation in NMDAR trafficking, postsynaptic expression, and regulation of synaptic plasticity.

Neurons treated with the SFK inhibitor SU6656 showed decreased NR2B phosphorylation (Zhang et al., 2008). Phosphorylation of NR2B Y1472 is increased when Cdk5 activity is inhibited pharmacologically or is absent (in Cdk5-/- brains) via the activity on Src tyrosine kinase (Zhang et al., 2008). Although Fyn has been identified as the predominant kinase responsible for the phosphorylation of NR2B in HEK 293T transfected cells, Y1472 NR2B phosphorylation is still present in Fyn mutant mice (Nakazawa et al., 2001), suggesting compensatory activity by other SFK family members like Src. Zhang et al(2008) confirmed this in transfected HEK 293T cells by showing that NR2B phosphorylation is increased by Src when Cdk5 is inactivated, whereas NR2B phosphorylation was not observed in HEK cells transfected with constitutively active Fyn. Thus, Src tyrosine kinase can phosphorylate NR2B Y1472 NR2B phosphorylation but is under chronic Cdk5-mediated suppression.
Regulation of NR2A subunits at the cell membrane also depends on tyrosine phosphorylation as tyrosine dephosphorylation triggers NR2A internalization (Vissel et al., 2001). The endogenous NMDAR agonist glutamate induces dephosphorylation of Y842 in the C-terminal tail of the NR2A subunit and causes a conformational change that promotes receptor internalization. The number of NMDARs would be stabilized at the cell surface of neurons because ambient glycine is just below the concentration to induce internalization. At times of high neuronal firing, as occur during seizures or the initiation of ischemia, the amount of extracellular glycine increases to activate this internalization mechanism, constituting a possible neuroprotective pathway.

Figure 1-3 NMDA receptor trafficking (Adapted from (Lau and Zukin, 2007))
1.6 Signalling pathways of Src

1.6.1 Signalling pathways of Src involved in synaptic plasticity

The Src family kinases are essential components of the multiple signalling pathways that modulate NMDA receptor surface expression and conductance. These pathways include receptor tyrosine kinase-mediated pathways; G-protein coupled receptor pathways, and signalling though small non-membrane associated G-proteins.

Receptor tyrosine kinase pathways include the EphB receptor-mediated activation of Src family kinases that regulate NMDA receptor function. Stimulation of neuronal EphB receptors increased tyrosine phosphorylation of NR2B subunits, and this response was suppressed by pharmacological SFK inhibitors. Furthermore, activation of EphB receptors has been shown to mediate Src activation and tyrosine phosphorylation-dependent enhancement of NMDA receptor function (Takasu et al., 2002).

Activation of G-protein-coupled receptor pathways can lead to Src-dependent NMDAR phosphorylation. This pathway signals through protein kinase C (PKC) and cell adhesion kinase-(CAK) to activate Src and up regulates NMDAR function. The PKC family of serine/threonine kinases increases both AMPA and NMDA responses in neurons. The PKC-mediated enhancement of AMPA is mediated through serine phosphorylation (S831) of AMPA subunit 1 (GluA1). Inhibition of PKC does not alter the Src-induced enhancement of NMDA responses but inhibition of Src depressed the potentiation of NMDAR currents by activated PKC.
Therefore Src and PKC are in the same pathway and PKC up regulates NMDARs through Src (Lu et al., 2000, Lu et al., 1999). Lu et al. provided the first evidence that tyrosine kinases are necessary for the induction, but not the maintenance, of LTP in area CA1 of the hippocampus. Activation of Src through PKC involves the non-receptor protein tyrosine cell adhesion kinase beta (CAKβ) which is highly expressed throughout the CNS, including the hippocampus. Activated CAKβ binds to the SH2 domain of Src and activates it by disrupting the intramolecular interaction (Figure 1). So, the PKC-CAKβ-Src pathway works upstream of NMDAR; stimulation of PKC leads to CAKβ activation, which then activates Src, leading to phosphor-activation of NMDARs (Kotecha et al., 2003, Kotecha and MacDonald, 2003). This pathway might be initiated by muscarinic receptors and the mGluR5 receptors (both are Gq-coupled receptors).

The Ras family of non-receptor GTP binding proteins inhibit Src and down-regulate NMDAR function. In H-Ras knockout mice, NMDAR-mediated synaptic responses are enhanced but AMPAR responses remain unchanged (Manabe et al., 2000). These mice show a higher phosphorylation of both NR2A and NR2B subunits and PTK activity is increased. Activated H-Ras suppressed Src activity and reduced NR2A phosphorylation, while exogenous H-Ras decreased Src activity and NR2A tyrosine phosphorylation in brain slices (Thornton et al., 2003, Salter and Kalia, 2004). Release of an excitatory neurotransmitter like glutamate depolarizes the postsynaptic neuron, resulting in the influx of Ca\(^{2+}\) through both ionotrophic receptors (mainly NMDARs) and voltage-gated Ca channels. Intracellular Ca\(^{2+}\) activates Ras family small G proteins through an unknown mechanism involving Src family tyrosine kinases. The association of Ras and Raf with other regulatory proteins then induce the phosphorylation and activation of Raf. Alternatively, Ca\(^{2+}\)/CaM can stimulate adenylyl cyclases, resulting in the accumulation of cAMP that in turn activates cAMP-dependent kinase PKA, which activates the Raf activator
Rap1. The ensuing activation of Raf leads to the sequential phospho-activation of MEK and MAPK. Activated MAPK has multiple targets, including the transcription factor CREB that mediate long-term adaptive changes in neurons through changes in gene expression. The resulting synthesis of new proteins mediates the long-term remodelling of the synapse believed to underlie long-term memory and the late sustained phase of LTP (L-LTP). Other potential targets of MAPK are cell adhesion molecules (CAMs), cytoskeletal elements, and ion channels (Wong et al., 1999).

1.6.2 The role of Src inhibitory peptide in synaptic plasticity

A specific role of Src in synaptic plasticity has been studied by the use of an inhibitory antibody (anti-src1) (Roche et al., 1995) and an inhibitory peptide, Src(40–58), (Yu et al., 1997), that has no effect on other members of the Src kinase family kinases. Both of these Src-specific inhibitors decreases synaptic NMDAR-mediated currents and each produced a decrease in NMDAR channel gating (Yu et al., 1997). Src(40–58) is the antigen for anti-src1 and corresponds to amino acids 40–58 within the unique domain of Src. These inhibitors are suggested to block Src-mediated upregulation of NMDAR activity by disrupting the interaction of the Src with NMDARs to modify receptor function (Gingrich et al., 2004). It has been demonstrated that, the Src-specific inhibitors prevent the increase in channel activity produced by the SFK-activating pYEEI peptide (Yu et al., 1997). In addition, the SFK activator pYEEI peptide increased synaptic AMPAR responses, which is prevented by Src(40–58). The pYEEI-induced increase in AMPAR responses can be blocked by chelating intracellular Ca2+, but this can not affect the increase of NMDAR currents. However, blocking NMDARs prevents the potentiation of AMPAR responses by pYEEI. Thereby, upregulation of NMDARs through Src is necessary for tetanus-induced LTP in CA1 neurons (Ali and Salter, 2001). In agreement with this model, the level of Y1472 phosphorylation of NR2B increased after tetanic stimulation in
area CA1 (Nakazawa et al., 2001) and tyrosine phosphorylation of NR2B increased after LTP induction in the dentate gyrus (Rosenblum et al., 1996). Also, PTPα, an activator of SFKs, has been shown to be important for in LTP induction in the hippocampus (Lei et al., 2002, Petrone et al., 2003) 39,98. The induction of LTP in area CA1 is impaired in mice with a targeted deletion of PTPα. This impairment is associated with a reduction in phosphorylation of Y1472 in the NR2B C-tail in the PTPα−/− mice (Petrone et al., 2003).

1.6.3 The possible role of Src signalling pathways in Williams-Beuren syndrome

Many growth factors induce proliferation and differentiation by binding to receptor tyrosine kinases. Receptor binding in fibroblasts can lead to receptor autophosphorylation and Src phosphorylation. In turn, c-Src phosphorylates the tyrosine residues Y248 and Y611 of transcription factor TFII-I, causing nuclear translocation and subsequent activation of several immediate early genes, including c-Fos.(Cheriyath et al., 2002, Hong et al., 2005). The TFII-I family of transcription factors consist of three members: TFII-I (or GTF2I), WBSCR11 (or GTF2IRD1) and GTF2IRD2. Both TFII-I and GTF2IRD1 are deleted in Williams-Beuren Syndrome (WBS) (Tassabehji et al., 2005) as part of a larger micro-deletion on chromosome 7q11.23. Isoform TFII-I (the first member identified in this group) is a basal transcription factor and also a transcription regulator. However GTF2IRD1 is only a transcription regulator. Individuals with WBS have craniofacial anomalies, vascular problems (like supravalvular aortic stenosis, SVAS), and mild to moderate mental retardation, specifically visuospatial and numerical impairments.
The GTF2IRD1 gene was associated with craniofacial and visiospatial symptoms in WBS. According to chromosomal deletion data in humans, however, the GTF2I gene encoding TFII-I is thought to contribute to aberrant social behaviours in WBS, including increased gaze and interest towards strangers. The facial features of WBS were associated with deletions of GTF2IRD1, TFII-I, and CYLN2 but not with deletions that preserve these genes (Karmiloff-Smith et al., 2003). It was also demonstrated shown that loss of both GTF2I and GTF1IRD1 in mice was responsible for craniofacial features, while heterozygous deletion of exons 2-4 in GTF2I caused hyper-sociability.

1.7 LTP and learning in the amygdala

Long-term potentiation is a possible neurocellular mechanism that contributes to learning and memory. This proposed relation is based on several properties of LTP. First, LTP induction is rapid following appropriate synaptic input (Nicoll and Malenka, 1999). Second, it is long-lasting. Indeed, LTP can last for hours in vitro and for days in behaving animals (Bliss and Lomo, 1973). Long-term potentiation consists of at least two temporal phases (E-LTP and L-LTP) that have kinetic and pharmacological properties that mirror aspects of memory. Among the most intriguing of these is the sensitivity of both L-LTP and long-lasting memory to inhibition of protein synthesis following LTP induction or behavioural training. Third, LTP is associative and input specific. It requires coincident pre-synaptic neurotransmitter release and action potential firing in the post-synaptic neuron (Sundberget al. 1977), thus fulfilling a theoretical requirement first proposed by Hebb (1949) to explain simple forms of associative
learning like classical conditioning. The input specificity can be observed in an experiment where LTP is induced in one pathway without affecting a second convergent input.

A voluminous body of work has focused on whether synaptic strength is enhanced during learning. LeDoux and colleagues were among the first to establish such a link. They first demonstrated that stimulating geniculate nucleus (MGN) fibres could evoke LTP in the lateral amygdala (LA). Furthermore, auditory-evoked EPSPs in LA could be potentiated by brief pairing with a painful shock, demonstrating that an LTP-like neuronal response could accompany cued fear conditioning. While learning is best defined as a change in behaviour resulting from experience, memory is best defined as a multistep process within the brain that consists of acquisition, consolidation, retrieval, and expression (McGaugh, 2000). The explicit acquisition and short term processing of information requires the hippocampus. For instance, the famous patient HM had profound memory impairments after bilateral resection of his medial temporal lobe including the hippocampus. He was unable to remember all episodes after his surgery, but retained some remote memories from long before his surgery (Scoville and Milner 1957). Similarly, animals with hippocampus lesions were unable to learn new information (Morris et al., 1982). Consolidation is defined as a process in which this newly acquired information is stored. During memory retrieval, previously encoded memories are brought to consciousness (Tronson et al., 2006) or at least expressed as observable behaviours. The amygdala is essential for the acquisition, storage, and retrieval of fear-related memories. During auditory fear conditioning in mice, information about the auditory and the aversive shock stimulus converge on the lateral amygdala and induces synaptic plasticity (Romanski and LeDoux, 1992, Fanselow and LeDoux, 1999). Furthermore, cortical areas that are both afferent and efferent to the amygdala participate in storage and encoding of specific aspects of fear memories (Fanselow and LeDoux 1999).
The origins of classical conditioning date to Ivan Pavlov’s famous experiments on dog gastric physiology in the 1920s. He paired an initial neutral auditory stimulus of a metronome repeatedly with an unconditional stimulus, food. Eventually, the tone elicited salivation in the dog without any food delivery, presumably because the tone becomes predictive of food delivery (Pavlov 1927). The classical conditioning is widely used as Pavlovian fear conditioning. Usually, a neutral conditioned stimulus (CS) is paired with an unconditioned aversive stimulus (US) that produces an unconditioned fear response (UR). Freezing is an innate fear response used as a behavioural memory read out. Freezing is a robust and rapidly encoded behaviour that depends on long-term synaptic plasticity in the amygdala (LeDoux, 2000, Maren and Quirk, 2004).

1.7.1 The structure and connectivity of the amygdala

The amygdala is an almond-shaped structure located within the temporal lobe, anterior to the hippocampus. It contains three groups of nuclei: 1) the basolateral group, including the lateral, the basal (or basolateral nucleus (BLA)) and basomedial nucleus, 2) the cortical like group, including the cortical nuclei and the nucleus of the lateral olfactory tract, and 3) the centromedial group, including the central and medial nuclei. There are also several cell masses in the connection area between amygdala and hippocampus. The amygdala receives inputs from the frontal, cingulate, insular, and temporal cortices, as well as from subcortical regions like the thalamus, hypothalamus, and brain stem (Amaral and Insausti, 1992).

The inputs from the cortex convey glutamatergic signals from sensory systems and from areas linked to memory in the medial temporal lobe. The amygdala receives sensory input from all
major modalities: olfactory, somatosensory, auditory, and visual. Few connections reach the amygdala directly from the primary somatosensory cortex; most afferents go through the dysgranular parietal insular cortex in the parietal lobe (Shi and Cassell, 1998). These projections target the LA, BA, and CeA (McDonald and Jackson, 1987). Nociceptive signals are transferred to amygdala via the thalamic nuclei, and the posterior internuclear nucleus (PIN) involved in fear conditioning pathway (LeDoux, 1994). Also, the amygdala receives highly processed polymodal information from the prefrontal cortex, the perirhinal cortex, and the hippocampus.

Different pathways exist inside the amygdala; unimodal sensory information enters the lateral part of the lateral amygdala (LA), whereas polymodal information from the medial temporal lobe enter the medial LA, which is thought to be a region for integrating sensory information with past experiences (Pitkanen et al., 2000). The LA sends inputs to basal and central nuclei. Basal nuclei project back to the LA. Also, cortical regions send glutamatergic projections to the basal nucleus which goes to central nucleus of the amygdala (CeA). The central nucleus is the main out nucleus, sending projections to other regions of the brain with few reciprocal connections with other amygdalar nuclei (Jolkkonen and Pitkanen, 1998). These cortical projections from the amygdala target all regions of the temporal and occipital lobes, as well as the parafrontal cortex (PFC) and anterior cingulate cortex (Price at al, 1996).

In addition to projects to and from the medial temporal lobe memory system, prefrontal cortex, and thalamus, the amygdala also projects to hypothalamus, forebrain, and the brain stem that coordinates both emotional behaviour and exerts global effects on cortical information processing. Conditioned fear results in enhanced synaptic transmission through the major amygdalar nuclei. The CeA transmits these outputs to the pons, midbrain, and medulla to influence autonomic response, to the hypothalamus, resulting in elevated blood pressure, and to the bed nucleus of the stria terminalis (BNST), triggering the release of stress hormones.
Projections to brain stem produce behaviours such as freezing, vocalization, startle responses, and cardiovascular changes (Rizvi et al., 1991, Bellgowan and Helmstetter, 1996). Projections from the amygdala to hypothalamus regulate reproductive, ingestive, and defensive behaviours. The basolateral nucleus sends projections back to the medial temporal lobe, thus influencing ongoing memory processing (Petrovich et al., 2001). The BLA projects strongly to the hippocampus but also connects to the nucleus accumbens, thalamus, and prefrontal cortex. In sum, the amygdala integrates environmental cues predictive of emotionally salient events and coordinates emotional responses mediated by the autonomic nervous system.

1.8 Amygdala dependent behaviours

1.8.1 Fear conditioning

The amygdala is essential for both the encoding and storage of fear memories (Campeau and Davis, 1995a, LeDoux, 2000). Sensory information encoding the unconditional stimulus (US) and any coincident neutral stimuli (conditioned stimuli) is conveyed by glutamatergic projections that terminate on neurons within both the basolateral and lateral nuclei (Fanselow and LeDoux, 1999). Neurons within the LA and BLA exhibit short-latency firing during US and CS presentation (Quirk et al., 1995, Maren et al., 1991). Temporal pairing of the US and CS can induce long-term potentiation of CS-evoked AMPA-mediated synaptic responses both in vitro and in vivo (McKernan and Shinnick-Gallagher, 1997), suggesting that NMDAR-dependent Hebbian synaptic plasticity mediates the US-CS association at the neuronal level. Similarly, tetanic stimulation also induces a form of LTP that can be blocked by NMDAR antagonists.
It is known that different parts of the amygdala have different functions. For instance, the BLA is involved in fear acquisition while the CeA is involved in fear expression. Lesioning the CeA eliminates the freezing response (Hitchcock and Davis, 1986). Also, the BLA appears to “store” the conditioned fear memory through an enhanced synaptic response to the conditioned stimulus. The activation of NMDAR (Rodrigues et al., 2001) and voltage-gated calcium channels (Bauer et al., 2002) are necessary for the formation of fear memories. For example, NR2B specific antagonists can block both cued fear memories and synaptic potentiation in the amygdala (Bauer et al., 2002, Li et al., 1995, Nakazawa et al., 2006). Significantly, mice lacking the Src phosphorylation site Y1472 of NR2B also show deficits in fear learning (Nakasawa et al., 2006, 2002).

In addition, a multitude of other signalling molecules are necessary for the formation of fear memories, as infusing pathway-specific blockers greatly attenuates cued fear memories. Indeed, inhibition of the protein kinase A (PKA) (Schafe and LeDoux, 2000), Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CAMKII) (Rodrigues et al., 2004), extracellular signal-regulated kinases (ERKs), and mitogen-activated protein kinases (MAPKs) (Schafe et al., 2000) disrupt protein synthesis required for long term amygdalar plasticity and cued-fear memory. Similarly, blocking protein synthesis using anisomycin in the amygdala blocked both LTP in the amygdala and the formation of fear memories (Schafe and LeDoux, 2000). In conclusion, there is substantial evidence that the amygdala is critical for acquiring, storing, and controlling the expression of conditioned fear memories. These features play an important role in rodent life by storing associations between environmental cues predictive of survival enhancing or threatening situations.
1.8.2 Social behaviours

The amygdala is involved in both social affiliation and social recognition behaviours (Borelli et al., 2009, Ferguson et al., 2001, Kang et al., 2009, Richter et al., 2005). In 1990, Brothers suggested that socio-emotional behaviour may be mediated by a network of three structures: the amygdala, the prefrontal cortex, and the temporal cortex (Brothers et al., 1990). The amygdala is involved in processing and generating socio-emotional behaviours (Adolphs and Spezio, 2006, Phelps, 2006). These conclusions were drawn from non-human and human primate lesion studies, as well as neuroimaging. Damage to the amygdala disinhibited approach characteristics and caused abnormal friendliness in humans (Adolphs et al., 1999). The amygdala is also required for the accurate identification of social cues from individual faces by extracting information from the eye regions (Adolphs et al., 2005). Moreover, when amygdala-lesioned patients were asked to distinguish between trustworthy and untrustworthy faces, they failed to identify an unapproachable face as untrustworthy (Adolphs, 1999). Similarly, autistic patients can only extract emotional facial expressions from the mouth region, whereas normal individuals extract information through eyes and mouth (Spezio et al., 2007a, Spezio et al., 2007b). We propose that the amygdala is not only involved in identifying dangerous situations or faces but also plays a role in identifying complex social stimuli to guide appropriate social behaviours.

In humans, it is not known which parts of the amygdala are important for identification of social stimuli because of the low resolution of imaging techniques (Zald, 2003). However, animal studies have shown that multiple regions of the brain interact during social recognition in mice. The main olfactory olfactory system (MOS), main olfactory bulb (MOB), and limbic structures
are involved in the processing of volatile stimuli. However, the accessory olfactory system (AOS), including the medial nucleus of the amygdala, the septum, the bed nuclei of the lateral olfactory tract, and the striata terminalis of the medial preoptic area are all involved in the processing of non-volatile stimuli. In summary, the amygdala participates in a network that processes social information and generates socio-emotional behaviours. Its particular function might be to attribute social meaning to faces and environmental situations (Cooke, 1998).

Rodent social recognition behaviours are highly dependent on the olfactory bulb system and the amygdala. The pathway connecting the olfactory bulb to the amygdala is of special interest in rodents due to their reliance on odours as social and emotional cues (in addition to signs of food and danger). In response to specific cues, both unconditional or conditioned, amygdala outputs coordination endocrine and autonomic responses to emotionally relevant environmental stimuli (Brennan and Zufall, 2006). Fos expression, which is indicative of neuronal activity, is enhanced in the basolateral and medial amygdala following social recognition tasks (Borelli et al., 2009).

The amygdala is essential for mental health and appropriate emotional processing of the inputs from the outside world, and amygdala dysfunction is implicated in psychological and neurodevelopmental disorders. The amygdala is the loci for emotional memory storage and can modulate the processing and storage of memories in other brain regions. In addition, abnormal social behaviour in neurodevelopment disorders such as autism and WBS relates to abnormal amygdala function.

People with Williams–Beuren syndrome (WBS) have unusual personality traits, including overfriendliness and charismatic speech rich in vocabulary. About 70% also suffer from attention deficit disorder and many experience phobias and anxiety. Hyperacusis (hypersensitivity to specific sound frequencies) is observed in most of WBS cases (Meyer-Lindenberg, 2009). Autism spectrum disorders are characterized by three core deficits: impaired
communication, impaired social interactions, and restricted, repetitive, and stereotyped patterns of behaviours (Canitano and Scandurra).

Post-traumatic stress disorder (PTSD) is characterized by a collection of emotionally painful and impairing symptoms occurring after a traumatic experience, usually involving repeated memories related to the trauma (flashbacks) and hyperarousal manifested as concentration deficits and exaggerated startle responses. The amygdala has been shown to be hyperreactive in patients with PTSD when presented with trauma-related stimuli (Damsa et al., 2005).

Abnormalities in amygdalar function have also been reported in autism. Social behaviours differ significantly between autistic patients and individuals with amygdala damage or WBS. Autistics individuals tend to avoid socialization and interpersonal communication, while patients with amygdala damage and WBS are overly friendly and show a profound interest in socialization and communication. Another main difference between these two disorders is the ability to maintain eye contact. Children with Williams-Beuren syndrome spend more time maintaining eye contact whereas children with autism fail to do so. Amygdalar function is central to these disorders, but it is important to stress that emotional and social behaviours are the product of a complex interplay between the amygdalar and other brain regions such as the prefrontal and medial lobe regions.

1.8.3 Hypothesis
Enhanced NMDA currents induced by Src-mediated NR2 phosphorylation serves to enhance or lower the threshold for NMDA-dependent LTP induction. Indeed, LTP in single neurons is blocked by injection of the Src inhibitor Src(40-58) (Lu et al., 1998), while a peptide that activates Src [EPQ(pY)EEPIA] can increase NMDA channel activity in inside-out patches when applied to the cytoplasmic surface of neuronal membranes. Furthermore, NR1 is co-immunoprecipitated from synaptic membranes with anti-Src antibodies (Yu et al., 1997). Since
LTP is a possible neurocellular mechanism required for some forms of memory, we hypothesized that the Src kinase pathway must play an important role in learning and memory. However, mutant mice that lack Src show more robust LTP in the CA1 area of the hippocampus than mice that lack Fyn, possibly due to functional redundancy and changes in dominant expression with brain maturation. Based on behavioural experiments that engage other brain regions, however, it is possible that Src is critical for other forms of memory, including social memories. In order to test our hypothesis, we decided to use the experimental three approaches outlined below based inhibition and disinhibition of Src activity.

1) We designed a cell permeant version of the Src peptide inhibitor by linking it to the HIV Tat-1 transduction domain to inject systemically into mice after development (at 12-14 wks old) and to examine the effect of Src inhibition on fear conditioning and social recognition behaviours. We proposed that Src inhibition will impair NMDAR-dependent learning based on the known role of Src in NMDAR dependent synaptic plasticity.

We also proposed that Src inhibition will reduce NR2B surface expression by clathrin mediated endocytosis through a cyclin-kinase 5 (Cdk5)-dependent pathway that is regulated mainly by Src (Zhang et al., 2008).

2) We took advantage of a point mutation within the kinase domain of Src that leads to a premature stop codon. This mutation predicts a deletion of ½ of the kinase domain. These mice were predicted have reduced Src activity without compensatory fyn activity because the mutant Src retains the domains important for anchoring to the membrane and for binding with interacting partners. These mice were also predicted to demonstrate both anti-autistic and WBS-like phenotype based on the evidence cited above (Section 2.6.2)
3) To study the behavioural and neurocellular effects of Src up-regulation, we examined mice deficient for the chronic Src inhibitor C-terminal Src kinase (Csk). We predicted that Csk (-/+ ) mice would display enhanced performance in some Src-dependent tasked due to up-regulation of NMDAR activity.

The overall aim of these approaches was to investigate the role of the Src tyrosine kinase pathway in NMDAR regulation and in social behaviours that may depend on NMDAR function. The role of Src in social behaviours could lead to deeper insights into the pathophysiology of neurodevelopmental disorders characterized by abnormal social behaviours.
Chapter 2

2 Materials and Methods

2.1.1 Subjects

Mice were housed in groups of 5 in transparent cages on wood chip bedding and provided with dry food pellets and water *ad libitum*. They were maintained on a 12:12 hour light-dark artificial cycle (lights on from 05.00 to 17:00) in a temperature (20±1C) and humidity (50-60%) controlled vivarium. Mice were tested during the light phase between 08.00 and 16.00 h. All animal procedures were approved by the Animal Management Committee of Mount Sinai Hospital and Toronto Centre for Phenogenomics in compliance with the requirements of the Province of Ontario Animals for Research Act 1971 and the Canadian Council on Animal Care.

For social recognition task, male juvenile mice of the same background were obtained from our own breeding colony (25–30) days old.

Src mutant mice were first discovered during a routine observation of our animal colony. The mice were significantly smaller relatively to their littermates and lacked incisors and some molar teeth. When maintained on a special mashed food diet $Sr^{thl/thl}$ mice survived at least until one year of age (the end of the observation period). Heterozygous $Sr^{thl/+}$ mice produced affected progeny at the expected Mendelian ratio.

Genome-wide association studies were performed on 20 affected mice using a panel of microsatellite markers positioned approximately 20 cM apart. For narrowing down a critical interval region both microsatellite and SNP markers were used. Genotyping has been performed at the TCAG facility (The Centre for Applied Genomics, Hospital for Sick Children, Toronto).
After placing the \textit{Src} gene within the critical interval region, we sequenced a \textit{Src} coding region from one affected mouse using primers spanning every exon of the gene. The primers design and sequencing was performed by the TCAG core facility.

Genotyping \textit{Src}(thl/thl) mice involved the amplification of a 180-bp PCR product using the primer pair: 5’-CTATCCTTCTATCAGGAATAACCAG-3’ and 5’-GTTCTCCCCTACTAGGATATTG-3’. PCR products from wild type mice lacked a Hpa II (Fermentas, Burlington, ON, Canada) restriction site that is created after the insertion of a C nucleotide into the \textit{Src}(thl/thl) mice genome. All experiments were done with 1.5-5 month old littersmates that were housed in groups of 4-5 per home cage.

For the Tat-Src (40-58) inhibitor studies, adult C57BL/6J mice were purchased from Jackson Laboratory (8-14 wks old).

129/Sv-\textit{Csk}\textsuperscript{mlSor}/J mice were a kind gift from Yu, X., originally purchased from the Jackson Laboratory (Bar Harbor, ME). These mice were crossed for 10 generation to 129P2 purchased from Harlan Laboratories. The behaviour procedures were performed under dim light conditions, except anxiety tasks that required bright light.

2.1.2 Drugs

Tat-Src (40-58), Tat-sSrc (40-58) and Dansyl-Tat-Src (40-58) were synthesized at the hospital for Sick Children Advanced Protein Technology Centre (Toronto) on a Novasyn-Crystal Peptide Synthesizer (NovaBiochem (U.K) LTD., Nottingham, U.K.) by Fmoc-chemistry on PEG-PS resin. The solvent used throughout the synthesis was freshly purified DMF. A solution of 20% piperididine in DMF was used for the removal of the Fmoc-protection group. For each 0.5 g of resin (0.1 mmole substitution) four times excess of Fmoc-amino acid activated with HATU and disopropylethylamine (1:1:2 mol/mol/mol) was uses for the coupling reaction.
The reaction time was one hour at room temperature. After the synthesis, the peptide-resin conjugate was washed two times with iso-propanol and another two times with diethyl ether and dried under reduced pressure. The dry peptide-resins conjugate was treated with 20 ml of TFA containing 4 ml thioanisole, 0.4 ml m-cresol, 2 ml 1, 2-ethanedithiol, and 4 ml bromotrimethylsilane at 0°C for two hours. After removal of the resin by filtration and TFA under reduced pressure, 50 ml of diethyl ether and 50 ml of 0.1% TFA solution were added to the residue. The aqueous layer was extracted three times with diethyl ether and freeze-dried. The crude peptide was dissolved in 0.1 TFA and desalted on a Sephadex G10 column. The peptide was then analyzed by reverse phase HPLC on a Bondclone C18 column (Phenomenex). Mass Spectrometry analysis was performed on a Biosystems/MDS Sciex MALDI Qstar XL quadropole time-of-flight (QqTOF) instrument. The peptides were dissolved in saline and administered i.v. at a dose of (9.3 nmol/g) 60 min before the training phase or testing phase of each experiment. Dose and route of administration was chosen in a pilot experiment where dansyl tagged Tat-Src (40-58) reaches peak concentration 75 min after an i.v. injection. Concentrations of the inhibitor would reach undetectable levels in the brain by 24 h post-injection.

2.1.3 Western blot

Dissected whole brain or amygdala tissue were homogenized in 300µl of RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) per gram tissue containing protease and phosphatase inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Homogenates were centrifuged at 15,000 × g for 20 min at 4°C, supernatants were collected, and protein concentrations were measured by a Bradford assay (Bio-Rad, Hercules, CA, USA). Protein samples (30 µg) were suspended in loading buffer (Bio-Rad, Hercules, CA, USA) containing 2-
mercaptoethanol (Sigma, Oakville, ON, Canada), incubated at 95°C for 5 min, and loaded onto an SDS-PAGE gel (6% and 8%) along with 5 μl of MagicMark XP Western Protein Standard (Invitrogen, Burlington, ON, Canada) to be separated (100 V, ~2 h) using a Bio-Rad electrophoresis system (Bio-Rad, Hercules, CA, USA). Proteins were then electrotransferred (30 V, overnight) onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked with 5% BSA (Bio-Rad, Hercules, CA, USA) in PBS-T solution (PBS, 0.1% Tween-20) for ≥1 h, and then incubated with one of the following primary antibodies: Src (Ab7950, Abcam), polyclonal antibodies against phospho Tyr-1472 and NR2B were a kind gift from Takanobu Nakasawa (Nakasawa et al., EMBO J, 2006) and Millipore, Csk antibody Monoclonal (anti-Csk, mouse, BD Biosciences).

Incubation with the primary antibody was completed in blocking solution overnight at 4°C. Following washes with PBS-T (3 × 10 min), the membrane was incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse IgG. The membrane was washed in PBS-T (3 × 10 min) and then processed for chemiluminescence using a Western blotting detection kit (GE Healthcare, Piscataway, NJ, USA). Equal loading was confirmed by immersing the membrane in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) prior to incubation with a rabbit anti-β-tubulin III polyclonal antibody (1:20,000 in blocking solutions for 1 h; Sigma, Oakville, ON, Canada).

Exposure of the membrane to photographic film permitted for visualization of protein bands that were then quantified by densitometric analysis using the Image J 1.41 software (http://rsbweb.nih.gov/ij/). Each densitometric value was normalized to its respective β-tubulin III loading control. The absolute intensity of the western blot bands is measured by multiplying the mean value and the pixel value of each band which provides a measurement of intensity and
band size. The absolute intensity of each sample is then divided by absolute intensity of the standard sample to measure the relative intensity.

Preparation of Synaptosomal fractions (p2) from AM and OB, and immunoblotting were performed as described by (Xu et al., 2008). Different brain areas were dissected and homogenized in homogenization buffer containing: sucrose (320mM), Tris-HCl (10mM, pH 7.4), NaHCO3 (1mM, pH 7.4), EDTA (2mM), sodium orthovanadate (1mM) and 1% (v/v) protease inhibitor cocktail. Homogenate (H) was subsequently centrifuged at 1000g for 15min to remove nuclei and other large debris (P1). The supernatant (S1) was centrifuged at 10,000g for 30min to obtain a crude synaptosomal fraction (P2). Western blot analyses were performed on (P2) fractions as mentioned above.

2.1.4 Dissociated neuronal amygdala cultures

Pregnant mice were sacrificed by cervical dislocation and dissociated neuronal amygdala cultures were prepared from postnatal day 1 (P1) mouse brain. Dissociated amygdala neurons were plated on poly-D-lysine-coated glass coverslips at a density of 115,000/25mm coverslip. Neurons were grown in Neurobasal medium supplemented with B27 and GlutaMax (Invitrogen) for 18–20 d in vitro (DIV). To analyze the effect of inhibition of Src, cultures were treated for 60 min with either 20 µM Tat-Src (40-58) dissolved in saline or with saline (control).

2.1.5 Immunocytochemistry

In order to visualize NMDAR surface expression, neurons were fixed after treatment with 4% paraformaldehyde, 5% sucrose in PBS for 10 min, washed with PBS, and then blocked with 10% normal goat serum (NGS) in PBS for 1 h. Surface NMDARs were stained overnight in 3% NGS in PBS with a mouse antibody that recognizes an extracellular epitope of the NR1 subunit (NR1, 1:200; BD PharMingen). After washes in PBS, neurons were permeabilized with
0.2% Triton X-100 in PBS with 10% bovine serum albumin (BSA) and incubated overnight with a rabbit NR2B antibody (1:100; Millipore) to label both surface and internalized NR2B-containing NMDARs. After extensive washes in PBS with 0.1% Triton X-100 cultures were incubated with anti-mouse (green; Cy2) and anti-rabbit (red; Cy5) secondary antibodies (1:200; Jackson Immuno Research). Images were captured using a Leica DM LFSA confocal microscope under identical conditions for all preparations (100 objective, 1024x1024 pixels format, identical camera gain and black level). Two different methods were used to analyze the NR2B surface expression. First, the intensity (Pearson’s correlation) was used to measure the colocalization intensity between surface NR1 and total NR2B. The method is described by Lavezzari et al (Lavezzari et al., 2004). Second, the level of surface expression of NR2B-containing NMDARs was determined by quantifying the number of surface dendritic puncta that were immunostained for NR2B compared with the number of puncta immunostained for both NR2B and NR1, using the method described by Zhang et al (Zhang et al., 2008). NR2B surface expression was defined as the ratio of surface receptor (NR2B+NR1) versus total receptor (NR2B). The degree of surface expression was expressed as percentage of mean ± SEM normalized to control and statistically compared using a one-way ANOVA with post hoc between treatment groups analyzed with Tukey’s post hoc multiple comparison test (MINITAB 13 for windows).

2.1.6 Electrophysiology

Extracellular excitatory field potentials (fEPSPs) in the lateral nucleus to basolateral nucleus pathway (LA-BLA) were recorded as described by Schimanski and Nguyen (2005): freshly isolated brains from 8-10 week old C57Bl6/J mice were submerged in ice cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 3.3 KCl, 26 NaHCO₃, 2 CaCl₂, 2
MgCl$_2$, 10 glucose (pH=7.35). Coronal slices (400 µM) were prepared from brain sections using a vibratome. Slices were maintained at room temperature in a submerged chamber containing aerated ACSF (95% O$_2$/5% CO$_2$) for a minimum of 3 hours before electrophysiological studies commenced. Individual slices were then transferred to a submerged recording chamber and superfused with aerated ACSF with the ionic composition as above except that KCl was raised to 4 mM and MgCl$_2$ reduced to 1.2 mM (superfusion ACSF). Slices were maintained at 29.5 ± 0.5 C. To measure fEPSPs in the LA-BLA pathway, a concentric bipolar stimulating electrode (platinum/Iridium, HFC) was inserted into the LA near the apex formed by the intersection of the internal and external capsules. The recording electrode was placed ventrally at the LA-BLA boundary to measure field potentials from the dendrites of BLA neurons (Schimanski and Nguyen, 2005). Electrophysiological responses induced by a S88 stimulator (Grass Instruments) were recorded using an Axopatch 1D amplifier (Axon Instruments). Only preparations showing a clearly discernable early presynaptic volley followed by a fEPSP of at least 2 mV maximum amplitude were used. The fEPSP slope, measured between ≈10-60% of the peak amplitude, was used to estimate synaptic strength. Initially, slices were superfused in an open bath, but switched to a closed re-circulating bath containing 40 ml superfusion ACSF with 10 µM bicuculine, and in experimental trials, with 20 µM of the Src inhibitory peptide Tat-Src (40-58). These drug-treated slices were pre-incubated with peptide for 40-50 minutes before the start of baseline recording, whilst control slices were pre-incubated in ACSF and bicuculine in the closed bath for the same duration. In both groups, the chamber remained in the closed configuration for the remainder of the experiment. Following pre-incubation, fEPSPs at 50% of maximum slope were recorded every 30 seconds for 15-20 minutes. The last 10 minutes of this recording period was used as the baseline for subsequent LTP measurements. To induce LTP, the LA was then tetanized by 5, 100 Hz trains lasting 1
second and separated by 10 seconds. Post-tetanic fEPSPs were recorded one every 30 s for the next 60 minutes.

2.1.7 BMD and Craniofacial analysis

Two month-old male mice (four mice per genotype) were dissected to obtain the skull for faxitron analysis and the femurs and tibiae for bone mineral density (BMD) determinations by Piximus. Faxitron images were printed at the same magnification and the distances between established landmarks measured (Vilmann et al., 1989, Kiliaridis et al., 1985, Lightfoot and German, 1998, Rice et al., 1997)

2.1.8 Learning and Memory tasks

2.1.8.1 Water Maze

To test spatial memory we used the Water Maze. Prior to the test mice were handled for 2 min per day for 7 consecutive days.

The Morris water maze consisted of a white plastic 117 cm diameter circular pool filled with water (with constant 26C temperature) made opaque by white latex paint. Around the outside of the pool 2D and 3D visual cues were displayed and we used dim lighting. The pool was divided up into four equal quadrants called northeast, southeast, southwest and northwest. A hidden platform (10 cm diameter) submerged 1cm below the water surface was placed at the center of one of the four quadrants (the target quadrants). A closed-circuit video camera was mounted directly above the center of the pool and connected to an image analyzer (HVS Image Ltd, Twickenham, Middlesex, UK), which digitized the path data. Mice were given 2 trials per day, with 30 sec inter trial interval for 6 consecutive days. During the visible water maze
the platform was flagged with a small cue on top the platform. The mouse was first placed on the platform for 30 sec then it was placed in the water at a pseudo-random start position and it was given a maximum of 60 seconds to find the platform. If the mouse did not find the platform within 60 seconds, it was placed back on the platform. After 30 seconds on the platform, this training procedure was repeated once more. The platform position remained at the same position during all trials. The hidden platform was performed in the same way as the visible platform, only the visible cue was removed from the platform. Two hour after the training on day 6 a probe trial was given to test spatial learning. Mice were placed in the pool at the opposite side of the platform position. The mice were then allowed to search for the platform for 60 seconds.

2.1.8.2 Fear Conditioning

Fear conditioning was performed in a testing chamber (25 cm high, 30 cm wide, 25 cm deep, MED Associates Inc., Georgia, VT), a removable grid floor of 36 stainless steel rods (3.2 mm diameter, 4.7 mm apart) connected to a constant current shock generator, and an amplifier and speaker. A 12-inch, 8-W fluorescent tube (GE Lighting Canada) illuminated the chamber interior. A computer running automated fear conditioning software (FreezeFrame, Actimetrics Software, Evanston, IL) administered foot shocks and auditory tones. Video images were recorded from the chamber, and the activity of subjects was recorded throughout the experiment. Immediately prior to training, the chamber was cleaned with 70% ethanol. A white cloth covered the front of the chamber.

Conditioning for Src mutant mice consisted of two pairing of an auditory tone with a continuous foot shock. Each mouse was placed inside the conditioning chamber for 2 min before the onset of a conditioned stimulus (CS; an 85 dB tone), which lasted for 30 s. A 2 s US foot shock (0.5mA) was delivered immediately after the termination of the CS. Each mouse remained in the chamber for an additional 120 s, followed by another CS–US pairing. Each mouse was returned
to its home cage after another 2.5 min. Approximately 24 hours later, each subject was returned to the chamber and percentage of freezing was monitored for 3 minutes in the conditioned context. The activity of each subject was recorded at 0.25 seconds intervals using the FreezeFrame automated fear conditioning software (Actimetrics Software), which can detect any kind of movement. The mean activity during the context exposure was calculated, subtracted by the mean baseline activity, and used as a measure of contextual learning. Four hours later, the context was changed by covering the grid floor with a sheet of white PerspexTM (polymethyl methacrylate), inserting two sheets of transparent Perspex into the chamber to give it a prism shape, cleaning the chamber with 1% vinegar, covering the front door with a striped black and white card, and turning on the ceiling lights. Each mouse was placed into the altered chamber, and allowed 3 minutes for exploration in the novel environment, after which the auditory tone of 3 minutes duration was delivered. The mean activity during cue delivery was calculated, subtracted by the mean activity in the novel context (prior to the presentation of the tone) was taken as a measure of cue-associated learning (Bolivar et al., 2001).

Each Tat-Src(40-58) or Control (scrambled peptide and saline) injected subject were removed from its home cage and placed into the chamber for 2 minutes prior to conditioning. Activity during this time was recorded as baseline. Conditioning consisted of a single pairing of an auditory tone with a continuous foot shock. The tone (3600 Hz; 80 dB) was delivered 2 minutes after the training session started and was 30 seconds in duration. The foot shock (0.75 mA scrambled) was presented during the last 2 seconds of the tone. The mouse was left in the chamber for 30 seconds and then returned to its home cage. Approximately 24 hours later, each subject was returned to the chamber and percentage of freezing was monitored for 5 minutes in the conditioned context. The activity of each subject was recorded manually by an observer using a stop watch. The mean activity during the context exposure was calculated, subtracted by
the mean baseline activity, and used as a measure of contextual learning. Two hours later, the context was changed by covering the grid floor with a sheet of white PerspexTM (polymethyl methacrylate), inserting two sheets of transparent Perspex into the chamber to give it a prism shape, cleaning the chamber with 1% vinegar, covering the front door with a striped black and white card, and turning on the ceiling lights. Each mouse was placed into the altered chamber, and allowed 3 minutes for exploration in the novel environment, after which the auditory tone of 3 minutes duration was delivered. The mean activity during cue delivery was calculated, subtracted by the mean activity in the novel context (prior to the presentation of the tone) was taken as a measure of cue-associated learning (Bolivar et al., 2001). The drugs were administered 60 min prior to training in each experiment.

Two different methods were used to measure pain threshold. Tail flick was measured by using of a hot-water bath system. The water was maintained at 55 ºC in a constant-temperature water bath. Mice were held in a restrainer, and the distal third of the tail was immersed in the bath. The time required for a mouse to remove its tail was measured by use of a stopwatch, and the tail-flick latency score was calculated as the mean of the last 2 of 3 trials. Trials were separated by 30 s intervals.

Shock threshold was assessed by placing the animal in the fear conditioning chamber and by delivering foot shocks starting at 0.075 mA and increasing by 0.05 mA every 30 s. The experiment was terminated at the shock intensity sufficient to induce audible vocalization.

2.1.8.3 Visual-object recognition

The visual spatial and novelty recognition task was performed in a transparent Plexiglas open field (41×41×31 cm) equipped with infrared beams to detect locomotor movements (model 7420/7430; Ugo Basile, Comerio, Italy). Four of the objects used in this task were similar in shape, color, and material (approximately 7×6×6 cm) for the spatial recognition
task and one object was different for the novel object recognition task. Animal behaviour was recorded by an observer and analyzed using The Observer 5.0 (Noldus, Wageningen, The Netherlands). The testing procedure was adapted from previously described protocols (Frick and Gresack, 2003, Mandillo et al., 2003, Roullet et al., 1996). On test day, each mouse was individually placed in the centre of the empty arena to habituate for a 5-min session. The mouse was then placed in a holding cage for 2 min. Two objects were placed in specific positions in the corner of the arena and another two objects were positioned in the centre of the arena. The mouse was returned to the centre of the arena and allowed to explore the objects for three continuous 5-min sessions (training phase). Training to object exploration was measured by recording the time spent exploring the objects across the sessions. A mouse was considered to be exploring an object if its snout was in contact with the object. At the end of the training phase, the mouse was again placed in the holding cage for 2 min and the position of two centre objects were changed to the corner position in order to assess the response to a spatial change. The mouse was returned to the arena, and the time spent exploring the displaced and non-displaced objects was recorded for 5 min (spatial change phase). Reaction to a spatial change was assessed by comparing the mean time spent exploring the displaced (DO) and non-displaced object (NDO). Reactivity to a novelty change was also examined. Directly after the spatial change phase, the test subject was returned to the holding cage for 2 min, during which one of the familiar non-displaced objects in the arena was replaced by a novel object in the same location. The mouse was returned to the centre of the arena for a 5-min period (novelty recognition phase). Measurements were taken as described for the previous phase, and the response to novelty change was evaluated by considering the mean time spent exploring the novel object (NO) and the three familiar objects (FO).
2.1.9 Spatial object recognition

The spatial object recognition task was performed in a transparent Plexiglas open field (41x41x31 cm). We used four similar funnels in this task to avoid mice from climbing on objects. Mice behaviour was recorded and analyzed using The Observer 5.0 (Noldus, Wageningen). The testing procedure was adapted from previously described protocols (Roullet et al., 1996).

On test day, each mouse was individually placed in the center of the empty open field for a 5-min session for habituation. The mouse was then placed in a holding cage for 2 min. Four objects were placed in specific positions near each corner of the arena. The mouse was placed to the centre of open field with objects and allowed to explore the objects for two continuous 5-min sessions (training phase). Object exploration was measured by recording the time spent exploring the objects across the sessions. A mouse was considered to be exploring an object if its snout was in contact with the object. At the end of the training phase, the mouse was again placed in the holding cage for 3 min and the position of two objects was changed from the centre to periphery to assess response to a spatial change.

The mouse was then returned to the open field, and the time spent exploring the displaced and non-displaced objects was recorded for 5 min. Reaction to a spatial change of the objects was assessed by comparing the mean time spent exploring the displaced (DO) versus non-displaced objects (NDO).

2.1.10 Social recognition

For social recognition experiments, we used the procedure described by Kogan et al (Kogan et al., 2000). Briefly, adult male mice were single caged prior to an experimental session. After 15 min a male juvenile mouse was added to the cage for an initial interaction trial
of 2 min. Twenty hours later, either the same or a novel juvenile was placed back into the adult’s cage for a 2 min test trial. Social investigation of the juvenile (mainly sniffing and licking of the anogenital region of the juvenile) by the adult mouse was observed continuously by an observer who scored the duration of investigation behaviour with the Noldus software (Observer 5.0, Noldus Information technology).

2.1.11 Sociability and social novelty recognition

We used the social approach task that was originally designed by Crawley (Nadler et al., 2004). In this task the tendency to spend time with another mouse versus a novel object (an empty wire cage), and the ability to discriminate and choose between familiar and new mouse is measured. The stranger mouse is under a small wire cage to prevent aggressive interactions and also support the availability of visual, auditory, and olfactory cues. We measured time spent sniffing each cage in each chamber. Also to control for general activity number of entries to each chamber was measured. The apparatus consisted of a clear Plexiglas box (53 cm length × 25.6 cm width × 23 cm height) divided into three chambers, the outer chambers were 19.5 cm in length and the central chamber 13 cm in length. The outer chambers were divided from the central chamber by clear Plexiglas partitions (7.3 cm width × 23 cm height) containing and opening (11 cm width × 23 cm height). An empty wire cage (Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, Ohio) was used to hold the stranger mouse. The wire cage was 11 cm in height, with a bottom diameter of 10.5 cm and bars spaced 1 cm apart. A beaker full of water was placed on the top of the cage to prevent from moving. This cage was located in the centre of each outer chamber throughout the experiment to permit social investigation. The test consisted of four experimental trails. First the test mouse was placed in the middle chamber to explore the arena, after 5 min the doors were removed to enable the mouse to go to outer chambers. Measures were taken of time spent and number of entries in each compartment by an observer.
Immediately after the habituation period the mouse was placed into the centre arena and the doors were closed, an unfamiliar mouse (mouse 1) was enclosed in the wire cage and placed in one of the outside chambers the location of mouse one was alternated between right and left side chambers across subjects. An empty wired cage was placed in the middle of the other outside chamber. Following the placement of the wired cages the doors of the centre arena were opened and the mice and the subject was allowed to explore the entire arena for 10 min. Time spend in each chamber, number of entries and time sniffing each wired cage was measured by an observer using Noldus software. At the end of this 10 min trail each mouse was further tested in a fourth session for 10 min to measure preference for spending time with a new mouse A new unfamiliar mouse was placed in the wire cage that had been empty during the previous session. The test mouse could choose between the first familiar mouse (mouse 1) and the novel unfamiliar mouse (mouse 2). Measures were taken as described before.

2.1.12 Social interaction test in neutral cage

Each mouse was placed in unfamiliar neutral cage (30 x 17 x 12 cm) which was an illuminated (280 lux) as previously described (File et al., 2001). The social interaction was measured when the test mouse encountered a weight- and age-matched unfamiliar adult wild-type 129Sv/C57B6 male opponent. Neutral cages were changed between experiments for each tested pair and the opponent control mouse was used only once. Scoring behaviour parameters started with the first interaction and lasted for 5 min. During this time we measured: social investigation including and non social behaviour such as self-grooming, cage exploration, and rearing.

2.1.13 Tube test

The tube is a measure of social behaviour dominance (Shahbazian et al., 2002, Spencer et al., 2005). In this test Src\(^{(+/+)}\) and Src\(^{(thl/thl)}\) mice were placed into opposite ends of a clear acrylic
cylindrical tube (3.5 cm diameter and 33 cm in length) and released at the same time. When they enter the tube, the mouse that backs out of the tube first is considered the submissive partners. The latency exiting the tube is measured and compared between groups.

2.1.14 Ultrasonic vocalizations recording

USVs were obtained with a D1000X ultrasound recorder (Pettersson Elektronik AB, Uppsala, Sweden) for 5 min at a sampling frequency of 250 kHz. The microphone was suspended from a Mating Chamber Addition put on top of the mating cage. Spectrographs (20-125 kHz) were generated by discrete Fourier transform (256 bins) and analyzed with Avisoft SASLab Pro Software v4.39 (Avisoft Bioacoustics, Berlin, Germany). A Vostro 1710 Laptop running Windows XP was used for analysis of sonograms by a trained observer blind to genotype.

In the male-female paradigm, mating-induced USVs and video recording were obtained. Wild type females were paired with either Src\(^{(+/-)}\) or Src\(^{(-/-)}\) males. The female mouse was removed from the home cage and placed in a clean standard polyethylene cage (22x30x15cm) with fresh bedding. The clean cage was brought into the testing room adjacent to the housing room illuminated by a 40 watt red bulb. The top of the cage was removed and replaced with the Mating Chamber Addition, which consisted of 4 polyethylene extension walls with an open top and with an arm suspended in the centre of the chamber. The microphone was suspended in the centre of the cage, 12 cm above the floor of the cage. A video camera outside the lateral wall was positioned to record behaviours synchronously for further analysis.

The female was left to habituate to the testing room for 5 minutes. Following habituation, the male was introduced to the female cage and pairing was allowed to continue for 5 minutes. Audio and video recordings were obtained for the entire duration of the pairing. After the trial, the male mouse and the female mouse were removed and returned to their appropriate home
cages in the room next door. A new female mouse and cage were then brought into the testing room, ready for the next trial.

In our female-female social reunion paradigm, same litter females were reunited following a period of separation. Src\(^{+/+}\) females were housed together in the same home cage and Src\(^{thl/thl}\) females were housed together in their own home cage. The day prior to testing, one of the mice was removed from the home cage and put into a clean standard polyethylene cage, the “host’s cage”. 20 hours following separation, the host’s cage was taken to the testing room lit by a 40 watt red light. The host was left to habituate for several minutes. Food, water and nesting were removed from the host’s cage to minimize distractions. The top of the cage was removed and replaced with the Mating Chamber Addition for the same purpose as in the male-female paradigm. Following habituation, a mouse from the home cage, “the visitor”, was introduced into the host’s cage and the reunion was allowed for 5 minutes. USVs and video recordings were obtained for the entire trial. Both females were then removed and returned to their original home cage in the housing room next to the testing room. A new female host was then brought into the room and left to habituate, ready for the next trial of female-female social reunion experiment. Total USV counts generated each minute were counted and grouped into three categories for the entire trial. The within subject repeated factor was Time (taken as minute bins). The between subject factor was wild type vs. knockout males and the measure used was total number of calls each minute and type of calls each minute. Video recording was also analyzed to determine sexual behavior in respect to USV production.

2.1.15 Motor performance

2.1.15.1 Rotarod

We used an Economex Rotarod apparatus (Columbus Instruments, Columbus, OH, USA). The original 3-cm ribbed plastic rotating axle is hanging at a height of 30 cm above base of the
apparatus. Mice were placed on top of the rod, facing away from the experimenter. In this orientation, forward locomotion opposite to rotation of the rod is necessary to avoid falling. During the stationary mode, each mouse is first observed for 60 seconds without any rotation, this session is to allow the animals to become accustomed to the apparatus. The axle is then adjusted for a constant motor speed of 5 r.p.m., and each mouse is observed for a total of 90 seconds at a fixed speed mode. Next, beginning at 5 r.p.m., the rotation gradually increased in increments of 0.1 r.p.m. at every second and the latency to fall off the axle is recorded in seconds for each mouse for the maximum period of 5 minutes called the accelerating speed mode. Mice were given two trials per day with a 60 min inter trial interval for 4 consecutive days. Average of the time spent on the rotarod was calculated for each day (Abramow-Newerly et al. 2006).

2.1.15.2 Grip strength

The maximal muscle strength of the fore limbs and both fore and back of each mouse was measured with an isometric transducer attached to a 3 mm diameter metal bar (Ugo Basile, Comerio, Italy). Each mouse gripped the bar with its fore paws, and was then slowly pulled backwards until it released the metal bar. The transducer measured the maximal grip strength in grams. Five trials were performed in each testing session, and the mean value was calculated. For the back limb measurements, each mouse was allowed to grab both its front and hind paws gently lower the mouse over the top of the grid so that both its front paws and hind paws can grip. The fore limb measurement was subtracted from front and hind paw measurement to obtain the hind paw grip strength.

2.1.15.3 Balance Beam test

Mice were acclimated to a round, 30 cm long; 3 mm wide beam elevated 28 cm above a padded base. A 60 W lamp at the starting platform served as an aversive stimulus, whereas the opposite
end of the beam entered a darkened escape box on the arriving platform. Transversal time and number of slips were measured as mice traversed the beam. All testing was performed in triplicate and median values were used for subsequent statistical analyses.

2.1.16 Acoustic startle response

To measure startle in response to a startling stimulus, each mouse was placed into the startle chamber and allowed to acclimatize for 15 min. The mouse was then presented with startle stimuli of varying intensities (70-120 dB), with 25-ms duration and an inter-stimulus interval of 25-30 s. Startle stimuli were presented in three blocks each composed of two demonstrations of the 11 stimulus intensities given in pseudorandom order. The average startle amplitude for each stimulus intensity was calculated from the three blocks.

2.1.17 Social transmission of food preference

Social transmission of food preferences (STFP) for olfactory memory was conducted as described previously (Kogan et al., 2000). Briefly, all mice were habituated for 2 days to eat powdered chow from plastic jars placed in the opposite corners of the home cage in the absence of standard food pellets. A demonstrator mouse was randomly chosen from each home cage and food deprived for 22 hr with free access to water. The next day, each demonstrator mouse received powdered chow mixed with either 2% cocoa or 1% cinnamon for 1 h, or until at least 0.2 g of powdered food was consumed; one half of the demonstrators received coca- and the other half cinnamon-flavoured food. The demonstrator mice were then returned to their home cages to interact with observer mice. After 30 min of interaction the demonstrator mice were separated from the observer group. The observer mice were food deprived overnight with free access to water and tested 24 h later for food preference. Observer mice were single caged and habituated to the novel environment. After 15 min the observer mice were given a free choice of
food flavoured with 2% Coca or 1% cinnamon; observer mice were allowed to eat freely. The position of the food jar with the cued flavour was balanced between cages to control for possible place preference. After 1 hour the food jars were removed and the amount of food eaten from each jar was determined by weight. The percent of cued flavoured food eaten was calculated over the total amount of consumed food.

2.1.18 Motor activity and anxiety like behaviours

Motor activity was measured in the open field. Also, anxiety like behaviours were measured as time spent in the centre of the open field, and as time spent in the open arms of elevated plus maze. Olfactory ability was assessed using a buried-food procedure following food deprivation. These procedures have previously been described in detail (Moy et al., 2007).

2.2 Statistical analysis

All the results were subjected to one-way ANOVA or repeated-measures ANOVA (RM ANOVA) between different groups. When the ANOVA detected significant treatment effects, pair wise differences between means for a given variable were evaluated using Tukey’s post hoc multiple comparison test, with significance set at p<0.05 (indicated by an asterisk in the figures). All statistics were calculated using MINITAB for Windows 13.32 (Minitab Inc., State College, PA, USA) and Graph pad prism. All values reported in the text and figures are expressed as mean ± standard error of mean (S.E.M.).
Chapter 3

3 « The role of Src in synaptic plasticity and learning/memory »

3.1 «Abstract»

The Src protein tyrosine kinase plays a central role in the regulation of N-methyl-D-aspartate receptor (NMDAR) activity by regulating NMDAR subunit 2B (NR2B) surface expression. In the amygdala, NMDA-dependent synaptic plasticity resulting from convergent somatosensory and auditory inputs contributes to emotional memory; however, the role of Src tyrosine kinase has not been investigated. We have synthesized a Src-derived peptide, Tat-Src (40-58), that crosses the blood-brain barrier following injection and accumulates intra-cellularly. Tat-Src (40-58) blocks the interaction of Src with NMDA receptors. Following injection, mice demonstrate impaired amygdala-dependent cued fear conditioning, as well as impairments in an amygdala-dependent non-associative social recognition task. The Src inhibitor decreased NR2B phosphorylation in amygdala tissue and reduced NR2B surface expression in cultured amygdala neurons with a concomitant reduction in NMDA multimer-containing dendritic puncta. In addition, pre-incubation of this inhibitory peptide blocked amygdalar long-term potentiation in the lateral to basolateral pathway in vitro. These results indicate that Src may regulate NMDAR trafficking in the amygdala. Furthermore, Src-dependent phosphorylation of NR2B suggests amygdala plasticity and amygdalar-dependent learning.
3.2 Introduction

Src is a non-receptor protein tyrosine kinase that is expressed widely throughout the central nervous system. Src plays an important role in up-regulating the activity of the NMDARs at glutamatergic synapses (Yu et al., 1997, Salter and Kalia, 2004). The NMDAR is a heterodimer of NR1 and NR2 subunits. The NR1 subunit is essential for pore formation and for the binding of several regulatory ligands, whereas NR2 subunits (NR2A-D) bind glutamate and determine the kinetic characteristics of specific NMDAR multimers (Monyer et al., 1994). Unlike the NR1 subunit, NR2A and NR2B are tyrosine phosphorylated by Src family kinases (Moy et al., 2007).

Recent studies have shown that NMDARs are subject to rapid activity-dependent trafficking between synaptic and extrasynaptic domains (Groc et al., 2006, Tovar and Westbrook, 2002, Jeyifous et al., 2009) and that this trafficking is dependent on subunit tyrosine phosphorylation (Goebel et al., 2005, Snyder et al., 2005, Braithwaite et al., 2006). Localization of NMDAR to the postsynaptic membrane depends on NR2B phosphorylation at Tyrosine 1472 (Nakazawa et al., 2002, Nakazawa et al., 2006, Zhang et al., 2008). While both Src family kinases Src and Fyn phosphorylate NMDARs, NR2B surface expression is regulated by clathrin mediated endocytosis through a Cyclin-kinase 5 (Cdk5)-dependent pathway that is regulated mainly by Src (Zhang et al., 2008).

The circuits mediating auditory fear conditioning consist of auditory and somatosensory inputs that converge onto neurons within the lateral nucleus of the amygdala (LA)(LeDoux, 2000). The LA then projects to the central nucleus of the amygdala (CE), both directly, and indirectly through the basolateral amygdala complex (BLA). Outputs from the central nucleus to the hypothalamus and brain stem then control the expression of fear responses such as freezing
Activation of NMDARs within the LA and BLA is necessary for the acquisition of fear memories (Bauer et al., 2002, Lee and Kim, 1998, Gewirtz and Davis, 1997). In addition, BLA and medial amygdala (MeA) activity are required for social recognition and affective responses to social cues (Fleming and Walsh, 1994) and NMDAR antagonists disrupts social recognition (Gao et al., 2009). An increase in Fos expression, indicative of neuronal activity, has been observed in various amygdalar nuclei following both fear and social learning (Fleming and Walsh, 1994, Kubota et al., 2004).

Here, we report that a Src peptide fragment ligated to the cell permeable Tat motif Tat-Src(40-58) and previously shown to block the interaction of Src with the NMDARs (Kalia et al., 2004), inhibited NR2B phosphorylation in vivo, decreased NR2B surface expression in cultured amygdalar neurons, decreased cued fear conditioning and social recognition, and impaired amygdalar long term potentiation (LTP). These results demonstrate that Src may regulate amygdalar synaptic plasticity and learning by controlling the surface expression of NMDARs at amygdalar synapses.

### 3.3 Results

#### 3.3.1 Src inhibition decreases the phosphorylation of NR2B at Y1472 and increases the activity-dependent internalization of NR2B/NMDARs.

To first assess if intravenously-injected Tat-Src (40-58) peptide could accumulate in the amygdala, mice were injected with a Tat-Src (40-58) peptide conjugated with Dansyl one hour prior to amygdala dissection (Figure 1). The Dansyl epitope was detected in the amygdala of
Dansyl-Tat-Src (40-58)-injected mice but not in the amygdala of mice injected with saline (Figure 1A). Furthermore, the tyrosine phosphorylation level of NR2B in Tat-Src (40-58)-injected mice demonstrated a significant decrease compared to saline-injected mice. Densomeric quantification of the western blots, probed using phospho antibody, showed that Tat-Src (40-58) treatment decreases the basal phosphorylation of Y1472 NR2B compared with control and Tat-sSrc(40-58) (Control intensity: 72.76 ± 5.59%, N=7; Tat-Src (40-58): 46.51 ± 9.462%, N=7; p<0.05, Tat-sSrc (40-58): 71.88 ± 5.447%, N=8; p<0.05, F(1,19)=0.026) (Figure 1B). Thus, the inhibitory peptide accumulates in the amygdala sufficiently to reduce Src-dependent phosphorylation of NR2B.

Because inhibition of Src decreased phospho-Y1472 NR2B in the amygdala, we predicted that inhibition of Src interaction with the NMDAR complex should also result in an increased NMDARs endocytosis (Zhang et al., 2008). To study the effect of Src inhibition on NMDA receptor internalization, we examined the effects of this peptide on NMDA subunit surface expression in neuronal cultures from the amygdala (Figure 2). Cultures were treated with peptide (20 µM) or saline for 1 hour, fixed, and then immunostained with an NR1 antibody that recognizes an extracellular epitope. This surface NR1 immunostaining was followed by immunostaining with an antibody to NR2B (Figure 2A). We then used both intensity of the surface colocalization (Pearson’s correlation) and the number of dendritic puncta containing colocalized receptor subunits to quantify NR2B surface expression (Figure 2B and 2C). There was a significant difference in surface colocalization between control and inhibitor-treated neurons (Control: 0.76 ± 0.00%, N=12; Tat-Src (40-58), 0.53 ± 0.01%, N=10; p<0.001, F(1,21)=166, ANOVA) (Figure 2B). Furthermore, in control cultures, 47.41 ± 3.04 (N=13) of NR2B puncta were also stained with NR1 antibody, whereas in cultures treated with the peptide inhibitor only 23.01 ± 1.81 (N=10) of NR2B puncta were also positive for NR1 (p<0.001,
Together these studies indicate that Src regulates the surface expression of NR2B-containing (i.e. functional) NMDARs multimers in the amygdala.

### 3.3.2 Src inhibition impairs cued fear conditioning

Cued fear conditioning is the most widely used behavioral task to assess amygdalar function (Phelps and LeDoux, 2005). Inhibitor injected mice showed impaired fear-related learning in cued fear conditioning. Mice were injected one hour prior to a single pairing of a tone (conditioned stimulus, CS) and a footshock (unconditioned stimulus, US) on the conditioning day. Prior to pairing, there was no significant differences between groups in baseline freezing (Control: 1.85 ± 0.84, N=13; Tat-Src (40-58): 0.97 ± 0.49, N=12; Tat-sSrc (40-58): 3.33 ± 1.51 N=8, P>0.1, F(2,30)= 1.48, ANOVA), freezing in response to unpaired tone (Control: 5.38 ± 1.94 N=13; Tat-Src (40-58): 3.33 ± 1.47 N=12; Tat-sSrc (40-58): 7.50 ± 3.97 N=8, P>0.2, F(2,30)= 0.72 ANOVA), or in post-shock freezing (Control: 28.46 ± 6.71 N=13; Tat-Src (40-58): 26.67 ± 6.59, N=12; Tat-sSrc (40-58): 24.58 ± 8.01 N=8; P>0.5, F(2,30)= 0.07, ANOVA)(Figure 3A), suggesting that the peptide did not alter general anxiety. At 24 h after pairing, the mice were tested for long-term contextual and cued fear memory. Subjects were placed in a novel chamber for 3 min prior to the presentation of the tone; both control and Tat-Src (40-58)-injected mice displayed only weak freezing in the novel chamber (Control: 9.13 ± 1.56 N=16; Tat-Src (40-58): 5.07 ± 1.69 N=15; Tat-sSrc (40-58): 4.37 ± 2.61 N=8, P>0.1, F(2,36)= 2.02, ANOVA) (Figure 3B). During the tone delivery, Tat-Src (40-58)-injected mice demonstrated much weaker freezing (33.33 ± 4.388, N=15) in comparison to mice injected with saline (66.98 ± 3.126, N=16, P<0.0001) and Tat-sSrc(40-58) (Tat-sSrc (40-58): 61.46 ± 5.20 N=8 P<0.0007, F(2,36)= 21.63, ANOVA) (Figure 3B), suggesting that Src inhibition significantly
decreased long-term memory of the CS–US association. In contrast, contextual fear was not affected by Src inhibition (Control: 37.80 ± 6.78, N=13; Tat-Src (40-58): 32.59 ± 6.03 N=12 and Tat-sSrc (40-58): 28.42 ± 5.05 N=8; \( P>0.5, F(2,30)=0.5, \text{ANOVA} \) (Figure 4A). It is also possible that Src inhibition decreases expression, rather than formation, of auditory fear memory. To examine this, we trained mice for auditory fear conditioning as before but injected Tat-Src(40-58) and Tat-sSrc(40-58) 60 min before auditory testing rather than before training. Figure 4B shows that injecting the inhibitor before testing does not decrease the expression of an auditory fear memory.

Freezing levels in both groups increased during the tone presentation (Tat-Src (40-58): 62.12 ± 6.330 N=9 and Tat-sSrc (40-58): 55.89 ± 4.885 N=9; \( P>0.5, F(1,16)=0.61, \text{ANOVA} \)). Therefore, Src inhibition does not decrease expression of an auditory fear memory (Figure 4B).

Because pain sensitivity can also affect the strength of associative learning, we measured the pain threshold in two different tasks. First, nociceptive response was assessed using the tail flick test, which engages supra-spinal pathways of the nociceptive system. Second, the minimum footshock intensity required to produce an audible vocalization response was determined. As shown in Table 1, tail flick latencies did not differ significantly between the groups; neither one-way ANOVA nor Tukey’s post-hoc analysis revealed difference between the two groups (Control: 3.87 ± 0.51, N=8; Tat-Src (40-58): 3.62 ± 0.56, N=8, \( p>0.5, F_{(1,15)}=0.11, \text{ANOVA} \)). Similarly, the shock threshold for vocalization was unaltered. (Control: 0.26 ± 0.01 mA, N=8; Tat-Src (40-58): 0.26 ± 0.01, N=8, \( p>0.5, F_{(1,15)}=0.26, \text{ANOVA} \))
3.3.3 Src inhibition impairs social recognition

To further investigate the role of Src in amygdalar learning, we studied a second amygdalar-dependent task that does not rely on aversive stimulation, social recognition (Richter et al., 2005). Mice were injected 45 minutes prior to a training trial that consisted of a 15 minute habituation to a new home cage, followed by a 2 min presentation of a novel juvenile (Presentation 1). The social interaction time was measured, after which the animals were returned to their home cages. Mice were then tested 1 day after this initial interaction using the same juvenile (Presentation 2). There was a significant reduction in the duration of investigation time in the saline-injected control group but not in the peptide-injected group, indicating that control mice were better able to recognize the pre-exposed juvenile (Control P1: 71.61 ± 6.25, N=10, Control P2: 46.90 ± 6.23 s, N=10; P<0.01, ANOVA, F_{(1,19)}= 8.96)(Tat-Src (40-58) P1: 58.47 ± 4.33 s, N=9, Tat-Src (40-58) P2: 53.31 ± 7.24 s, N=9, P>0.5, F_{(1,17)}=3.81) (Tat-sSrc (40-58) P1: 75.14 ± 3.91 N=8, Tat-sSrc (40-58) P2: 43.89 ± 5.23s, N=8 ,P<0.01, F_{(1,15)}=22.87)(Figure 5A.). As a control, we examined the response to a novel (non-pre exposed) juvenile presented after P2. In this test, there was no significant reduction in the investigation duration (Control: 74.41 ± 6.74 s, N=9, P>0.6, ANOVA F_{(1,19)}=0.5; Tat-Src (40-58): 64.65 ± 4.44 s, N=10, P>0.6, F_{(1,17)}=8.7 ANOVA and Tat-sSrc (40-58): 74.00 ± 6.857s, N=8, P>0.8 F_{(1,15)}=0.02) (Figure 5B.), indicating that the reduction at P2 was specific to familiar juveniles. Thus, inhibitor injected mice are unable to form robust long-term memories for social recognition.
3.3.4 Impaired synaptic plasticity in Inhibitor incubated slices:

A long-lasting Hebbian synaptic potentiation (LTP) within lateral (LA) and basolateral (BLA) nuclei is a key neurocellular sequela of cued fear conditioning (Maren, 1999b). Therefore, we examined LTP in the amygdala *in vitro* following one hour pre-incubation with either saline or 20 µM Tat-Src (40-58). In saline-treated slices, tetanic stimulation (consisting of five, 1 second trains of 100 Hz) to the LA-BLA pathway induced a long-lasting potentiation (at 55-60 min. post-tet.: 166 ± 14 % of baseline) (Figure 6). In contrast, LTP was greatly diminished in slices pre-incubated with peptide (at 55-60 min. post-tet.: 119 ± 5% of baseline; p<.02) whilst the early post-tetanic potentiation (PTP) was unaltered. Furthermore, Tat-Src (40-58) caused no significant reduction in the baseline population EPSP slope prior to tetanization (Control slices: 0.75 ± .07 mV/ms; Peptide-treated slices: 0.66 ± .10 mV/ms). For these data we conclude that Src activity is required for the LTP at LA to BLA synapses but not for basal neurotransmission.

3.4 Discussion

Blocking the interaction of the Src tyrosine kinase with the NMDAR complex impaired auditory conditioned fear memory and social recognition. Inhibition of Src-NMDAR interaction also attenuated NR2B phosphorylation and decreased NR2B surface expression in the amygdala. Furthermore, at the lateral to basolateral nucleus pathway (LA-BLA), inhibition of Src impaired long-term potentiation, a form of synaptic plasticity that is vital for encoding CS-US associations (Maren et al., 2001). In sum, these data indicate that Src-mediated phosphorylation of NMDARs might be necessary for two vital forms of amygdala-dependent learning.
The amygdala is essential for both the encoding and storage of fear memories (Campeau and Davis, 1995b, LeDoux, 2000). Sensory information encoding the US and CS is conveyed by glutamatergic projections that terminate on neurons within both the basolateral and lateral nuclei (Fanselow and LeDoux, 1999, Maren, 1999a). Neurons within the LA and BLA exhibit short-latency firing during US and CS presentation (Quirk et al., 1995, Maren et al., 1991). Temporal pairing of the US and CS can induce long-term potentiation of CS-evoked AMPA-mediated synaptic responses both in vitro and in vivo (McKernan and Shinnick-Gallagher, 1997), suggesting that NMDAR-dependent Hebbian synaptic plasticity mediates the US-CS association at the neuronal level. Similarly, tetanic stimulation also induces a form of LTP that can be blocked by NMDAR antagonists (Maren and Fanselow, 1995, Gean et al., 1993). The NR2B subunit is expressed on the majority of thalamoamygdala dendritic spines (Radley et al., 2007) and antagonism specific to NR2B-containing NMDARs can block both cued fear memories and synaptic potentiation (Bauer et al., 2002, Li et al., 1995, Nakazawa et al., 2006). Significantly, mice lacking the Src phosphorylation site Y1472 of NR2B also show deficit fear learning (Nakazawa et al., 2006, Nakazawa et al., 2002). Thus, our results underscore the importance of the Src-NR2B interaction in the regulation of amygdalar synaptic plasticity and learning. Testing for auditory short term memory could distinguish between learning performance or memory deficits in the fear conditioning task. By controlling the surface expression of NR2B-containing NMDARs, Src-mediated phosphorylation regulates the threshold for NMDA-dependent synaptic plasticity and learning. Indeed, blockade of conditioned fear and LTP suggest that basal Src activity is necessary for proper expression of these behaviors, possibly by maintain sufficient numbers of postsynaptic NMDA receptors.

The peptide Src inhibitor Tat-Src (40-58) was injected systemically so as to avoid confounds of neural damage associated with direct needle injection into the amygdala. Thus,
while Tat-Src (40-58) blocked LTP in the LA-BLA pathway, it is possible that disrupted transmission or plasticity within other circuits, either within or outside the amygdala, could contribute to the observed deficits in conditioned fear. A decrease in transmission through the nociceptive could interfere with encoding of the US-CS association; alternatively suppression of amygdala output or effects within those brain stem nuclei controlling fear behaviour may also account for the observed response. The attenuation of the freezing response in inhibitor-injected mice was not due to a deficit in pain perception, however, because injected mice have had normal post-shock freezing, freezing threshold and tail withdraw latency to hot water. Second, inhibitor-injected mice exhibited no impairment in context-dependent freezing relative to controls, indicating that downstream synaptic pathways mediating the freezing response were not markedly affected. Third, Src inhibition did not alter expression of an auditory fear memory. Therefore, the auditory fear memory impairment can not be attributed to pain perception, an increase in overall fear memory, a non specific increase in anxiety, or a general change in auditory processing.

Social recognition tasks take advantage of the innate tendency of an adult rodent to investigate non-familiar over familiar conspecifics and, like fear conditioning, is amygdalar-dependent (Meredith and Westberry, 2004, Markham and Huhman, 2008, Petrulis, 2009). C-Fos expression, which is indicative of neuronal activity, is enhanced in the basolateral and medial amygdala following social recognition tasks (Borelli et al., 2009). In the present study, mice were not able to form a robust long-term memory for social recognition following inhibition of the Src-NMDAR interaction. Thus, it is possible that NMDA-dependent plasticity within the BLA could facilitate social recognition. It is well known that an increase in synaptic strength
lasting longer than 1-3 hours, termed L-LTP, requires protein synthesis in amygdala (Huang and Kandel, 2007) and that protein synthesis inhibitors block long-term (but not short term) social recognition (Richter et al., 2005). Given that the Src inhibitor was injected systemically, however, it is again possible that Src inhibition within other pathways in the amygdala contributes to the observed deficits in social memory, particularly as we have not investigated synaptic plasticity of NR2B trafficking in the medial nucleus. Local injections of the inhibitor into the medial amygdala and other relevant pathways are required to isolate the relevant loci.

The Src kinase is anchored within the NMDAR complex through an adaptor protein called NADH dehydrogenase subunit (ND2) (Kalia et al., 2004). Blocking the interaction between the Src unique domain and ND2 releases Src from the NMDAR complex, thereby inhibiting Src-mediated phosphorylation. To understand the neurocellular mechanisms and role of this inhibition in amygdala learning, we measured NR2B phosphorylation levels in the amygdala of inhibitor-injected mice and NR2B internalization in primary amygdala cultures. There was a decrease in NR2B Tyr-1472 phosphorylation one hour after injection, while NR2B surface expression was decreased in amygdala cultures in the same time frame. Moreover, the number of membrane clusters co-expressing NR1 and NR2B was reduced, indicating that Tyr-1472 phosphorylation is necessary for proper localization of NMDARs at postsynaptic sites. Whether Tyr-1472 phosphorylation by Src directly regulates NR2B endocytosis is not known, but one possibility is that phosphorylation of Y1472 NR2B down-regulates the binding of NR2B to the endocytosis motif AP-2. The C terminus of the NR2B subunit contains a clathrin adaptor AP-2 binding site and YEKEL internalization motif. Tyrosine-1472 is within this motif; moreover, tyrosine phosphorylation inhibits the binding of AP-2 and promotes surface expression of NMDARs (Prybylowski et al., 2005, Zhang et al., 2008). Although Fyn, not Src, has been suggested to be the major tyrosine kinase responsible for the phosphorylation of NR2B
(at least in transfected HEK cells) (Nakazawa et al., 2001), NR2B phosphorylation is not abolished in Fyn knock out mice. This suggests that Src family tyrosine kinases other than Fyn can also phosphorylate NR2B. Indeed, NR2B phosphorylation is modulated by cycline-dependent kinase IV (Cdk5) and PSD-95 in a Src-dependent but Fyn-independent manner (Zhang et al., 2008), suggesting that Src may be responsible for the remaining phosphorylation of NR2B in Fyn mutant mice.

These results provide a plausible mechanism for the regulation of amygdala-dependent learning by the Src protein kinase. By maintaining NR2B surface localization on the postsynaptic dendrites of amygdalar neurons within the LA and BLA, Src supports the induction of LTP concomitant with US-CS pairing. Further illumination of the role of Src signalling in amygdalar learning could facilitate the development of novel treatments for psychiatric diseases characterized by aberrant fear responses.
3.5 Table and figures

<table>
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<th>Control</th>
<th>Tat-Src(40-58)</th>
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<tr>
<td>Tail flick latency (seconds)</td>
<td>3.87 ± 0.51 N=8</td>
<td>3.62 ± 0.56 N=8</td>
</tr>
<tr>
<td>Vocalization threshold (mA)</td>
<td>0.26 ± 0.00 N=8</td>
<td>0.26 ± 0.00 N=8</td>
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Data presented are average (±SE), N=8 for each group

Table 3-1 Nociception and vocalization threshold in control and Tat-Src(40-58)-injected mice.

Tail flick latencies and shock threshold to vocalization was not affected by Src inhibition.
Figure 3-1 The Tat-Src (40-58) peptide penetrates the blood-brain barrier following i.v. injection and accumulates in amygdalar neurons where it inhibits NR2B phosphorylation.

A) An antibody against dansyl detects stains in amygdala cells 1 hour after the intravenous injection with Dansyl-Tat-Src (40-58) (9.3 nmol/g) whilst no staining was observed in the amygdala of saline-injected mice. B) Pre-injection of Tat-Src (40-58) decrease phosphorylation of NR2B at residue Y1472 in the amygdala. The level of phosphorylation of NR2B at residue Y1472 was monitored in amygdala tissue 1 hour after i.v. injection with Tat-Src (40-58) at 9.3 nmol/g) or saline and Tat-sSrc(40-58). Densiometric quantification demonstrated that Tat-Src (40-58) treatment decreased the phosphorylation of Y1472 compared with control (* p<0.05).
The Tat-Src (40-58) peptide accumulates intracellularly in cultured amygdala neurons and inhibits surface expression of NR2B.

A) The surface expression of NR2B were evaluated in neuronal amygdala cultures treated for 1 hour with control medium or with medium containing 20 µM Tat-Src (40-58). Neurons were immunostained for NR1 (green) to visualize surface NMDARs before being permeabilized and immunostained for NR2B (red). An overlay of NR1 and NR2B (yellow) immunostaining reveals co-localization of NR1 with NR2B. Quantification of NR1/NR2B colocalization. For the statistical analysis, confocal images from 10-12 cells were collected at 100x magnification. All data are presented as mean ± SEM of Pearson’s correlation (%) normalized to control (*p<0.001, ANOVA). Quantification of NR2B internalization. There was a significant decrease in NR2B surface expression in Tat-Src (40-58)-treated neurons as determined by the number of puncta containing NR1 and NR2B (*p<0.001, ANOVA) (B-C).
Figure 3. Pre-injection of Tat-Src (40-58) impaired auditory fear conditioning at 24 hours post-training but did not affect baseline responses to novel context, novel tone or foot shock (general anxiety).

A) Freezing (as a % of observation time) in a novel conditioning chamber (Baseline) for saline-injected (open bars), Tat-Src (40-58)-injected mice (black bars) and Tat-sSrc (40-58)-injected mice (striped bars). After recording the baseline freezing for 120 s, a tone was presented for 30s (Novel tone), after which mice were given a footshock and left inside the chamber for another 30s (After shock).

B) Cued fear memory 24 h after pairing. Pre-CS is freezing in the absence of the tone (Pre-CS) in a novel context while CS is % freezing during tone presentation (**P<0.005, ANOVA).
Figure 3-4 The effects of Src inhibition are specific to the formation of auditory fear memory.

Src inhibition does not impair the formation of a context fear memory (A) or expression of an auditory fear memory 24 h after pairing (B).
Figure 3-5 Long-term social memory is impaired in inhibitor injected mice.

Social recognition was assessed in two successive 2 minute interaction trials with inter-trial intervals of 24h. On the training day, mice were injected 45 minutes prior to training. Mean investigation duration is shown for training and test trials. A) Familiar juveniles were exposed to both groups at test trials. There was significant reduction in investigation duration, indicating long-term social memory in the saline-injected and Tat-sSrc (40-58)-injected mice (open bars, **P<.005 and striped bars, ***P<.005) but not in the Tat-Src (40-58)-injected group (closed bars) B) Novel juveniles were exposed to both groups after the test trial; there was no significant difference in interaction times between these second novel juveniles and those present 24 hours earlier.
Figure 3-6 Incubation in the Src inhibitory peptide Tat-Src (40-58) markedly reduced long-term potentiation in the lateral to basolateral amygdala pathway *in vitro*.

A) Sample field excitatory postsynaptic potentials (fEPSPs) recorded 1 minute before and 60 minutes following tetanization for a control slice (demarcated by a closed circle at left) and a Tat-Src (40-58)-treated slice (open circle, right). B) Average fEPSP slope for control (closed circles) and Tat-Src (40-58)-treated slices (open circles) as a percentage of baseline fEPSP slope. While tetanic stimulation induced similar initial increase in fEPSP slope, LTP decayed to near baseline in the presence of Tat-Src (40-58) C) Average LTP from each slice determined from the 20 sample sweeps recorded 50-60 minutes post-tetanus (*P<.02*).
Chapter 4

4  «Association of Src mutation in mice with altered social behaviours and learning/memory deficits

4.1  Abstract

Neurodevelopmental disorders, such as autism spectrum disorders (autism) and Williams Beuren syndrome (WBS), are linked to synaptic abnormalities. Src is a non-receptor protein tyrosine kinase that is expressed widely throughout the central nervous system and is involved in diverse biological functions like cell growth, differentiation, and signalling. Despite the well-documented role of Src in synaptic plasticity its role in social behaviour, motor function and cognition remains elusive. Here, we identified mice with a point mutation within the kinase domain of Src leading to a premature stop codon. This mutation predicts a deletion of ½ of the kinase domain. At birth, Src\textsuperscript{(thl/thl)} mice are indistinguishable from their littermates, but by 12 days of age can be easily identified by their small size, lack of incisors, and variable numbers of molar teeth; craniofacial anomalies are observed at 8 weeks of age in these mice. Western blot analysis indicate an almost complete loss of Src protein expression in the brain. We show that Src\textsuperscript{(thl/thl)} null mice exhibit hyper sociability and hyperactivity in the open field. These mice have impaired visio-spatial learning, amygdala dependent learning, and motor learning. Src mutant mice show increased ultrasonic vocalization during social interaction and increased startle response to loud tone. Interestingly, the behaviour phenotype observed in our mutant mice replicates several features of Williams Beurens Syndrome (WBS) and opposes some autistic like behaviours.
We suggest that Src may play a role in modifying the cytoplasmic localization of a molecule that is deleted and involved in the pathophysiology of WBS, called GTFII-I (Lucena et al.). Interestingly, Csk, a down regulator of Src, has been shown to be part of the micro deletion region in some autistic patient. All together Src regulates most of the behaviour phenotypes observed in WBS possibly by altering the signalling pathway directly linked to this syndrome.

Introduction

Neurodevelopmental disorders, such as autism spectrum disorders (autism) and Williams Beuren syndrome (WBS), are both linked to synaptic abnormalities. (Bear et al., 2008, Betancur et al., 2009, Berg et al., 2007) At the moment, Src is one of the most studied kinases but its implication in behaviour is poorly understood. Src tyrosine kinases mediate a broad spectrum of physiological responses including cell cycle control, proliferation, differentiation, migration and survival (Thomas and Brugge, 1997), when activated in response to signals from diverse cellular receptors and extracellular stimuli. Src is highly expressed in the mammalian central nervous system (CNS) and is involved in neuronal differentiation and neurite outgrowth (Kuo et al., 1997a, Hoffman-Kim et al., 2002). NMDA receptor is the first ion channel shown to be modulated by Src (Wang and Salter, 1994). It is also known that SFKs are involved in PDGF and EGF signalling and can modulate the activity and localization of the TFII-I transcription factor which is one gene part of a large micro deletion in Williams beuren syndrome (WBS). Interestingly, the cytoplasmic tyrosine kinase (Csk), a Src function inhibitor, mapped to 15q24 in human genome has been shown to be located on the micro deletion region of people with autism spectrum disorders (McInnes et al.). In addition, the autism spectrum phenotype is associated with duplication of the WBS region (Berg et al., 2007).

Here, we characterized mice with Src mutation for craniofacial, behavioral, and biochemical phenotypic abnormalities relevant to neurodevelopmental disorders with altered
social behaviours, such as ASD and Williams Beuren syndrome. We conclude that Src may be a connecting bridge between WBS and autism spectrum disorders which could facilitate the development of novel treatments for abnormal social behaviours.

4.2 Results

4.2.1 Novel mutation in the Src gene leads to the lack of incisors phenotype.

During a routine observation of our animal colony we noticed two 12 days old “toothless” pups (one male and one female) that could be distinguished from their littermates by small size and a lack of incisors. Both parents lacked the observed phenotype suggesting it was inherited as an autosomal recessive trait. These mice were of the mixed S129 genetic background derived from R1 embryonic stem cell line. To identify a mutation responsible for this phenotype, we crossed the parents with C57BL6/J mice, intercrossed the resulting progeny, and performed genome wide linkage analysis on 20 (S129xC57BL6/J) F2 “toothless” (Thl) mice. Only one marker, D2Mit411, of S129 origin showed significant linkage with the lack of incisors trait (data not shown). To identify boundaries of the critical interval region containing mutation of interest, Thl1-Thl10 mice were genotyped for additional markers surrounding D2Mit411 (Figure 1). It allowed us to narrow down position of the corresponding mutation to an approximately 8.5 Mb interval between D2Mit285 (Ensembl position: 152548882) and rs27332130 (Ensembl position: 161201634). One of the genes within the critical interval region, Src (Ensembl position: 157115730), caught our attention due to a known role of Src protein in the bone remodelling process (Lowe et al., 1993). Moreover, similarly to our “Thl” mutants, Src-null mice lack incisors. To evaluate whether “Thl” is a novel mutation in the Src gene we
sequenced *Src* coding region and discovered an insertion of a C nucleotide into exon 12 (Figure 2). Genotyping of both groups of mice produces a 1000-bp product with a HpaII restriction site in *Src*<sup>(thl/thl)</sup> mice, created by the C nucleotide insertion. Cutting the PCR product with HpaII produces four products in *Src*<sup>(thl/thl)</sup> mice (89,38,35,18-bp) and three products in wild type mice (106,38,35-bp) (Figure 4).

The nucleotide insertion leads to a frame shift and a premature stop codon. The mutation predicts a deletion of 3/4 of the kinase domain; however immunoblot of brain lysates of the *Src*<sup>(thl/thl)</sup> mice does not identify the Src protein (Figure 3). In addition to their small size and the lack of incisors *Src*<sup>(thl/thl)</sup> mice have variable numbers of molar teeth (usually one or two are absent, sometimes from the upper jaw sometimes from the mandible). The *Src*<sup>(thl/thl)</sup> mice are born at the expected mendelian ratio.

### 4.2.2 Craniofacial analysis

Because visual inspection revealed that the *Src*<sup>(thl/thl)</sup> mice also exhibited craniofacial anomalies, faxitron images were used for cephalographic analysis (Figure 5). All of the parameters analyzed showed a significant reduction in length for the Src mutant mice, with the exception of the Mx1-Mx2 distances, which tended to be reduced, and Go-Mn, which was not affected (Table 1). Defects in incisor and molar tooth formation and eruption were also evident, similar to what has been described in Src null mouse (Soriano et al., 1991).

### 4.2.3 Cognitive and behavioural phenotypes

#### 4.2.3.1 Social interaction and social recognition

To explore sociability in the *Src*<sup>(thl/thl)</sup> mice, we performed a variety of social interaction tests, including social choice, direct social interaction and tube tasks.
4.2.3.2 Social approach task and short term social recognition

We used a three-chambered apparatus in which consisted of two different trials, in the first trial, the subject mouse was given the choice to spend time with a social object (wired cage with a mouse inside) or spend time with a non-social object (empty wired cage) inside. Src\(^{(thl/thl)}\) mice spent significantly more time with the social object when compared to Src\(^{(+/+)}\) mice (Src\(^{(+/+)}\) = 180.6 ± 30.89 N=9, Src\(^{(thl/thl)}\) = 310.6 ± 34.91 N=8, p<0.05, F(1,15)=7.8) (Figure 6A). Although there were significant preference for social objects in both groups but the preference for social object was greater in Src\(^{(thl/thl)}\) mice (Time for Social object: Src\(^{(+/+)}\) = 180.6 ± 30.89 N=9, Time for non social object: Src\(^{(+/+)}\) = 79.08 ± 13.29 N=9, p<0.01, F(1,16)=9.11), (Time for Social object: Src\(^{(thl/thl)}\) mice = 310.6 ± 34.91 N=8, Time for non social object: Src\(^{(thl/thl)}\) mice = 61.43 ± 17.83 N=8, p<0.0001, F(1,14)=40.42) (Figure 6A). However in the test for social novelty preference, only Src\(^{(+/+)}\) mice showed a significant preference for non familiar social subject (mouse 2) in comparison with time spent with familiar social subject (mouse 1) (Time for mouse 1: Src\(^{(thl/thl)}\) = 60.90 ± 13.10 N=9, Time for mouse 2: Src\(^{(thl/thl)}\) = 153.1 ± 26.30 N=9, p<0.01, F(1,16)=9.85) (Figure 6B). Src\(^{(thl/thl)}\) mice were not able to distinguish between mouse 1 and mouse 2 suggesting an impairment in short term social recognition in Src\(^{(thl/thl)}\) mice. (Time for familiar Social object: Src\(^{(thl/thl)}\) = 192.8 ± 29.00 N=8, Time for non familiar social object: Src\(^{(thl/thl)}\) = 141.0 ± 27.56 N=8, p>0.05, F(1,14)=1.68) (Figure 6B). Number of entries into each compartment was not different among groups in each session (Figure 6B and 6D)

4.2.3.3 Direct social interaction test

Mice were scored for active social behaviour in a 10 min direct social interaction test. Subject mice were introduced along with control wild type mice to a neutral new cage. We
found that Src\(^{(thl/thl)}\) mice spent more time involved in active social approaches than Src\(^{(+/+)}\) mice (Src\(^{(+/+)}\): 8.730 ± 1.663 N=9; Src\(^{(thl/thl)}\): 64.72 ± 12.21 N=9, p<0.001, F(1,16)=20.66) (Figure 7A).

### 4.2.3.4 Tube test

In this test an experimental mouse is placed at one end of a tube and a control wild type mouse at the other end. They are both released at the same time and the mouse that backs out of the tube first is considered the loser. When Src\(^{(+/+)}\) mice and Src\(^{(thl/thl)}\) mice were facing each other, Src\(^{(thl/thl)}\) mice won approximately 80% of their matches (Src\(^{(+/+)}\): 20.00 ± 9.177 N=20; Src\(^{(thl/thl)}\): 80.00 ± 9.177 N=20, p<0.001, F(1,38)=21.38) (Figure 7B). Both genotypes showed dishabituation towards a new smell on trial 7 of an olfactory test (p>0.05, ANOVA) which is an indication of an intact olfactory system. Results from all the social interaction tests, taken together, provide evidence for the Src gene being involved in abnormal sociability phenotypes and short term social recognition.

### 4.2.3.5 Mating induced USVs

Src\(^{(thl/thl)}\) mutant and Src\(^{(+/+)}\) mice emitted similar numbers of ultrasonic calls during the 5 minutes encounter with a female mouse. Repeated measure analysis of variance revealed no significant difference between groups across time (p>0.05) (Figure 8A).

### 4.2.3.6 Social Reunion induced USVs

Most of the female calls during female-female social reunion paradigm occurred in the first 2 minutes of the 5 minute trial. Src\(^{(thl/thl)}\) females, sang more in both the first min (Src\(^{(+/+)}\): 9.273 ± 3.550 N=11, Src\(^{(thl/thl)}\): 160.9 ± 41.04 N=10, p<0.001, F(1,19)=14.96, ANOVA) and second min (Src\(^{(+/+)}\): 16.64 ± 10.79 N=11, Src\(^{(thl/thl)}\): 78.00 ± 23.33 N=10, p<0.05, F(1,19)=
6.06, ANOVA) when compared to $Src^{(+/+)}$ females (Figure 8B). A repeated measure analysis of variance over the first 2 minutes of number of USV calls revealed a significant difference between groups (Time interval: $F=6.32$, $DF=1$, $P<0.05$; Genotype: $F=11.44$, $Df=1$, $P<0.01$; Time interval and genotype interaction: $F=9.3$, $Df=1$, $P<0.01$).

There was a difference observed in both flat (Type 1) and frequency-modulated (Type 3) USVs between wild type and $Src^{(thl/thl)}$ female mice. When comparing type of calls there were more type III calls produced, followed by type I and type II calls in $Src^{(thl/thl)}$ female mice. Type I calls were significantly higher in $Src^{(thl/thl)}$ mice during the first minute of recording only ($Src^{(+/+)}$: 4.545 ± 2.060 $N=11$; $Src^{(thl/thl)}$: 37.20 ± 11.99 $N=10$, $p<0.01$, $F(1,19)=7.91$, ANOVA) (Figure 8C). For type II calls on the other hand, no significant differences were observed between groups ($P>0.05$) (Figure 8D). However, the number of calls were significantly higher in $Src^{(thl/thl)}$ female mice during the first ($Src^{(+/+)}$: 3.273 ± 1.137 $N=11$; $Src^{(thl/thl)}$: 109.6 ± 37.53 $N=10$, $P<0.01$, $F(1,19)=8.86$, ANOVA) and second minute ($Src^{(+/+)}$: 4.636 ± 2.439 $N=11$; $Src^{(thl/thl)}$: 51.30 ± 15.76 $N=10$, $P<0.01$, $F(1,19)=9.42$, ANOVA) (Figure 8E).

### 4.2.3.7 Exploratory activity and anxiety behaviour

We assessed exploratory activity and anxiety-related behaviours using the open-field test, as well as the elevated plus maze task since children and mouse models of WBS show abnormalities in these behaviours.

In the Open-field test, there was an overall difference among genotypes in the total distance travelled during the first 15 minutes (Time interval: $F=2.4$, $DF=2$, $P=0.09$; Genotype: $F=9.7$, $Df=1$, $P<0.01$; Time interval and genotype interaction: $F=1.79$, $Df=2$, $P=0.17$) (Figure 9A). $Src^{(thl/thl)}$ mice spent less of the total distance travelled in the centre of the arena, compared to WT mice ($Src^{(+/+)}$: 31.19 ± 6.761 $N=10$; $Src^{(thl/thl)}$: 12.89 ± 3.160 $N=16$, $p<0.05$, $F(1,24)=...
7.59)(Figure 9B). Suggesting increased anxiety like behaviour for this task in for $\text{Src}^{(thl/thl)}$ mutant mice. However, we did not observe any difference in elevated plus maze; another test for anxiety. The percentage time spent in the open arm was similar in both groups ($\text{Src}^{(+/+)}=4.105 \pm 1.169$ N=13; $\text{Src}^{(thl/thl)}$: 4.641 ± 1.352 N=18, p>0.05, F(1,29)= 0.08)(Figure 9C).

### 4.2.3.8 Sensory and motor function

We assessed motor coordination, strength and motor skill learning in $\text{Src}^{(thl/thl)}$ mice by using the balance beam, grip strength and rotarod task.

Mice were also analyzed in a raised-beam which is a balance testing paradigm. $\text{Src}^{(thl/thl)}$ mice took significantly longer time to cross the beam ($\text{Src}^{(+/+)}=5.538 \pm 0.8444$ N=13, $\text{Src}^{(thl/thl)}$: 11.50 ± 1.390 N=12, P>0.01, F(1,23)= 13.93) and had increased number of food slips while crossing the beam ($\text{Src}^{(+/+)}=0.5000 \pm 0.2303$ N=12, $\text{Src}^{(thl/thl)}$: 1.917 ± 0.3128 N=12, P>0.01, F(1,23)= 13.30) (Figure 10A and 10B).

We also tested grip strength in the same mice to determine if differences in the grip strength could account for the differences in the balance beam results. This test revealed a significant decrease in grip strength for both forelimb ($\text{Src}^{(+/+)}=98.97 \pm 4.367$ N=16, $\text{Src}^{(thl/thl)}$: 80.69 ± 5.196 N=12, P<0.05, F(1,26)=7.32) and a combination of forelimb and hind limb ($\text{Src}^{(+/+)}=162.7 \pm 8.624$ N=16, $\text{Src}^{(thl/thl)}$: 122.5 ± 9.607 N=12, P<0.01, F(1,26)= 9.56) in $\text{Src}^{(thl/thl)}$ mice (Figure 10C and 10D).

We found an overall significant difference in the amount of time mice spent walking on the rotating rod on day 3 and 4 (Day3: $\text{Src}^{(+/+)}=177.9 \pm 12.52$ N=12, $\text{Src}^{(thl/thl)}$: 137.1 ± 10.02 N=12,P<0.05, F(1,22)= 0.019 ;Day4: $\text{Src}^{(+/+)}=182.7 \pm 14.50$ N=12, $\text{Src}^{(thl/thl)}$: 124.7 ± 12.78 N=12,P<0.01, F(1,22)= 9.02) but not on day 1 and 2 (Day1: $\text{Src}^{(+/+)}=98.42 \pm 11.97$ N=12, $\text{Src}^{(thl/thl)}$: 89.58 ± 8.700 N=12,P>0.05, F(1,22)= 0.09; Day2: $\text{Src}^{(+/+)}=131.6 \pm 12.26$ N=12,
Src\(^{\text{thl/thl}}\): 126.2 ± 12.66 N=12, P>0.05, F(1,22)= 0.36). This confirms the role of Src in motor function; increasing rotation (motor demand) causes the rotarod test to be more sensitive to detect motor dysfunction. (Figure 10E).

### 4.2.3.9 Auditory startle response

In the analysis of peak value of the startle response with the \(\text{Src}^{+/+}\) and \(\text{Src}^{\text{thl/thl}}\) mice there was a significant enhancement of startle response at 115 (\(\text{Src}^{+/+}=242.0 \pm 41.36\) N=8, \(\text{Src}^{\text{thl/thl}}: 471.1 \pm 83.82\) N=8, \(P<0.05, F(1,13)=6.01\)) and 110 dB (\(\text{Src}^{+/+}=293.2 \pm 13.69\) N=8, \(\text{Src}^{\text{thl/thl}}: 410.3 \pm 36.92\) N=8, \(P<0.01, F(1,13)= 8.85\)) level startling stimulus. However, at the 70-105 dB levels, the \(\text{Src}^{\text{thl/thl}}\) mice exhibited a similar startle magnitude when compared with \(\text{Src}^{+/+}\) mice \((P>0.05)\) (Figure 11).

### 4.2.3.10 Learning and memory and attention deficits

Learning and memory processes were examined using tests such as fear conditioning and Moris water maze to evaluate long term memory function and visual object recognition to assess short term memory. Both water maze and object recognition measure visio-spatial learning which is impaired in individuals with WBS.

### 4.2.3.11 Moris water maze

Visio-spatial learning was first assessed using the Morris water maze task. A probe trial given after 6 d of training showed a significant difference between genotypes when the number of target platform crossings was compared between mutants and wild type. \(\text{Src}^{+/+}\) mice showed significantly more crossings of the target platform position as compared with the other positions (Target platform location: 4.417 ± 0.5143 N=12, Average of non target
locations: 2.722 ± 0.2747 N=12, p<0.01, F(1,22) = 8.44). Whereas $Src^{(thl/thl)}$ mice did not show any preference for the target location (Target platform location: 1.417 ± 0.4167 N=12, Average of non target locations: 2.333 ± 0.3761 N=12, p>0.05, F(1,22) = 2.67)(Figure 12B).

The learning deficit of $Src^{(thl/thl)}$ mutants was not due to impaired motor performance or decreased motivation to escape, as all mutants showed similar escape latencies in the visible and the hidden platform task (p>0.05) Two way ANOVA,(Figure 12A). Moreover, the swim speed of $Src^{(thl/thl)}$ mutants was similar to that of wild-type mice. However, it is important to mention that mice with impairments in motor function may experience a different motivational force in the Morris watermaze, given that motor disfunction was observed in other tasks.

### 4.2.3.12 Fear conditioning

We measured levels of freezing during the context and cued fear conditioned stimulus (CS) During the context test, $Src^{(thl/thl)}$ mice displayed significantly similar bouts of freezing relative to the WT. In the CS test, Src mice displayed fewer freezing responses relative to WT. These findings suggest that Src mice, have impaired learning and/or memory performance. Cued fear conditioning is the most widely used behavioral task to assess amygdala function (Phelps and LeDoux, 2005). $Src^{(thl/thl)}$ mice showed impaired fear-related learning in cued fear conditioning. Mice were conditioned to 2 pairs of a tone (conditioned stimulus, CS) and a footshock (unconditioned stimulus, US) on the training day. Prior to pairing, there was a moderate difference between groups in baseline freezing ($Src^{(+/-)}$: 3.14 ± 0.7288 N=24; $Src^{(thl/thl)}$: 7.869 ± 1.326 N=22, p<0.001, F(1,44)= 10.21, ANOVA), freezing in response to unpaired tone ($Src^{(+/-)}$: 5.788 ± 1.686 N=24; $Src^{(thl/thl)}$: 13.97 ± 2.742 N=22, p<0.05, F(1,44)= 6.70, ANOVA). However, the post-shock freezing was similar between groups ($Src^{(+/-)}$: 29.21 ±
3.925 N=25; \(Source^{(+/-)}: 28.98 \pm 3.241 N=24, P>0.05, F(1,44)= 0.04\) (Figure 12A), suggesting that \(Src\) mutations alter general anxiety to some extent. At 24 h after pairing, the mice were tested for long-term contextual and cued fear memory. Subjects were placed in a novel chamber for 3 min prior to the presentation of the tone; both groups of mice displayed only weak freezing in the novel chamber (\(Source^{(-/-)}: 5.788 \pm 1.686 N=24; Source^{(thl/thl)}: 13.97 \pm 2.742 N=22; P<0.05, F(1,44)= 6.70, ANOVA\) (Figure 12B). During the tone delivery, \(Source^{(thl/thl)}\) mice demonstrated weaker freezing (38.48 \pm 4.258 N=24) in comparison to \(Source^{(+/-)}\) mice (57.68 \pm 3.978 N=25, P<0.001, F(1,47)= 10.88 (Figure), suggesting that \(Src\) function is important for long-term memory of the CS–US association. In contrast, contextual fear was not effected in \(Source^{(thl/thl)}\) mice (Source\(^{(+/-)}\): 50.14 \pm 5.654 N=25; Source\(^{(thl/thl)}\): 42.08 \pm 4.498 N=24, P>0.5 F(1,47)=1.23, ANOVA).

Because pain sensitivity can also affect the strength of associative learning, we measured the pain threshold. The minimum footshock intensity required to produce an audible vocalization response was determined. The shock threshold for vocalization was unaltered. (\(Source^{(+/-)}\): 0.2950 \pm 0.005000 N=10; \(Source^{(thl/thl)}\): 0.2900 \pm 0.006667 N=10, p>0.5, F(1,18)=0.36, ANOVA), suggesting that they have similar sensitivity to painful stimuli.

### 4.2.3.13 Visual object recognition task

The exploration time of the displaced objects (DO) and novel objects (NO) was evaluated. \(Source^{(thl/thl)}\) mutant mice demonstrated an inability to selectively react to a spatial and novelty change in the environment.

There was a significant difference between two groups in exploratory preference for two displaced object in the spatial recognition phase (\(Source^{(+/-)}\): 81.08 \pm 2.605 N=12; \(Source^{(thl/thl)}\): 58.11 \pm 3.206 N=15, p<0.001, F(1,25)= 28.81, ANOVA) and novelty (\(Source^{(+/-)}\): 87.06 \pm 1.445 N=12;
Discussion

Here we report that, $Src^{(thl/thl)}$ mice exhibit craniofacial abnormalities and growth retardation. We also observed differences in behaviour phenotypes similar to people and mice with WBS. Sociability and social vocalization were increased in three different social affiliation tasks in $Src^{(thl/thl)}$ mice. They showed hyperactivity in the open field and spent significantly less time in the centre of the open field. Also, motor function was decreased in three different motor performance tasks. $Src^{(thl/thl)}$ mice showed enhanced startle respond to loud stimulus and impaired cued fear conditioning and visio-spatial memory in the morris water maze. $Src^{(thl/thl)}$ mice were not able to pay attention and learn a visual object recognition task. All of the observed behaviour phenotypes resemble behaviour abnormalities seen in people with WBS. The enhanced social interaction, communication, and enhanced startle response can be also considered as anti-autistic behaviours. The hyperactivity and lack of concentration are indications of attention deficits in these mice.

People with WBS have particular personality traits, overfriendliness and charismatic speech rich in vocabulary. About 70%, also suffer from attention deficit disorder and many experience phobias and anxiety. Hyperacusis is seen in most of WBS cases. Autism spectrum disorders are characterized by three core deficits: impaired communication, impaired social interaction, and restricted, repetitive and stereotyped patterns of behaviours.

We hypothesized that decreased Src function could cause both anti-autistic and WBS like phenotypes in mice because of three main reasons: one, TFII-I activity and localization is modulated by Src in fibroblasts; and two, the craniofacial abnormalities are highly similar.
between WBS and Src mutant mice. In order to test this hypothesis, we decided to perform a wide range of behaviour, anatomical, and molecular analysis resembling phenotypes of both autism and WBS neurodevelopmental disorders.

When growth factors are activated upon an extracellular signal in fibroblasts, c-Src becomes activated which leads to its autophosphorylation and increased kinase activity. c-Src then phosphorylates the tyrosine residues Tyr$^{248}$ and Tyr$^{611}$ of TFII-I which causes translocation of the transcription factor TFII-I into the nucleus to activate various signal-induced gene including c-Fos. (Cheriyath et al., 2002, Hong et al., 2005). The TFII-I family transcription factors consist of three members: TFII-I (or GTF2I), WBSCR11 (or GTF2IRD1), and GTF2IRD2. TFII-I and GTF2IRD1 are deleted in Williams Beuren Syndrome (WBS)(Tassabehji et al., 2005) as part of a larger micro deletion on chromosome 7q11.23. TFII-I or GTF2I (the first member identified in this group) is a basal transcription factor and also regulates transcription. However, GTF2IRD1 has so far only been shown to regulate transcription. Individuals with WBS have craniofacial features, vascular problems (like supravalvular aortic stenosis, SVAS), mild to moderate mental retardation, specifically visuo-spatial and numerical abilities impairments.

The GTF2IRD1 gene is suggested to be associated with cranio-facial and visio-spatial symptoms in WBS. However, the GTF2I gene is thought to contribute to WS social behaviours including increased gaze and interest towards strangers according to small chromosome deletion in humans. GTF2I is also associated with general intelligence. WBS facial features are associated with atypical deletions, but the genes have not been completely defined. The facial abnormalities in WBS have been associated with deletions that include GTF2IRD1,
Novel mutation in the Src gene leads to the lack of incisors phenotype.

Craniofacial analysis

Craniofacial cephalomoetric measurements support data in humans and what was shown in the recent mouse model of WBS with shortened face and skull base. Src mutants have incisors that fail to erupt. From the faxitron images it appears that the incisors do not form in this mutant with the maxillary incisor being more affected.

Socialbility, social recognition and ultrasonic vocalization

Individuals and mice models with WBS show hypersociability specifically, children with WBS are not afraid of strangers and as adults continue to display extreme friendliness and outgoing behaviour towards strangers and tend to have charismatic speech rich in vocabulary. To explore sociability in the Src\textsuperscript{(thlthl)} mice, we performed a variety of social interaction tests, including social choice, direct social interaction and, tube tasks. USVs were recorded to measure vocalization during the direct social interaction task.

Using three different behavioral tests to study social interactions, we have shown that Src\textsuperscript{(thhthl)} exhibit an increased social interaction. In the first test measuring the time with a social versus non social subject we showed that mutant mice spent significantly more time exploring the social subject. Measures of motor activity demonstrated no significant difference in number of entering each compartments eliminating the fact that the increased sociability could be a side product of increased activity. Src\textsuperscript{(thhthl)} mice failed to show a preference for the novel encounter in the social short term recognition section of this task, where the mice have the option to explore a
novel mouse versus a familiar mouse, which is usually an indication of impaired social
recognition. But, it is also possible that the decreased social recognition behaviour is masked by
an increase in sociability.

The increased sociability was also observed in a direct social interaction task, which was
performed in a neutral home cage. Interestingly, Src\(^{(thlhl)}\) mice showed an increased social
dominance in the tube test not as a result of aggression but more as a result of overfriendliness.
The Src\(^{(thlhl)}\) were passively standing in the middle of the tube, gazing at the wild type mice
without showing any aggressive behaviours. The wild type mice tried to escape by moving
under the mutant mice, however, they were backing out of the tube after several unsuccessful
attempts. This behaviour could resemble the increased gaze in individuals with WBS.

Similar to an increased social behavior we also observed an enhanced number of USV
calls in a social reunion paradigm of female mice. In particular, there was an increase in the
number of flat and frequency modulated calls produced by Src\(^{(thlhl)}\) females in the first 2 minutes
compared to Src\(^{(+/+)}\) females whereas the number of broken calls was not affected. Also number
of USVs was unchanged in the mating-induced experiment. Therefore, mutations in the Src gene
increased social calls and not reward seeking behaviors such as courtship and mating.

Here is important to mention that, since Csk phosphorylates Src on Tyr530 in humans
and Tyr528 in mice, leading to Src kinase inactivation in a closed conformation under basal
condition, one could predict that the deletion in Csk genes observed in people with autism
disorders could reduce Src basal phosphorylation on Tyr530 therefore increasing Src activity.
For this reason, we suggest that decreased Src function could cause both anti-autistic and WBS
like phenotypes, such as increased sociability and communication.

**General activity and anxiety in the open field**
We assessed exploratory activity and anxiety-related behaviours using the open-field test, as well as the elevated plus maze task, since children and mouse models of WBS show abnormalities in these behaviours and anxiety and hyperactivity are common features in WBS syndrome. Src\textsuperscript{(thh/hl)} mutant mice differed significantly from their respective control mice in having increased locomotor activity and behavioral measures considered to reflect anxiety in the open field test. However, Src\textsuperscript{(thh/hl)} mutant mice failed to show anxiety phenotype in the elevated plus maze.

**Motor performance**

Individuals and mice models with WBS, have decreased motor coordination and balance and show difficulties in learning new motor skills. We assessed motor coordination, strength and motor skill learning in Src\textsuperscript{(thh/hl)} mice, by using the balance beam, grip strength and rotarod task. Behavioral tasks involving complex motor coordination like walking or balancing on a beam involve the combination of several sensory and motor regions of the brain such as motor and pre-motor cortex, brain stem and cerebellum, as well as the function of peripheral muscles guided by the signals from the spinal cord and peripheral nervous system. In order to evaluate abnormalities in motor coordination of Src\textsuperscript{(thh/hl)} mice which may be the result of cerebellar defects, we performed a battery of established motoric tests: accelerating rotarod assays, and beam task for balance. Src\textsuperscript{(thh/hl)} showed no motor defect during first two trails of the rotarod indicating intact motor function in this task, however they were not able to improve their motor skill during the third and forth day which indicates a motor learning deficit in these mice.

Rotarod assay is a direct assay of cerebellar function in motor learning and coordination. Src mutant mice were also impaired in a test for coordination and balance, called the balance beam. Finally, grip strength was reduced in Src mutants when compared to wild type mice.
Auditory startle response

The majority of WBS people show hypersensitivity to loud noises, which can be explained by abnormal auditory processing in these individuals.

The current study examined startle reactions to various intensities of auditory stimuli in the Src\(^{(thlhl)}\) mutant mice. Src\(^{(thlhl)}\) mutant exhibited greater responses to high intensity stimuli compared to Src\(^{(+/+)}\) mice (110 and 115 dB). This could be due to increased sensitivity to loud sound (decreased auditory threshold) or a decrease in the sound level necessary to provoke a startle response (decreased response threshold) in the mutant mice. However, both Src\(^{(thlhl)}\) and Src\(^{(+/+)}\) mice were less responsive to low intensity stimuli similarly (75-105 dB). An explanation for the elevated startle in response to high intensity stimuli in Src\(^{(thlhl)}\) mice is that there is an abnormality in secondary brain regions that modulate the primary startle response. This could occur either through intrinsic disturbance, possibly due to altered synaptic connections, or indirectly, through an increase in arousal rather than a change in the primary sensory response of the auditory circuit. This explanation is supported by the fact that over-responsiveness to sensory stimuli has been shown in humans with WBS syndrome and decreased responsiveness has been shown in people with autism.

Attention and cognitive impairments

Individuals with WBS exhibit mild to moderate developmental delay and cognitive impairment. Most of them suffer from attention deficits. Learning and memory processes were examined using tests such as fear conditioning and Moriss water maze to evaluate long term memory function and visual object recognition to assess short term memory and attention. Both water maze and object recognition measure visio-spatial learning which is impaired in individuals with WBS
Visio-spatial abilities were tested in the water maze. A probe trial given after 6 d of training showed a significant difference between genotypes when the number of target platform crossings was compared between mutant and wild type mice. The learning deficit of Src\(^{thlthl}\) mutants was not due to impaired motor performance or decreased motivation to escape, as all mutants showed similar escape latencies in the visible and hidden platform task. Moreover, the swim speed of Src\(^{thlthl}\) mice was similar to that of wild-type mice (data not shown).

Amygdala-dependent learning was assessed using cued fear conditioning. Src\(^{thlthl}\) mice froze significantly less than Src\(^{+/+}\). The observed defect of auditory fear memory may be due to an increase in salience of the auditory CS during training. As mentioned before when we used the auditory startle response to test the effects of Src mutation on hearing and sensorimotor processing, the response of Src mutant mice was not different when compared to wild types at 85 dB; the same tone intensity that was used for fear conditioning. Therefore, the impaired auditory fear memory in mice with Src mutation cannot be attributed to changes in simple auditory processing in the startling stimulus provided during this task.

### Conclusion

Src mutant mice show some phenotypic overlap with WBS like and anti-autistic like behaviours. Also, since the absence of Csk, a negative regulator of Src, is observed in microdeletion patients with autism. We suggest that Src is a great therapeutic target for WBS or any other neurodevelopmental disorders with abnormal social behaviours.
4.3 Figures

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Figure 4-1 Lack of incisors phenotype maps to a region on Chromosome 2 containing *Src* gene.
Original toothless (Thl) mouse founder was noticed on S129 genetic background. Whole genome scan in (S129xC57Bl/6) F2 toothless mice demonstrated linkage to D2Mit411. Thl1-Thl10 mice were genotyped for additional markers to refine critical interval region (S – homozygous for S129 allele, H – heterozygous for S129 and C57Bl/6 alleles). Critical interval region containing Src gene is highlighted.

![Mutant Src](Image)

**Mutant Src**

CATGGCCCTATGTGGAGCCGGATGAACCTATGT

| Met | Ala | Tyr | Val | Glu | Pro | Asp | Glu | Leu | Cys |

**WT Src**

CATGGCCCTATGTGGAGCGGGATGAACCTATGTG

| Met | Ala | Tyr | Val | Glu | Arg | Met | Asn | Tyr | Val |

Figure 4-2 Mutant mice have an insertion of C nucleotide in exon 12 of the Src gene
1 WT
2 Src^{thl/+}
3 Src^{thl/thl}

Src (60 kDa)

Beta-tubulin
Figure 4-3 Src protein can not be detected in lysates from $Src^{thl/thl}$ and $Src^{thl/+}$ mice

Immunoblot analysis of brain lysates from WT, $Src^{thl/+}$ and $Src^{thl/thl}$. 50 mg of protein from the corresponding samples were resolved on a 10% acrylamide gel and transferred onto a nitrocellulose membrane. Src protein was visualized using antibody Ab7950 (Abcam). WT Src and $Src^{thl}$ bands are marked with an asterisk and an arrow, respectively. Schematic diagrams of WT and mutant Src are also shown.
C nucleotide insertion in exon 12 of the gene generates an unique site for the HpaII restriction enzyme. Presence of the mutation can be determined by PCR followed HpaII digestion. Generated products are resolved on a 4% agarose gel.
Figure 4-5 Craniofacial dysmorphology and growth retardation in Src\(^{thl/thl}\) mice.

Faxitron images of wild type (A, C) and Src\(^{thl/thl}\) (B, D) mice in lateral (A, B) and superior (C, D) views with landmarks used for cephalometric analysis indicated and described in Materials and Methods. Analysis of data is summarized in Table Y. Weight of Src\(^{+/+}\) and Src\(^{thl/thl}\) mice.
Table 4-1: Means and standard error of the mean (SEM) of linear variables of the skull in Wild type and src mutant mice

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Wild type</th>
<th>Mutant</th>
<th>p-value</th>
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<td>110.8</td>
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<tr>
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<td>0.7</td>
<td>221.0</td>
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<tr>
<td>Ps-Inc</td>
<td>113.1</td>
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Figure 4-6 Social approach behaviors are increased and social recognition is impaired in Src\textsuperscript{thl/thl} mutant mice

Src\textsuperscript{thl/thl} mutant mice spent more time sniffing a social cage versus a non-social cage (A) however number of entries into each compartment was not different between groups (B). During the second phase of this test to measure social recognition, Src\textsuperscript{thl/thl} mutant mice did not show a preference for a novel mouse versus a familiar mouse from the first phase (C) however number of entries into each compartment was not different between groups (D). Data are presented as mean ± SEM.; *p < 0.05, **p < 0.01 and ***p < 0.001, compared to the chamber with empty cage, between genotypes and within genotypes respectively (A), and within genotype **p < 0.01 (C)
Enhanced sociability in a direct social task and social dominance in $Src^{thl/thl}$ mutant mice with intact olfaction

In a direct social interaction test, $Src^{thl/thl}$ mutant mice showed an increased frequency of interactions with a stranger mouse (A) $Src^{thl/thl}$ mutant mice showed social dominance over their wild type littermates in a tube test. Data are presented as mean ± SEM.; *** $p < 0.001$, between genotypes. $Src^{thl/thl}$ mutant mice were able to habituate to smell overtime and dishabituate toward a novel smell.
Figure 4-8 Enhanced social reunion female USV calls in $Src^{thl/thl}$ mutant mice

A) Time courses of total number of USVs in wild-type and $Src^{thl/thl}$ mutant mice when introduced to females for a 5 minute trial. A gradual decline in total number of calls is observed across time but total number of calls still high, with no significant difference between wild-type and mutants.

B) Time courses of total number of USVs in wild type and $Src^{thl/thl}$ mutant females when reunited with a litter mater for a 5 minute trial. A gradual incline in total number of calls is observed across time with a significant difference between wild-type and $Src^{thl/thl}$ mutant females in total number of USVs produced in first and second minute.

C) There is a significant difference in the number of flat USVs produced by wild-type vs. $Src^{thl/thl}$ mutant at minute 1.

D) There was no significant difference in the number of broken USVs produced by wild-type vs. $Src^{thl/thl}$ mutant females across time.

E). There is a significant difference in the number of frequency modulated USVs produced by wild-type vs. $Src^{thl/thl}$ mutant at minute 1 and at minute 2.

Data are presented as mean ± SEM. for each time point, *$p < 0.05$, **$p < 0.01$, between genotypes. The solid and the dashed bars represent wild-type and $Src^{thl/thl}$ mutant mice respectively.
**B**

% Time in the centre

- **Src**<sup>+/+</sup>
- **Src**<sup>(thi/thi)</sup>

**C**

% Time in the open arm

- **Src**<sup>+/+</sup>
- **Src**<sup>(thi/thi)</sup>
Figure 4-9 $Src^{thl/thl}$ mutant mice showed hyperactivity in the open field

$Src^{thl/thl}$ mutant mice showed an increase in total distance travelled in the open field (A). Percentage of time spent in the centre of the arena versus the entire open field was decreased in $Src^{(thl/thl)}$ (B) (*p<0.05). However, percentage time spent in the open arm versus the close arm of the elevated plus maze showed no significant differences among groups (p>0.05) (C).
Figure 4-10 Balance and motor strength deficiency in \( Src^{thl/thl} \) mice and the inability to learn motor skills

Quantitative analysis of raised balance beam, grip strength, and rotarod task in wild-type and \( Src^{thl/thl} \) deficient mice. (A) \( Src^{thl/thl} \) mice (empty bars) needed significantly more time to cross the balance beam than did wild-type (black bars) (B) \( Src^{thl/thl} \) mice had significantly more hind feet slips than wild-type mice. (D) Grip strength analysis revealed significantly reduced grip strengths in \( Src^{thl/thl} \) mice compared to wild-type animals. (E) Rotarod analysis of motor functioning in wild-type and \( Src^{thl/thl} \) mice. Mice were tested on an accelerating rotarod with the same speed for four consecutive days. The latencies from rotation onset until the mice fell off the rod were measured. Wild-type mice (solid line) managed to stay significantly longer on the accelerating rotarod than \( Src^{thl/thl} \) mice (dashed line) on day 3 and 4. The falling latencies were similar in both groups on day 1 and 2. Falling latencies are compared within genotype
between day 1 and Day 4. Data are presented as mean ± SEM.; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4-11 Auditory startle in response to high startling stimulus is increased in Src<sup>thl/thl</sup> mutant mice

Both the Src<sup>+/+</sup> and Src<sup>thl/thl</sup> mice exhibited similar startle amplitude responses to the low intensity acoustic stimuli (70-105 dB). Src<sup>thl/thl</sup> mice showed higher responses to the high intensity stimuli (110-115 dB). *p<0.05
Figure 4-12 Impaired spatial memory in \( \text{Src}^{(thl/thl)} \) mutant mice.

\( \text{Src}^{(+/-)} \) and \( \text{Src}^{(thl/thl)} \) mice were assessed in the Morris water maze procedure. (A) Mean latency to reach a target platform in a visible platform session (day 1 and day2), and in a hidden-platform acquisition phase (days 2-6). Spatial memory retention was assessed in the probe trials administered 2 h after days 6. Mean number of platform crosses (B) during probe trial 1 (frequency of platform crosses) Data are shown as mean ± SEM. n =12 wild-type and 12 mutant mice; **\( p < 0.005 \), ***\( p < 0.001 \).
Figure 4-13 Src\(^{(thl/thl)}\) mice showed impaired auditory fear conditioning, however context was not affected. A subtle increase in freezing was observed during baseline and novel tone presentation in Src\(^{(thl/thl)}\) mice.

(A) Freezing (as a percentage of total time) in a novel conditioning chamber

(Baseline) Src\(^{(thl/thl)}\) and Src\(^{(+/-)}\) mice. After recording the baseline freezing for 120 sec, a tone was presented for 30 sec (novel tone), after which mice were given a foot shock and left inside the chamber for another 30 sec (After shock). (B) Cued fear memory 24 h after pairing. Pre-CS is freezing in the absence of the tone (Pre-CS) in a novel context while CS is percentage freezing during tone presentation. Data are presented as mean ± SEM.; *p < 0.05, **p < 0.01.

(C) Src mutation does not impair the formation of a context fear memory.
Figure 4-14 Mice that lack Src have a deficit in short term object recognition.

Mean time (sec) spent exploring the displaced and non-displaced objects in the spatial change session. (A) Mean time (sec) spent exploring the novel item and three familiar objects in the non-spatial change session (B). Data are expressed as mean ± SEM. n = 12 wild-type and 15 mutant mice; ***p < 0.001.
Chapter 5

The role of Src upregulation in social learning and memory

5 « Impaired olfactory social memory in 129P2 inbred mouse strain is rescued by reduced Csk expression

5.1 Abstract

The C-terminal Src kinase (Csk) is an essential signaling factor guiding CNS development. In the adult brain, Csk-mediated control of Src may also modulate glutamatergic synaptic transmission and N-methyl-D-aspartate receptor (NMDAR)-dependent synaptic plasticity. The regulation of NMDA-dependent plasticity by a myriad of kinase cascades has been investigated intensively during spatial and fear learning, while little is known about the regulatory kinases and role of NMDA-dependent plasticity during equally critical forms of social learning. We assessed social memory in Csk(+/+) and Csk(+/-) mice backcrossed onto 129P2, an inbred strain with wild type impairments in social memory. Reduced Csk expression in Csk(+/-) mice was associated with increased NMDAR subunit 2B (NR2B) phosphorylation in the amygdala (AM) and olfactory bulb (OB), and with markedly improved social recognition memory and social transmission of food preference (STFP). In contrast, phosphorylation of NR2B was increased by 4% in the hippocampus of 129P2/Csk(+/-) mice, and the poor spatial object recognition memory of wild type 129P2/Csk(+/+) mice was not rescued by reduced Csk expression. The Csk pathway appears to be a critical signaling cascade regulating social learning and memory, and
presents a possible therapeutic target in diseases like autism that are characterized by aberrant social behaviors.

5.2 Introduction

The C-terminal Src kinase (Csk) acts to suppress Src family kinase activity and serves as a critical regulator of Src-mediated signaling in vivo. Genetic ablation of Csk leads to embryonic lethality (E9-10) and to severe defects in neuronal tube formation (Imamoto and Soriano, 1993). The expression of Csk is down-regulated in the adult brain, however, with the exception of the olfactory bulb (Kuo et al., 1997a). Despite this restricted adult expression, Xu et al. (2008) demonstrated that Csk regulates excitatory synaptic transmission and plasticity in the hippocampus, and interacts with NMDAR subunit 1 (NR1) in crude synaptosomal membrane fractions P2 and LP1. Moreover, a recombinant Csk vector depressed NMDAR responses in acutely isolated CA1 pyramidal neurons, an effect that was blocked by the Src (40-58) peptide inhibitor (Imamoto and Soriano, 1993).

Recombinant Src increases the open probability of NMDARs (Yu et al., 1997), and can increase NMDAR-mediated currents in acutely isolated CA1 neurons (Lu et al., 1998, Huang et al., 2001) by phosphorylating receptor subunits NR2A and NR2B (Salter and Kalia, 2004). Furthermore, the intracellular application of both anti-Csk and Src activator peptide led to enhanced NMDAR and AMPAR currents and the occlusion of subsequent LTP (Xu et al., 2008). A link between Src kinase activity and several forms of learning and memory has been established (Purcell et al., 2003, Kalia et al., 2004). We surmised that reduced Csk expression in the adult brain could lead to enhanced NMDAR phosphorylation and enhanced NMDAR-dependent learning and memory. While most studies investigating the relation between kinase signaling, NMDA receptor function, and behavior have focused on learned fear, object
recognition, or spatial memory, various forms of social behavior, including social recognition and transfer of food preference, are also vital for survival. We characterized social memory in male 129P2 mice in several different amygdala and olfactory-dependent social learning tasks (Richter et al., 2005).

Social communication in rodents is based on odors produced by exhaled gases, pheromones, feces, and urine (Sanchez-Andrade and Kendrick, 2009)

The olfactory system is closely connected to both the hippocampus and amygdala, two neural structures central for social and emotional behaviors. The social recognition pathway starts with detection of pheromones by the vomeronasal organ that projects to the accessory olfactory bulb (AOB) and then to the medial (MeA) and cortical (CoA) nuclei of the amygdala (Richter et al., 2005). Olfactory recognition memories require activation of ionotropic glutamate receptors (NMDA and AMPA), followed by nitrous oxide release (Gao et al., 2009, Sanchez-Andrade et al., 2005). Blocking either NMDA or AMPA receptors in the amygdala or olfactory bulb will disrupt long-term memory formation (Brennan and Keverne, 1997)

Several inbred strains, including C3H/HeJ, AKR/J, A/J, and 129S1/SvImJ, do not show any preference for social novelty (short-term social recognition) (Moy et al., 2007). While 129P2 exhibited normal olfaction, they demonstrated impaired short-term and long-term social recognition, as well as poor socially transmission of food preference (STFP). These aberrant olfactory social behaviors were rescued in 129P2/Csk(+/−)mice, possibly by Src-mediated enhancement of NMDAR activity and synaptic plasticity.

5.3 Results

5.3.1 Western blots

The expression of Csk was measured in the olfactory bulb, amygdala, and hippocampus in both 129P2/Csk(+/−) and 129P2/Csk(+/−) mice. Expression of Csk was detected in all three tissues but
was highest in the olfactory bulb (Figure. 1A) as shown previously (Kuo et al., 1997a). The Csk kinase can reduce NMDA receptor currents through downregulation of Src-mediated receptor phosphorylation (Imamoto and Soriano, 1993), suggesting that reduced Csk could increase phosphorylation of NMDAR subunits. Indeed, densitometric quantification of the Western blots probed using phospho anti-pY1472 (Figure. 1B) revealed that reduced Csk was associated with enhanced basal phosphorylation of NR2B at Y1472 compared to WT mice in both the olfactory bulb (WT intensity: 43.45 ± 5.670 N=6; Csk(+/-): 71.85 ± 9.181, N=6; p<0.05) (Figure 1C) and amygdala (WT intensity: 39.41 ± 7.250, N=4; 129P2/Csk(+/-): 74.22 ± 11.37 N=4; p<0.05) (Figure 1D) 4% increase in the hippocampus (WT intensity: 93.15 ± 0.5674, N=4; 129P2/Csk(+/-): 97.38 ± 0.9875, N=4; p<0.05) (Figure. 1E).

5.3.2 Activity in an open field

Exploration in an open field can assess both motor capacity and affective state. We measured total distance traveled and horizontal activity as indices of motor ability, as well as vertical activity, number of rearings, and time spent in the central field as indicators of basal anxiety. The 129P2/Csk(+/-) did not differ from wild type littermates in total distance travelled (Figure. 2A), horizontal activity (Figure. 2B) or in the time spent in the center region of the open field (WT: 0.7481% ± 0.3658, N=18; 129P2/Csk(+/-): 1.560% ± 0.6719, N=14; F(1,21)=1.26, P>0.05) (Figure 2C).

5.3.3 Elevated plus maze

The 129P2/Csk(+/-) mice spent significantly more time exploring the open arms of the elevated plus maze than wild type mice (WT= 6.547 ± 2.688%, N=12; Csk(+/-)= 24.04 ± 6.884%, N=11; F(1,21)=5.96, p<0.05), suggesting a decrease in one anxiety-like endophenotype in Csk(+/-) mice (Figure 3B). Similar to the open field test, however, the total number of arm entries indicated no difference in overall activity (Figure 3A).
5.3.4 Social approach tests

5.3.4.1 Social choice and social novelty tests

The social choice test assesses the general tendency of a mouse to approach a conspecific under non-threatening conditions relative to the time spent exploring an inanimate object. In the three compartment chamber, we measured the total time spent sniffing a wire cage containing a stranger mouse in one compartment and the time spent sniffing an identical but empty wire cage in the opposite compartment (Moy et al., 2008). We observed a significantly higher preference for social approach (mouse) over exploration of the empty wire cage (object) in both wild types (stranger mouse: 157.6 ± 26.85 s; object: 84.78 ± 15.89 s; N=14, p<0.05, F(1,26)=5.45) and Csk(+/-) mice (stranger mouse: 169.1 ± 31.09 s; object: 57.47 ± 13.86 s; N=12, p<0.005, F(1,22)=10.75) (Figure 4A).

In the test for social novelty preference, only 129P2/Csk(+/-) mice demonstrated a significant preference for the novel stranger (mouse 2) over the pre-exposed familiar mouse 1 as evidenced by the increase in time spent sniffing the wire cage containing the novel mouse. In fact, 129P2 wild type mice did not appear to distinguish between the first and second mouse as indicated by interaction time (familiar mouse 1: 73.00 ± 9.388 s; novel mouse 2: 80.08 ± 11.72; N=22, p>0.05, F(1,24)=1.71), suggesting an impairment in short term social recognition memory in the 129/P2 inbred strain. This deficit was rescued in Csk(+/-) mice (familiar mouse 1: 65.82 ± 17.98 s; novel mouse two: 138.2 ± 20.69 s; N=11, p<0.05, F(1,20)=6.97) (Figure 4B).

As previously reported (Moy et al., 2004), the number of entries into each outer side chamber (containing a mouse or empty cage) was the least sensitive index of social approach in the choice tests (Table 1), and is usually used as a control measurement for exploration of the new environment. The number of entries into each chamber was not different between genotypes across the three different sessions. The entry measure provided evidence that all of the subjects
tested in the present study explored the social test box. Therefore, the lack of preference for social novelty in 129P2/Csk(+/+) could not be attributed to hypoactivity (Table 1).

5.3.4.2 Social recognition test

The social recognition test relies on the known reduction in interaction time between two mice over repeated presentations and increased interactions between unfamiliar conspecifics. In this version of the social recognition test, an adult mouse was first habituated to a new home cage for 15 minutes, followed by a 2 minute presentation of a novel juvenile mouse (presentation 1, P1). The adult mouse was then re-exposed to the same juvenile 24 h later (presentation 2, P2). There was a significant reduction in the duration of investigation time between P1 and P2 in the 129P2/Csk(+/-) mice (P1: 94.93 ± 5.018 s; P2: 67.96 ± 5.262 s; N=12, P<0.005, F(1,22)=13.76) but not in the 129P2/Csk(+/-) wild type mice (P1: 89.12 ± 5.519 s, P2: 87.41 ± 5.881 s; N=16, P>0.05, F(1,30)=0.04), indicating that 129P2/Csk(+/-) mice were better able to recognize the pre-exposed juvenile (Figure 5). Thus, social recognition memory was improved in 129P2/Csk(+/-) mice.

5.3.5 Social transmission of food preference

Social transmission of food preference (STFP) is an innate form of olfactory memory that allows mice to gage food safety by the health of conspecific demonstrators. The observer mouse interacts with a healthy demonstrator mouse that has recently eaten a novel food. When observer mice are presented with a choice between the food eaten by the demonstrator and another novel food, observer mice prefer the food eaten by a healthy demonstrator. Mice were presented with 2 unfamiliar flavors of powdered food (rodent chow flavored with 2% cocoa or 1% cinnamon). The 129P2/Csk(+/-) mice strongly preferred the flavor previously consumed by the demonstrator mouse (the cued food) as measured by weight consumed in a free choice test (80.45 ± 5.468 %
of food intake, N=12, p<0.05, F_{(1,21)}=7.31) while wild type mice showed no preference for the cued food (47.03 ± 11.47%, N=11, p>0.05) (Figure 6A).

5.3.6 Tests of Olfaction

As the social memory and STFP tests rely on the ability of mice to discriminate odors, it was important to determine if all mice had similar olfactory function. For this purpose, we measured the latency to find a buried food pellet. Both genotypes were able to find the buried food in equal time (WT: 2.255 ± 0.3755 s, N=12; 129P2/Csk(+/-) = 1.878s ± 0.1606 N=8; p>0.05, F_{(1,18)}=0.61). Thus, differences in olfactory-dependent social memory tests were not due to differences in olfactory sensitivity (Figure 6B).

5.3.7 Displaced object recognition

In the displaced object recognition task, many strains of mice, including C57Bl/6, will spend significantly more time investigating familiar objects that have been displaced from an earlier encounter, indicating a memory for the spatial organization of objects. Wild type 129P2 mice exhibited impaired detection of a spatial change in previously explored objects, and this deficiency was not rescued in 129P2/Csk(+/-) mice. An initial fifteen minute free exploratory period revealed no innate preference for any individual objects in either WT or Csk(+/-) 129P2 mice (Figure 7A), or in the positive control strain C25Bl/6 (data not shown). In contrast to C57Bl/6 mice, neither 129P2 genotype demonstrated any exploratory preference for the displaced over the non-displaced objects, indicating that this particular deficit was not rescued by reduced Csk expression (Figure 7B).
5.4 Discussion:

Fear conditioning and spatial navigation are by far the most frequently studied rodent behaviours, and much is known about the neural substrates, signalling cascades, and the synaptoplastic mechanisms that underlie these learned behaviours. Associating places or cues with food or danger has an obvious survival benefit. In addition, mammals learn necessary survival skills from other members of the species through social learning, but the neuronal pathways, and modulatory signals have not been investigated with the same rigour. Our findings suggest that decreased Csk expression leads to enhanced NR2B phosphorylation in brain regions important for NMDA-dependent social memories. These social memory paradigms may help define behavioural endophenotypes relevant to the study of aberrant socialization in humans.

Enhanced NR2B phosphorylation could decrease the threshold for learning. Indeed, the Src family kinases are a well-studied example of a neuronal signal that enhances NMDA activity, NMDA-dependent synaptic plasticity, and memory under various learning situations. The C-terminal Src kinase (Csk) acts to inhibit Src, suggesting that Csk inhibition or genetic ablation could improve memory through Src disinhibition. To test whether Src kinase disinhibition could improve behavioural phenotypes in the 129P2 background strain, 129P2/Csk(+/+) and 129P2/Csk(+-) littermates were first compared on tests of motor activity, anxiety, and olfactory sensitivity, as these traits or abilities are either necessary for social behaviour (olfaction) or greatly influence performance (motor activity, anxiety). While 129P2/Csk(+/+) and 129P2/Csk(+-) mice exhibited similar mobility and anxiety in the open field and similar olfactory thresholds, NMDA-dependent social choice, novelty preference/discrimination, social recognition, and transfer of food preference were all rescued by reduced Csk activity. Concomitant with improved social memory, reduced Csk activity in Csk (+/-) mice was associated with elevated NR2B phosphorylation at Y1472, possibly leading to increase synaptic
plasticity in relevant neuronal circuits within the olfactory bulb-amygdala pathway. In contrast, the hippocampus-dependent displaced object recognition task was not rescued, nor was hippocampal NR2B tyrosine phosphorylation greatly increased, suggesting that NMDA phosphorylation could enhance certain forms of learning and underscoring the possible role of amygdalar NR2B phosphorylation in social memory.

The 129P2/Csk(+/−) mice showed a decreased anxiety-like phenotype in the elevated plus maze but not in the open field test. Previous studies suggested that an imbalance between excitatory and inhibitory neurotransmitter systems leading to glutamatergic hypoactivity can explain fearfulness in olfactory bulbectomised OB rats (Kelly et al., 1997). Enhanced NR2B phosphorylation did reduce innate anxiety in Csk heterozygotic mice in the elevated place maze. However, no effect was observed in the open field. None-the-less, general anxiety did not appear to contribute to the reduced social behaviours in wild types as they demonstrated the same exploratory tendencies in tests of socialization and object recognition.

Olfaction is thought to be one of the most important senses for rodents, governing social recognition and discrimination, foraging, and protection against toxic ingestion. Wild type 129P2 mice were impaired in several dimensions of sociability, neither preferring social novelty nor showing long-term social recognition memory. Downregulation of Csk rescued the aberrant STFP and both short-term and long-term olfactory social memory in the 129P2 inbred strain, possibly by increasing the NR2B phosphorylation in the amygdala and olfactory bulb. Indeed, both sensory memory tasks are known to be NMDA-dependent, indicating that Csk is an important regulator of NMDA-dependent olfactory memory formation. Similarly, forebrain-specific over-expression of the NR2B subunit in the amygdala, the CA1 region of the hippocampus, the striatum, and the cortex of transgenic animals resulted in superior memory on a variety of tasks (Tang et al., 1999), including STFP. Since NR2B phosphorylation was
increased by 4% in the hippocampus and could not rescue the aberrant displaced object recognition memory in mice, we suggest that reduced Csk protein levels specifically upregulated NMDAR function in the amygdala and olfactory bulb, resulting in enhanced olfactory social recognition in 129P2/Csk(+/−) mice.

Our study demonstrates the importance of choosing the optimal strain to study the contribution of individual genes to a specific behavior or group of behaviors. We chose a strain that is a poor performer in several tasks of social memory so that a possible rescue could be observed. Similarly, strains that perform well in many behavioral tasks are ideal for detecting phenotypic impairments, while average performing strains are beneficial if the behavioral outcome of a genetic manipulation is uncertain. We performed a battery of different cognitive tasks dependent on the hippocampus, amygdala, and olfactory bulb using the deficient 129P2 strain. Reducing Csk expression could also enhance other behavioral tasks, such as fear conditioning, and object recognition. To explore this possibility, the genetic background strain could be changed to one with behavioral deficits in these tasks or the training time could be reduced so that only the superior learner mice could learn the task.

We do not yet know the allelic combinations that confer these abnormal behavioral phenotypes in 129P2 mice. Here we argue that increased NR2B activity rescued learning impairments in this strain, suggesting a role for WT deficits in NMDA-dependent plasticity. In the future, it is essential to perform QTL and microarray analysis on the 129P2/Csk(+/+) and 129P2/Csk(+/−) mice to identify targets involved in olfactory social memory (Schimanski and Nguyen, 2004). Also, it would be of great interest to investigate whether this mouse strain shows impaired synaptic plasticity in the amygdala and OB and if this impairment is rescued by CSK reduction. These mice could also be used in QTL analysis to determine whether QTLs for
amygdala or OB LTP and QLTs for amygdala and OB-dependent learning and memory overlap. If this were the case, a causal relationship between LTP and memory might be strengthened.

Differences in neurocellular organization may also underlie these functional deficits in social behaviours and social learning in 129P2 mice. It has been shown that Csk phosphorylates SFKs, including Src and Fyn, and that this suppresses the ability of Fyn and Src to phosphorylate downstream targets. Expression of Csk is normally highest in the embryonic brain, while Fyn activity is low (Inomata et al., 1994). During myelination, Csk levels decrease substantially compared to those in the embryonic brain. The brain of fyn-/- mice had fewer oligodendrocytes and reduced numbers of myelin sheaths (Sperber et al., 2001). Given the integrated activities of SFKs and their upstream regulators in development, it is possible that Csk plays some indirect role in myelination, a subject of a future study.

In addition to the importance of Csk in synaptic plasticity, learning, and memory, Csk might be an interesting target molecule to regulate social tendencies and behaviors. Similar to our 129P2 mice, 129S1/SvImJ (Moy et al., 2007) mice exhibited a preference for sniffing a social subject versus a nonsocial object in the three chamber social task, indicating normal social approach. However, these mice failed to show a preference for social novelty in the three chamber social approach task. Failure to display preference for social novelty by this inbred strain could be a relevant endophenotype for the symptoms of autism. Aberrant social behaviors in people with autism may emerge as indiscriminate approaches to strangers in addition to an overall lack of social approach (Loveland et al., 2001). A lack of preference for social novelty in mice might, therefore, model autism-spectrum behaviours where these individuals prefer to remain with familiar people.

Our results provide evidence of enhanced NR2B phosphorylation in the amygdala and olfactory bulb, and improved olfactory social memory in 129P2 mice when Csk protein levels were reduced during development and adulthood. Further investigation of this pathway could facilitate the development of new treatments for diseases characterized by aberrant social and social recognition behaviors, such as autism.
5.5 Figures
**Figure 5-1 Representation of Csk expression and NR2B phosphorylation in different brain regions**

Csk protein expression levels in the olfactory bulb, amygdala and hippocampus in wild type and heterozygote Csk mice (A). NR2B phosphorylation at residue Y1497 is increased in the amygdala and the olfactory bulb in 129P2/Csk(+/−) (B). NR2B phosphorylation in the amygdala (C) NR2B phosphorylation in the olfactory bulb (D) NR2B phosphorylation in the hippocampus. NR2B phosphorylation levels in the olfactory bulb, amygdala and hippocampus in wild type and heterozygote Csk mice (E).
A. Total distance traveled (cm)

B. Horizontal Activity

C. Number of Rearings

Legend:
- Csk (+/+)
- Csk (+/-)
Figure 5.2 Activity measurements are similar between 129P2/Csk(+/+) and 129P2/Csk(+/−) mice.

Each data point represents activity for 5 min. A) Total distance traveled B) Horizontal activity C) Number of rearing
Anxiety like behaviour is decreased in the elevated plus maze but not in the open field. Percentage time spent in the centre of the open field is not significantly different between groups (A) However 129P2/Csk(+-) mice spent more time in the open arm on the elevated plus maze when compared to 129P2/Csk(+/+) *P<0.05 (B) Each time point represents activity during 5 min.
A

Sniffing Time (s)

Empty cup  Mouse 1  Empty cup  Mouse 1

Csk (+/+)  Csk (+/-)

B

Sniffing Time (s)

Mouse 1  Mouse 2  Mouse 1  Mouse 2

Csk (+/+)  Csk (+/-)
Figure 5-4 Short term social recognition was rescued in 129P2/Csk(+/−) mice

Time spent sniffing each cage during the test for (A) sociability and (B) preference for social novelty in 129P2/Csk(+/+) and 129P2/Csk(+/−) mice. Both groups showed a preference for a social subject over a non-social empty cage (A) Only 129P2/Csk(+/−) showed a preference for social novelty (B) **P<0.005
<table>
<thead>
<tr>
<th>Sessions</th>
<th>Number of Entries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Csk(+/+)</td>
</tr>
<tr>
<td><strong>Habituation</strong></td>
<td></td>
</tr>
<tr>
<td>Chamber 1</td>
<td>6.571428571</td>
</tr>
<tr>
<td>Chamber 2</td>
<td>6.214285714</td>
</tr>
<tr>
<td>Centre Chamber</td>
<td>12.78571429</td>
</tr>
<tr>
<td><strong>Sociability</strong></td>
<td></td>
</tr>
<tr>
<td>Social Chamber</td>
<td>23.85714286</td>
</tr>
<tr>
<td>Non Social Chamber</td>
<td>16.28571429</td>
</tr>
<tr>
<td>Centre Chamber</td>
<td>16.35714286</td>
</tr>
<tr>
<td><strong>Short term social novelty recognition</strong></td>
<td></td>
</tr>
<tr>
<td>Social Chamber 1</td>
<td>18.57142857</td>
</tr>
<tr>
<td>Social Chamber 2</td>
<td>20.07142857</td>
</tr>
<tr>
<td>Centre Chamber</td>
<td>18.21428571</td>
</tr>
</tbody>
</table>
Table 5-1

Number of entries into each side chamber during the test for (A) sociability and (B) preference for social novelty recognition in 129P2/Csk(+/+) and 129P2/Csk(+-) mice. There are no significant differences in number of entries among groups.
Figure 5-5 Long-term social memory is impaired in 129P2(Csk+/+) mice and restored in 129P2(Csk+/-) littermates.

A) Familiar juveniles were exposed to both groups at test trials. There was significant reduction in investigation duration, indicating long-term social memory in the 129P2/(Csk+/-) mice, **P<.005** but not in the 129P2/(Csk+/+) mice and not with a novel juvenile.
Figure 5-6 Social olfactory memory enhancement in 129P2(Csk+/-) mice measured by the social transmission of food preference test.

129P2(Csk+/-) mice showed an enhanced preference for familiar food, but 129P2(Csk+/+) did not show any preference. Data are presented as of percent preference intake of familiar food.
Reduced Csk expression can not restore spatial object recognition deficit in 129P2(Csk+/+) mice.

A) Mean duration (sec) of object exploration in the training phase (10 min) of object recognition. B) Mean time (sec) spent exploring the displaced and non-displaced objects in the spatial change session.
Chapter 6

6 « Final discussion»

The non-receptor tyrosine kinase Src is a central mediator of social learning in mice. This conclusion is supported by our results documenting the behavioural effects of a Src inhibitory peptide and behavioural analysis of Src and C-terminal Src kinase (Csk) mutants. The Src signalling pathway is an important focus for both basic research probing the mechanisms of social behaviour and studies aimed at elucidating the pathophysiology of human psychiatric disorders characterized by aberrant socialization.

For the in-vivo inhibition of Src, we used a multidisciplinary approach to investigate Src-mediated regulation of NMDAR subunit 2B (NR2B) surface expression, NMDA-mediated plasticity, and social learning. Furthermore, upregulation of Src through knock out of the inhibitory Csk rescued aberrant social learning in a mouse strain model of reduced socialization. Inhibition of Src–NMDAR interaction attenuated NR2B phosphorylation and decreased NR2B surface expression in the amygdala. Blocking the interaction of the Src tyrosine kinase with the NMDAR complex impaired auditory conditioned fear memory and social recognition. Furthermore, at the lateral to basolateral nucleus pathway (LA-BLA), inhibition of Src impaired long-term potentiation, a form of synaptic plasticity that is vital for encoding CS-US associations (Maren et al., 2001). In sum, these data indicate that Src-mediated phosphorylation of NMDARs is necessary for two vital forms of amygdala-dependent learning.

These results are in agreement with our original hypothesis proposing a central role for Src in NMDAR-dependent plasticity and learning. Our results contradict a proposed role in hippocampus-dependent learning and memory, however, and suggesting that Src exerts it
greatest known impact on social learning rather than spatial learning. The disparate influence of Src in these two broad forms of behaviour may be explained by distinct expression patterns of Src and Fyn (Table 6-1); while Fyn is abundantly expressed in the hippocampus, it is only a minor Src family kinase in the amygdala, suggesting that it cannot compensate for the absence of Src.

**Table 6-1**

Mouse brain expression values were derived from the Allen Brain Atlas (http://www.brain-map.org/) and reflect average expression levels over a particular region normalized to maximum possible expression (which is derived from a set of ubiquitously expressed house-keeping gene

<table>
<thead>
<tr>
<th>Expression level</th>
<th>Src</th>
<th>Fyn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebellum</td>
<td>72</td>
<td>29</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>100</td>
<td>24</td>
</tr>
<tr>
<td>Hippocampal region</td>
<td>100</td>
<td>76</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>56</td>
<td>4</td>
</tr>
<tr>
<td>Midbrain</td>
<td>53</td>
<td>3</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>100</td>
<td>42</td>
</tr>
<tr>
<td>Amygdala region</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>Striatum</td>
<td>96</td>
<td>8</td>
</tr>
<tr>
<td>Thalamus</td>
<td>100</td>
<td>38</td>
</tr>
</tbody>
</table>

Our results demonstrating a unique role for Src-NMDAR2B signalling in the amygdala confirm several previous studies of NMDAR2B subunit modification using genetic and pharmacological approaches. Mice harbouring a knockin Y1472F mutant exhibited decreased receptor surface NMDAR expression, impaired cued-dependent fear learning, and decreased long term synaptic plasticity in the amygdala (Nakazawa et al., 2006). The genetically modified mouse harbouring the Src point mutation, Src<sup>(thl/thl)</sup>, demonstrated gross development
abnormalities and growth retardation. The localization of TFII-I was also altered in this Src mutant.

We observed differences in behavioural phenotypes similar to people with WBS. Sociability and social vocalization were increased in three different social affiliation tasks in $Src^{(thl/thl)}$ mice. They showed hyperactivity in the open field and spent significantly less time in the centre of the open field (a behavioural endophenotype of anxiety). Also, motor function was decreased in three different motor performance tasks. The $Src^{(thl/thl)}$ mice showed an enhanced startle response to loud stimuli, impaired cued fear conditioning, and poor visiospatial memory in the Morris water maze. The $Src^{(thl/thl)}$ mice were also unable to learn a visual object recognition task. All of these behavioural phenotypes resemble those observed in people with WBS. The enhanced social interaction, communication, and enhanced startle response can be also regarded as anti-autistic behaviours, while the hyperactivity and the impaired short term memory are indications of attention deficits in these mice.

The $Src^{(thl/thl)}$ point mutation is within the kinase domain, leading to a premature stop codon. This mutation predicts a deletion of $\frac{1}{2}$ of the kinase domain. It was predicted that fyn would not compensate for reduced Src activity because the mutant Src protein would retain regions important for anchoring to the membrane and for binding to target proteins. These mice were also predicted to demonstrate impaired learning and memory. In contradiction to our hypothesis, Western blot analysis indicate an almost complete loss of Src protein expression in the nucleus and cytosol, a result that might stem from the introduction of a premature stop codon upstream of a special nonsense-mediated mRNA decay (NMD) signal (Chang et al., 2007). Nonsense-mediated decay mechanisms can distinguish between premature and normal stop codons by a second signal downstream of the stop codon that is located within a sliceable downstream intron in mammalian genes. The spliceosome leaves an imprint with the second
signal on the spliced mRNA that remains even when it enters the cytoplasm to be read by ribosomes Codon 343 is in exon 12, upstream of the spliceable intron containing the signal for NMD (Carter et al., 1996, Cheng et al., 1994, Thermann et al., 1998, Ulbrich and Isacoff, 2007), suggesting that the mutant Src mRNA was degraded. In the hippocampus, Fyn mediated phosphorylation may have maintained sufficient NMDAR function to preserve the synaptoplastic mechanisms of hippocampus-dependent learning. Such compensatory response did not occur in the amygdala, however, as NMDAR phosphorylation was markedly reduced.

**Limitations**

An important limitation of these experiments is the systemic method of Src inhibitor administration and the global nature of the Src\(^{thl/thl}\) point mutation. Therefore, it cannot be concluded that the behavioural effects observed were due solely to neuronal and synaptic changes within the amygdala alone. In light of the amygdala’s extensive afferent and efferent connections, it is probable that changes in synaptic signalling in other brain regions, either downstream of amygdalar output or independent of amygdalar function, contribute to the behavioural changes concomitant with Src modulation. Local injections into the BLA amygdala and other relevant structures and anatomically targeted mutations are required to isolate the circuitry involved.

Based on control experiments, a few alternative possibilities can be eliminated, however. Attenuation of the freezing response in inhibitor-injected mice was not due to a deficit in pain perception because injected mice have exhibited normal post-shock freezing, shock-freezing threshold, and tail withdraw latency to painful stimuli (hot water). Also, inhibitor-injected mice exhibited no impairment in context-dependent freezing relative to controls, indicating that downstream afferent synaptic pathways mediating the freezing response were not markedly
affected by Src inhibition. Moreover, inhibition of Src after fear conditioning did not alter
test expression of an auditory-evoked fear response. Therefore, the auditory fear memory
impairment can not be attributed to pain perception, a general decrease in expression of fear
response, a non-specific decrease in anxiety, or to a general change in auditory processing.

The Src inhibitor decreased the duration of LTP within the LA-BLA pathway, but it is
not known if other pathways, upstream or downstream of the amygdala, where similarly
affected. We chose to record from the basolateral pathway because extracellular field EPSPs in
this pathway were much easier to measure than those in the LA input pathway. The alternative
would be to use intracellular or whole cell patch clamp recording, but we deemed this method to
be impractical because the inhibitor peptide (which was difficult to synthesize and expensive)
required at least 90 min preincubation, and this would necessitate difficult 2.5-3.5 h patch
clamp recordings. Furthermore, we had to use a closed-recirculating bath (due to the expense of
the peptide) and the pump caused vibration. Therefore, we do not exclude the possibility of Src
inhibition in the lateral amygdala since the peptide was injected systematically or perfused over
the whole amygdalar slice.

Possible mechanisms for altered social behaviours induced by Src modulation and future
research directions

Introduction: Mouse models of WBS

The full spectrum of WBS symptoms is conferred by the loss of a region of human
chromosome 7q11.23 that spans 25 genes. The major symptoms in patient with WBS include
increased sociability and anxiety, motor deficits, cardiovascular anomalies, and growth
retardation. In mice, specific clusters of these physical and behavioural anomalies are associated
with loss of different subregions of the homologous region. Lie et al. (1999) were able to specify
behavioural and molecular phenotypes to subregions by narrowing the deletions to either the
centromeric half (distal deletion (DD): lack Limk1 to Fkbp6) or the telomeric half (proximal deletion (PD): lack Gtf2i to Limk1) of the WBS deletion region (Li et al., 2009). The PD region includes GTF2I, GTF2Ird1, Cyln2, and Limk1, while the DD region includes Limk1, Eln, and Fzd9. Deletion of each of these genes has been associated with some WBS symptom in humans. Unfortunately, visuospatial learning and ultrasonic vocalizations were not measured in this study. Therefore, we could not compare our Morris water maze and ultra sonic vocalization data to Li’s deletion model.

Knockout mice based on the human microdeletion data have also been generated to characterize the contribution of single genes to WBS. These mice can illuminate the contributions of the various structural and signalling proteins to the pathophysiology of WBS. A Limk1 knockout mouse was produced by Meng et al. (2002). The Limk1 gene was absent in both PD and DD deletions (Li et al., 2009). The Limk1 protein is a regulator of actin dynamics and growth cone motility, and is expressed specifically in the central nervous system. The regulation of actin is essential for dendritic spine formation and neurite outgrowth critical for maintaining and modifying synaptic connections. Knockout mice had a grossly normal nervous system, possibly as a result of the functional redundancy of Limk2. However, these mice exhibited abnormal spine morphology, hyperactivity, enhanced fear conditioning, and impairments in the reversal phase of the water maze task (Meng et al., 2002). Hemi-insufficient Cyln2 mice (Hoogenraad et al., 2002) showed some phenotypes resembling WBS, such as mild growth retardation and impaired motor coordination. They also showed impaired contextual fear conditioning, while cued conditioning was normal, indicating functional deficits in the hippocampal rather than the amygdala. Moreover, they had decreased hippocampal LTP, supporting the fear conditioning results. The Cyln2 gene encodes a cytoplasmic linker protein (CLIP) involved in the regulation of microtubule dynamics and in the formation and/or turnover.
of gap junction (Tassabehji, 2003). In contrast, the Fzd9-hemizygous mice did not show any developmental or behavioural phenotypes associated with WBS. They had normal gross anatomical hippocampal organization, but showed an increase in apoptotic cell death in the developing dentate gyrus, which was partially compensated by an increase in precursor cells (Zhao et al., 2005). General transcription factor II (GTF2I) and GTF2IRD1 are interesting candidate genes as evidenced by WBS symptoms in humans with small chromosomal deletions that include these genes. These transcription factors are responsible for the transduction of extracellular signals into changes in the expression of a variety of genes. The GTF2IRD1 mutant mice (Young et al., 2008) exhibited deficits in cued but not contextual fear conditioning. These mice were less aggressive and showed increased social interactions, making them the first mouse model of WBS with increased sociability. The GTF2I heterozygous mice demonstrated increased social interaction and impaired social habituation similar to the hypersociability phenotype observed in WBS patients. In addition they showed normal learning and memory in the Morris water maze, but showed impaired novel object recognition. In terms of anxiety, these mice were not different from their wild type littermates (Sakurai et al.). While several genes are involved in the craniofacial and cognitive symptoms of WBS, GTF2I deletion appears to be one of the main causal factors (Enkhmandakh et al., 2009, Tassabehji et al., 2005)

**Possible mechanism and future directions**

It has been reported that TFII-I regulates the surface expression of TRPC3 calcium channels (Caraveo et al., 2006). It has been suggested that TFII-I exists in two alternate conformations, opened and closed. The open state is stabilized by tyrosine phosphorylation mediated by TKF members such as Btk or Src. The open (phosphorylated) TFII-I can bind to PLC-γ and thereby prevent the binding of TRPC3 to PLC-γ. This inhibits TRPC3-mediated calcium influx because activated PLC-γ can promote agonist-induced calcium entry by
stimulating surface expression of TRPC3 channels (Caraveo et al., 2006). Conversely, dephosphorylation shifts TFII-I into a close state that promotes PLC-γ binding to TRPC3 and stimulates TRPC3 surface expression. In addition to TFII-I, a functional Src kinase is required for TRPC3 activation. Therefore, in addition to playing a role in the TFII-I-TRPC3 pathway, Src is necessary for TRPC3 activation. This is interesting given the association of both Src and TFII-I genes in the pathophysiology of WBS and the fact that defects in calcium metabolism have been associated with many neurobehavioral and neurodegenerative disorders (Splawski et al., 2004). In order to study the relationship between Src, TFII-I and TRPC3 we will examine the subcellular localization of these three molecules. We predict that TFII-I is more abundantly located in the nucleus than the cytosol in the whole brain lysates of Src mutant mice. Since a decreased amount of TFII-I in the cytosol could lead to enhanced localization of TRPC3 in the membrane, we will study the localization of TRPC3 channels. We hypothesize that the expression of TRPC3 will be increased at the membrane of the brain lysates from Src mutant mice enhancing the possibility of more calcium influx into the cells, which could underlay the mechanism for the observed behavior abnormalities seen in the Src mutant mice.

Similar to TFII-I, a functional Src kinase is known to be required for TRPC3 activation. Therefore, in addition to playing a role in TFII-I-TRPC3 pathway, Src has an individual responsibility to activate TRPC3. This is interesting given the association of both Src and TFII-I genes in the pathophysiology of WBS (Hirota et al., 2003). Our studies may help to define the underlying mechanism of this disorder, because defects in calcium transport have been associated with other neurobehavioral disorders (Splawski et al., 2004) The Src gene is highly expressed in the cerebral cortex, hippocampus, olfactory bulb, amygdala, thalamus, striatum, and cerebellum. The function of Src in regulating TFII-I-TRPC3 pathway in these brain regions
could be the underlying mechanism of cognitive deficits in WBS individuals and mice. Further research is needed to link Src to TRPC3 function.

Interactions between the amygdala and prefrontal cortex (PFC) are critical for stimulus-reinforcement association learning (Pears et al., 2003). In social cognition, PFC–amygdala interactions has been proposed to link sensory representations of stimuli with social decisions based on motivational value (Adolphs, 2003). Lesions of the PFC are associated with social disinhibition (Rolls et al., 1994) and impaired ability to detect social cues in humans. The disinhibition and impairments in social behaviours seen in individuals with WBS can be related to abnormal PFC and/or amygdala function. Moreover, it was suggested that the interaction between the PFC and amygdala is greatly reduced in WBS patients, which could lead to reduced reactivity to social cues and an increased tendency to approach strangers. During maturation, aversive consequences of these dysfunctional social interactions would become increasingly apparent but could not be translated into appropriate emotional valence or behavioural adjustments by the hypofunctional amygdala-PFC circuit. Indeed, amygdalar-PFC interactions are critical for various forms of extinction, so conditioned behaviours may prove particularly inflexible.

Recent studies have shown that NMDARs are subject to rapid activity-dependent trafficking between synaptic and extrasynaptic domains (Groc et al., 2006, Tovar and Westbrook, 2002, Jeyifous et al., 2009) and that this trafficking is dependent on subunit tyrosine phosphorylation (Goebel et al., 2005, Snyder et al., 2005, Braithwaite et al., 2006). Insertion of NMDARs into the postsynaptic membrane depends on NR2B phosphorylation at Tyrosine 1472 (Nakazawa et al., 2002, Nakazawa et al., 2006, Zhang et al., 2008). We have shown that a Tat-Src (40-58) inhibitor blocks the interaction of Src with NMDA receptors and led to decreased
NR2B phosphorylation and reduced NR2B surface expression in amygdalar neurons. In addition, peptide injection impaired amygdala-dependent cued fear conditioning and a non-associative social recognition task. It has been shown that NR2B surface expression is regulated by clathrin-mediated endocytosis through a cyclin dependent kinase 5 (Cdk5)-dependent pathway that is ultimately Src (Zhang et al., 2008). These data indicate that Src activity is a major determinant of NMDA surface expression; in turn, the surface expression of NMDARs is the most critical factor determining the threshold for most forms of long-term potentiation that underlie amygdalar associative learning.

It has been reported that hippocampus-specific NMDA receptor knockout mice exhibit memory deficits that were rescued by environmental enrichment (Rampon et al., 2000). Moreover, NMDAR antagonists can block LTP and impair memory formation (but not expression) (Bashir et al., 1991). Rescue of emotional and social learning by environment enrichment has not be studied in detail and warrants further investigation. Furthermore, the role of Src in the effects of environment enrichment is still largely unexplored.

The biogenic amine system exerts global control over mood and emotional regulation. A Src-dependent reduction in NMDAR signalling could alter emotional behaviours by altering the metabolism of these neurotransmitters. The 5-HT signalling pathway in the prefrontal cortex plays an important role in regulating anxiety like behaviours (Graeff and Zangrossi). It has been suggested that the reduced social anxiety in individuals with WBS is caused by disregulation of the prefrontal cortex pathway (Meyer-Lindenberg et al., 2005) Young et al (2008) has shown that levels of the 5-HT metabolite 5-HIAA were significantly higher in the prefrontal cortex and amygdala of Gtf2ird1 knockout mice, while 5-HT levels remain unchanged, suggesting increased release and turnover of 5-HT in these brain regions (Young et 2008). In Gtf2ird1−/− mice, 5-HT1A-induced inhibitory currents were increased in
layer V pyramidal cells of the prefrontal cortex. This enhanced inhibition of layer V neurons disinhibited the raphe, which in turn caused more 5-HT release in the prefrontal cortex (Proulx et al.). The interaction of amygdala and prefrontal cortex plays an important role in social recognition and anxiety. Further, it has been shown that the prefrontal cortex modulates the amygdala in WBS (Meyer-Lindenberg et al., 2005). The NMDAR is one of the targets of 5-HT1A receptors in PFC neurons. Activation of 5-HT1A receptors caused a reduction in NMDAR currents in these neurons (Yuen et al., 2005), suggesting that the interaction of 5-HT1A with NMDAR in the PFC could play a significant role in regulating cognitive and emotional behaviours.

Functional NMDAR are released from the ER after NR1 and NR2 subunits assemble together to form a complex. These receptors are transported to dendritic spines via kinesin motor protein which is linked to NR2B containing vesicles (Setou et al., 2000). It has been demonstrated that 5-HT1A receptors modulate NMDAR currents and knock-down of the kinesin motor protein eliminated the 5-HT1A effect on NMDAR currents. Stimulation of 5-HT1A receptors preferentially affects NR2B-containing NMDAR channels. The kinesin motor delivers at least 30% of NR2B in dendrites (Guillaud et al., 2003), suggesting that the 5-HT1A modulation of NMDA receptors is dependent on the microtubule network and involves the transport of NR2B-containing vesicles along microtubules by the molecular kinesin motor. It has also been shown that NMDAR function in the PFC is downregulated when local 5-HT1A receptors are activated by serotonin. This pathway is dependent on microtubulin dynamics that are regulated by CaMKII and ERK. The 5-HT1A receptor is known to reduce social anxiety. Full and partial 5-HT1A receptor agonists increase social interactions and evoked anti-aggressive effects in rodents (de Boer et al., 2000, Picazo et al., 1995) that can be prevented by pre-treatment with the 5HT1A receptor antagonist WAY 100635 (Andrews et al., 1994, File et
al., 1996). Also 3, 4 methylenedioxyamphetamine (MDMA), which facilitates social approach, is believed to act by indirect stimulation of the 5HT1A receptor. Based on this evidence it could be argued that NR2B modulation in specific brain regions could alter social interaction. Thus, a possible reduction of NR2B phosphorylation in our mice in the PFC could cause reduced social anxiety. It is also known that acute MDMA treatment impaired learning retention due to a reduction of NR2B and CAMKII membrane expression. To further investigate these possibilities one could measure the NR2B phosphorylation in different brain regions such as the amygdala and the prefrontal cortex in the Src(thl/thl) mice. Social interaction behaviours and NR2B phosphorylation could also be assessed after administration of a 5-HT antagonist. If our hypothesis revealed to be true, the increase social interaction in the Src(thl/thl) mice should decrease and the NR2B phosphorylation should be increased after the 5-HT antagonist administration.

We also examined the behavioural effects of Src family kinases upregulation in Csk(+/−) mice. The 129P2 inbred strain demonstrates a variety of aberrant social behaviours, although the mechanisms are still unclear. To test whether decreased SFKs inhibition could rescue these behavioural phenotypes in the 129P2 background strain, we crossed Csk mice to the 129P line and compared 129P2/Csk(+/+) and 129P2/Csk(+/−) littermates in tests of motor activity, anxiety, and NMDAR-dependent social olfactory recognition and spatial object recognition tasks. In addition, we examined NMDAR phosphorylation and surface expression. Decreased Csk expression led to enhanced NR2B phosphorylation in the olfactory bulb and amygdala, brain regions important for NMDA dependent social memories. The anxiety-like phenotype was decreased in the elevated plus maze but not in the open field. Social olfactory recognition memories were greatly improved as revealed by enhanced short- and long-term social
recognition and social transmission of food preference. In contrast, no improvement in a spatial recognition task was observed.

Usually, genetic manipulations altering LTP impairs learning and memory (Sweatt 2003, mechanism of memory). Enhancement of learning has been rarely reported, however. One notable example is a mouse that overexpressed NMDAR2B in the forebrain under the control of the CaMKIIα promoter. The overexpression of NR2B led to enhanced NMDAR activation, a reduced frequency threshold for LTP, improved learning and memory in the Morris water maze, enhanced contextual and cued fear conditioning, superior novel object recognition, and more rapid extinction of learned behaviours, indicating enhanced behavioural flexibility (Tang et al., 1999, Tang et al., 2001). These results support our theory that enhanced NR2B phosphorylation in the amygdala and olfactory bulb could decrease the threshold for social olfactory learning in 129P2/Csk(+/−) mice. Furthermore, changes in the NR2A/NR2B ratio has been suggested to be common mechanism for altering the plasticity threshold, as sleep deprivation and olfactory learning are both manipulations that increase the induction threshold for LTP and raise the ratio of NR2A to NR2B subunits. Age-related changes in learning may also involved reduced NR2B expression in adult animals and humans (Hestrin, 1992, Sheng et al., 1994). This suggests that NR2B could be critical for regulating the threshold for plasticity and memory formation in our Csk(+/−) mice. Hebb’s (1949) rule emphasized that stimulus pairing during association learning strengthens the synapses between simultaneously active neurons. The enhanced activity of NMDAR, which is a synaptic coincidence detector (Stevens and Sullivan, 1998, Bear and Malenka, 1994) could enhance LTP thereby learning and memory in 129P2/Csk(+/−) mice, although this remains to be determined. For example it has been shown that eIF2a phospho-knock in mice have both enhanced LTP and better memory (Costa-Mattioli et al., 2007).
While enhanced NMDAR-mediated plasticity could contribute to the normalized behavioural phenotype of 129P2/Csk(+/1), the underlying allelic combinations that cause these abnormal behaviour phenotypes are not known. Therefore, it is essential to perform QTL and microarray analysis on the 129P2/Csk(+/+) and 129P2/Csk(+-) mice to identify possible targets involved in olfactory social memory.

Quantitative trait locus (QTL) mapping and cloning use genetic polymorphisms between strains to define loci that underlie quantitative traits. Thus, genes associated with QTLs are those that influence quantitative behavioural, emotional, and physical traits. In QTL analysis, a set of genetic markers is mapped against the difference between two inbred strains. For example, we could crossbreed 129P2 (impaired social recognition) with BL6 (intact social recognition) and identify QLTs that segregate with the observed behavioural changes. This involves phenotyping F2 crosses in which the associations between specific markers and the phenotypes of interest are investigated (Nguyen and Gerlai, 2002). Of course, these types of experiments are challenging because behavioural traits are polygenic and vary significantly from trial to trial within the same animal. None-the-less, such a strategy has been employed successfully. For instance, performance of C57BL/6 × DBA mice in the Morris water maze was correlated with genes associated with PKC activity in the hippocampus (Wehner et al., 1990). Furthermore, a QTL on chromosome 1 was involved in the latency to find the hidden platform during training trials, and a QTL on chromosome 5 was involved in spatial preference during the probe trial in C57BL/6 × DBA mice (Steinberger et al., 2003, Wehner et al., 1997).

In addition to QTL analysis, other types of genetic analyses, such as gene expression analysis using microarray techniques, can be used to identify genes involved in social recognition. These techniques can be used to simultaneously screen the expression levels of several thousand genes at once. However, when using gene chip microarrays, it should be kept
in mind that false positives can arise during analysis. To confirm the microarray results, quantitative RT-PCR and in situ hybridization should be performed to confirm differential expression of target genes (Henry et al., 2003). Together, QTL analysis and gene expression may enable the identification of alleles or mutations associated with specific impairments in social recognition and social memory. Identification of inbred mouse strains with phenotypic variance in tests of social recognition is an important prerequisite to identify allelic combinations or mutations that contribute to social recognition and social learning.

To date, only a few inbred strains that possess specific sociability and cognitive impairments have been identified. A useful start would be to extend the behavioural phenotyping of the 129P2 strain using several additional tests of amygdalar- or olfactory-dependent learning and memory to confirm that their memory deficits are indeed end-products of AM and OB dysfunction. Then, this strain could be studied in depth to probe the genetic, cellular, and molecular mechanisms of these impairments. This strain could also be used for QTL analysis to determine whether QTLs for LTP or for AM- and OB-dependent learning and memory overlap (Nguyen and Gerlai, 2002). If this were the case, a causal relationship between LTP and memory would be strengthened. In sum, QTL analysis has provided insights into mouse loci that may contribute to particular learning and memory phenotypes. The identification of trait-related genes within the QTLs should be the next focus of this research project.

It would also be of great value to investigate whether this mouse strain shows impaired synaptic function or plasticity in the amygdala and OB, and if impairments are rescued by CSK reduction. In general, mouse models in which hippocampus- or amygdala-dependent memory is impaired can be used to determine whether synaptic plasticity in these regions has a role in mediating these impairments. In conclusion, apart from the implication of Src in the phenotypes
observed in WBS, our data are relevant for the study of social behaviour and could stimulate a much greater emphasis on social behaviours and learning in the neurosciences.
References


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