

NCS-1 in the Dentate Gyrus Promotes Exploration, Synaptic Plasticity, and Rapid Acquisition of Spatial Memory

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SUMMARY

The molecular underpinnings of exploration and its link to learning and memory remain poorly understood. Here we show that inducible, modest overexpression of neuronal calcium sensor 1 (*Ncs1*) selectively in the adult murine dentate gyrus (DG) promotes a specific form of exploratory behavior. The mice also display a selective facilitation of long-term potentiation (LTP) in the medial perforant path and a selective enhancement in rapid-acquisition spatial memory, phenotypes that are reversed by direct application of a cell-permeant peptide (DNIP) designed to interfere with NCS-1 binding to the dopamine type-2 receptor (D2R). Moreover, the DNIP and the D2R-selective antagonist L-741,626 attenuated exploratory behavior, DG LTP, and spatial memory in control mice. These data demonstrate a role for NCS-1 and D2R in DG plasticity and provide insight for understanding how the DG contributes to the origin of exploration and spatial memory acquisition.

INTRODUCTION

The remarkable plasticity of the trisynaptic hippocampal network is suggested to underlie several neural processes including new acquisition of spatial memory (Goodrich-Hunsaker et al., 2008; Kandel, 2006; Nakazawa et al., 2004; Scoville and Milner, 1957; Squire et al., 2004) and novelty exploration (Lever et al., 2006). While the three best-studied anatomical hippocampal subregions, CA1, CA3, and the dentate gyrus (DG), must act cooperatively to produce a functional hippocampus, strong evidence demonstrates that each subregion performs its own specific, specialized operations. For example, region CA1 is involved in the temporal pattern association and temporal pattern completion aspects of memory formation (Klausberger and Somogyi,

2008), while subregion CA3 supports spatial pattern association and spatial pattern completion (Kesner, 2007b). The DG, on the other hand, orthogonalizes sensory information cumulated from the entorhinal cortex (EC) and, together with area CA3, underlies spatial pattern separation (Kesner et al., 2004). Detection of spatial novelty depends on both area CA3 and the DG (Kesner et al., 2004) and the perirhinal cortex (Kumaran and Maguire, 2007). Because novelty detection is a prerequisite for novelty exploration, plasticity mechanisms within these regions are ideally suited to serve a role in the generation of exploratory behavior, a possibility we explore here. Enhancement of plasticity via genetic manipulation in the forebrain can improve long-term spatial memory formation (Malleret et al., 2001), but analogous experiments have not been described for more anatomically restricted regions within the hippocampus. Here we report the emergence of specific increases in exploration, facilitated plasticity, and enhanced rapid acquisition of spatial memory following inducible, modest overexpression of neuronal calcium sensor 1 (*Ncs1*) selectively in the adult DG of a transgenic mouse line (DGNCS-1 mice).

NCS-1 plays a critical role in several forms of neuromuscular physiology and short-term neuroplasticity (Burgoyne, 2007; Hilfiker, 2003) including axonal development in *Lymnaea stagnalis* (Hui et al., 2007), Kv4 current modulation in the mouse myocardium (Guo et al., 2002), P/Q-type calcium channel activity-dependent facilitation in rat Calyx of Held (Tsujimoto et al., 2002), neurotransmitter release in *Xenopus* and *Drosophila* (Pongs et al., 1993; Wang et al., 2001), and long-term depression (LTD) in the perirhinal cortex (Jo et al., 2008). In mammals, *Ncs1* is highly expressed in the DG and is dynamically regulated during in vivo perforant path long-term potentiation (LTP) (Genin et al., 2001), but whether the increase in *Ncs1* mRNA is a result of LTP or a factor in its induction or maintenance is unknown. Here we show that DGNCS-1 mice have a lower threshold and higher ceiling for LTP in the corticohippocampal medial perforant path (MPP).

This is exciting because LTP is widely considered a molecular model for learning and memory in mammals, and *Ncs1* is already known to control memory in *C. elegans* (Gomez et al., 2001), an

invertebrate system. Though the neural circuits important for NCS-1-mediated memory in *C. elegans* were established, molecular mechanisms remain elusive and no role for NCS-1 in learning and memory in mammals has been reported. An enhancement in rapid acquisition of spatial memory in DGNCS-1 mice is a compelling demonstration of a role for NCS-1 in learning and memory in higher organisms, where the nervous system is more complex (Lein et al., 2007) and physiological correlates exist (Bliss and Collingridge, 1993).

NCS-1 binds dopamine type-2 receptors (D2R), regulates D2R phosphorylation through an interaction with G protein-coupled receptor kinase 2 (GRK2), and controls D2R surface expression in HEK293 cells (Kabbani et al., 2002). However, there is no evidence for an in vivo role for NCS-1 in D2R regulation. Here we show increased surface expression of D2R selectively in the DG molecular layer of DGNCS-1 mice, where dopaminergic modulation of synaptic plasticity may be critical to memory formation (Korz and Frey, 2007; Kovacs et al., 1979; Manahan-Vaughan and Kulla, 2003). For example, pharmacological antagonism of D2R reduces basolateral amygdala-DG LTP (Abe et al., 2008) and pharmacological activation of D2R improves working memory (Wilkerson and Levin, 1999) and alleviates scopolamine-induced passive-avoidance amnesia (Sigala et al., 1997). Yet, pharmacological antagonism of D2R can impair Morris water maze learning if administered immediately posttraining (Setlow and McGaugh, 2000), and how D2R is regulated during memory acquisition is unclear. Many of these studies employed systemic injections that affect the entire CNS, whereas the function of D2Rs is likely anatomically distinct. In DGNCS-1 mice, changes to D2R surface expression in the DG MPP may directly facilitate plasticity and enhance spatial memory because these effects were blocked by a small cell-permeant D2R/NCS-1 Interfering Peptide (DNIP) designed to compete with NCS-1 binding to the D2R.

Because human *NCS1* expression is associated with psychiatric disorders (Koh et al., 2003) and D2R is a major target for antipsychotic treatment, it is also of clinical interest to investigate what role the interaction between NCS-1 and D2R plays in the mammalian nervous system. Here we restrict our focus to the DG and provide insight for how this specialized brain area controls exploration and spatial memory.

RESULTS

Generation of Mice with Inducible *Ncs1* Overexpression in the DG

We generated mice with inducible overexpression of rat *Ncs1* selectively in DG granule neurons using the *rtTA2-M2* system (Michalon et al., 2005) (Figure 1A). Both the *tetO-ncs* (GenBank construct submission FJ756409) and *rtTA2* (construct described in Michalon et al., 2005) lines were created on the identical background strain in order to exclude potential differences between genotypes arising from contaminating donor alleles (Armstrong et al., 2006). By using the rat gene to create the *tetO-ncs* mice, silent single nucleotide divergence between mouse and rat provided a simple reverse-transcriptase-based discernment of exogenous versus endogenous *Ncs-1* mRNA while conserving the NCS-1 amino acid sequence (Figure 1B). *Camk2a-rtTA2*

mice (*rtTA2*) bred to an rTA-responsive-teto-LacZ reporter line showed prominent rTA2 expression in the dorsal DG cells of the hippocampal formation (Figure 1C). Rat *Ncs1* expression was doxycycline (dox)-dependent and only occurred in double transgenic mice (DGNCS-1) positive for both the *rtTA2* and *tetO-ncs* transgenes (Figure 1D). Quantitative westerns of microdissected brain tissue (Figure S10 available online) showed a 40% increase in NCS-1 selectively in the DG (Figures 1E and 1F). Similarly, immunofluorescence analysis of confocal z-stacks from the MPP demonstrated a 40% increase in NCS-1 reactivity (Figures 1G–1I). Immunohistochemistry on frozen sagittal sections also demonstrated a subtle increase in NCS-1 levels selectively in the DG (Figure 1J). Because the rat and NCS-1 proteins are identical, posttranslational modifications and subcellular targeting of the exogenous protein should track the endogenous protein. Throughout all experimental analyses, the other two transgenic genotypes (*tetO-ncs* and *rtTA2*) were not statistically different from wild-type (for example: in NCS-1 DG quantification: $p = 0.16$, $p = 0.26$; in rearing in the safe environment: $p = 0.16$, $p = 0.55$; in time spent in the target quadrant of the Morris water maze: $p = 0.84$, $p = 0.47$), and the three genotypes were pooled for comparison against DGNCS-1 mice (Figures S1A and S1B).

Ncs1 Overexpression Enhances Exploration in a Safe Novel Environment

Prior to any experimentation, subjects were handled for a minimum of 10 min per day for 10 consecutive mornings. Experiments were conducted on DGNCS-1 and littermate control subjects on dox for at least 6 days. This excluded any effects due to minor weight loss (see Figure S1D). Moreover, all experiments were conducted during the same circadian window to reduce phasic variation (Cain et al., 2008; Valentinuzzi et al., 2008).

The hippocampus is suggested to be important for rearing behavior under certain conditions (Lever et al., 2006). Thus we compared rearing behavior in two separate environments using two separate cohorts of animals. In a dimly lit, nonfearful environment, DGNCS-1 subjects spent nearly twice as much time rearing compared to their littermate controls (Figure 2A). DGNCS-1 mice also spent less time inactive (still) in this environment (Figure 2C), suggesting that more time was spent exploring. We know the increase in rearing and decrease in activity is not a general effect on locomotion because there were no differences between the genotypes in any of the standard measures when we examined a second cohort of animals in an overhead-lit, fearful environment (Figures 2B and 2D), where rearing is escape-oriented.

To further explore this tendency for increased exploration, we designed an experiment similar to the radial arms maze in which the latency and number of exploratory events for novel environments in either dim or bright lighting could be easily determined. We term this experiment the “New Frontier Exploration Test” (see Supplemental Data). In the dimly lit version of this task, DGNCS-1 mice demonstrated a nearly 2-fold increase in the number of frontiers visited and a nearly 2-fold decrease in the latency to explore one or all four new frontiers (Figures 2E and 2G). Yet, no genotypic differences in either measure were observed when bright overhead lighting was used (Figures 2F and 2H).

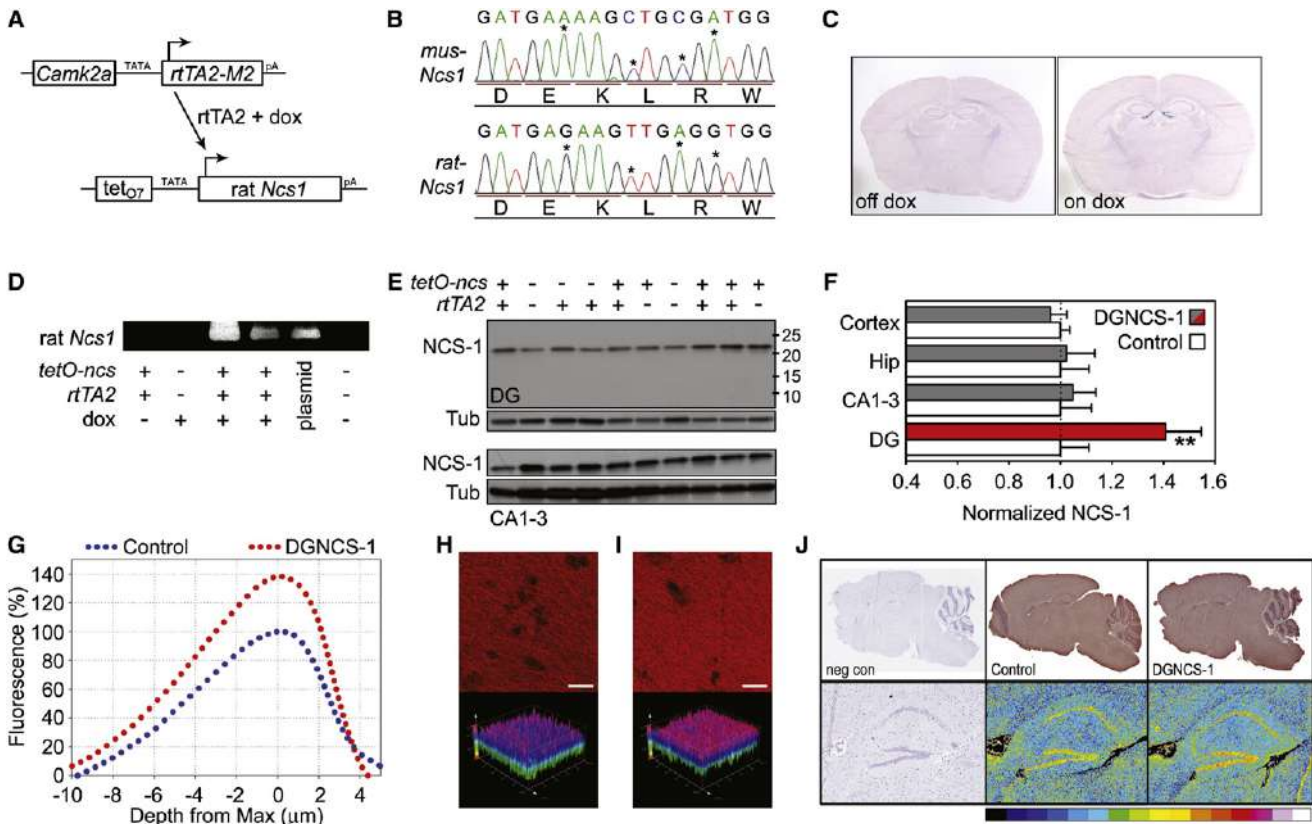


Figure 1. Generation of DG NCS-1 Mice

(A) Schematic representation of the rTA2 system and transgenic constructs used to generate the *tetO-ncs*, *rTA2*, and DG NCS-1 lines.
 (B) Representative sequencing chromatograms of endogenous mouse *Ncs1* mRNA amplicon (upper chromatogram) compared to rat *Ncs1* mRNA amplicon from a DG NCS-1 animal on dox (lower chromatogram). Asterisks indicate silent single nucleotide divergence.
 (C) β -gal staining of coronal adult mouse brain sections from rTA2/LacZ on and off dox.
 (D) Selective amplification of rat *Ncs1* RNA from hippocampal extracts of DG NCS-1 mice on dox. No expression was observed in littermate controls or in DG NCS-1 hippocampii off dox.
 (E) Hippocampal subregion CA1 and CA3 and DG lysates blotted for NCS-1 and β -tubulin (Tub).
 (F) NCS-1 normalized to Tub from various isolated brain regions of DG NCS-1 and littermate control mice on dox (n = 6).
 (G) Quantitative z-stack immunofluorescence of NCS-1 in the MPP of DG NCS-1 animals compared to littermate controls.
 (H and I) NCS-1 immunofluorescence images from the DG MPP. Representative layer of maximum fluorescent intensity in **(H)** control and **(I)** DG NCS-1 slices with intensity plots shown below (n = 4). Scale bar = 18 μ m.
 (J) Immunohistochemical staining of NCS-1 demonstrates a subtle increase in staining selectively in the DG of DG NCS-1 slices.
 Hip, hippocampus. **p < 0.01. Error bars = SEM.

DG NCS-1 subjects also spent more time exploring the holes of the hole board test (Figure 2I) and spent longer exploring the very first hole they encountered, a behavior repeated for each time they encountered a new hole (Figure 2J). Littermate control and DG NCS-1 subjects demonstrated similar anxiety as measured by the light/dark box (Figures 2K and 2L), indicating that this behavior does not confound the exploration tasks.

Ncs1 Overexpression in the DG Enhances Synaptic Plasticity

Elevation of DG NCS-1 did not affect basal synaptic transmission along the MPP (Figures S2A and S2B), the major excitatory innervation of the hippocampus and the primary source of sensory information relayed from the EC. Synaptic responses possessed double exponential decay (not shown) as well as

short-term paired-pulse depression (PPD) (Figure S2C), confirming electrode placement along the medial and not lateral perforant path (Bramham and Sarvey, 1996; Hanse and Gustafsson, 1992; McNaughton, 1980). Because the *Ncs1* homolog, *Freq*, affects calcium-dependent neurotransmitter release in *Drosophila* and *Xenopus* (Pongs et al., 1993; Wang et al., 2001), we measured PPD, a short-term form of presynaptic plasticity known to be sensitive to calcium, but found no differences (Figure S2C), corroborating a lack of *Ncs1* overexpression in EC-DG presynaptic terminals. When we stimulated to induce LTP along the MPP by applying an above-threshold stimulus (four 100 Hz trains), DG NCS-1 slices demonstrated more robust plasticity than those of their littermate controls (Figure 3A). Bath application of the NMDAR antagonist D-APV abolished LTP in DG NCS-1 mice (Figure 3A).

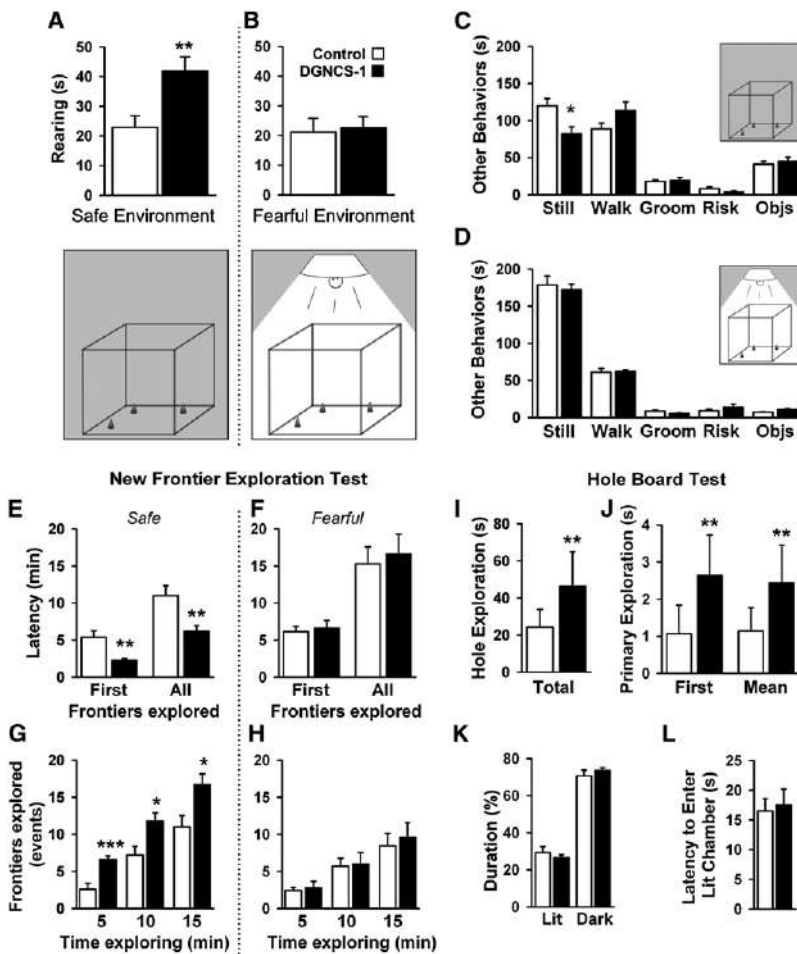


Figure 2. Elevation of NCS-1 in the Adult DG Selectively Promotes Exploratory Behavior in Dimly Lit Environments

(A) Exploratory rearing in a dimly lit, safe novel environment was increased in DGNCs-1 mice compared to their littermate controls. (DGNCs-1, $n = 8$; Control, $n = 16$).

(B) Rearing in a brightly lit, fearful environment remained unchanged between the genotypes, indicating that fear-motivated rearing was unaffected. Separate cohorts of mice were used for each of the environment types. (DGNCs-1, $n = 12$; Control, $n = 11$.)

(C and D) Time spent on other behaviors in the (C) safe and (D) fearful environment. (DGNCs-1, $n = 8$; Control, $n = 16$.)

(E–H) New Frontier Exploration Test. Under dimly lit conditions, the DGNCs-1 group had (E) a lower latency to reach any or all frontiers and (G) more total frontier visit events. (F and H) Under bright lights, no genotypic difference was observed. (DGNCs-1, $n = 8$; Control, $n = 11$.)

(I and J) Hole board test. (I) DGNCs-1 mice spent more total time exploring holes. (J) DGNCs-1 mice spent more time exploring a hole during primary encounter of the first and all subsequent holes. (DGNCs-1, $n = 9$; Control, $n = 10$.) (K and L) Light/Dark Box. DGNCs-1 and littermate control subjects (K) spent equivalent time in each chamber of the light/dark box and (L) demonstrated equivalent latencies to move into the brightly lit chamber. (DGNCs-1, $n = 9$; Control, $n = 10$.)

* $p < 0.05$, ** $p < 0.01$. Error bars = SEM.

Ncs1 Overexpression Leads to Faster and Longer-Lasting Displaced Object Recognition Learning and Memory

Due to the role of the DG in conjunctive encoding (O’Reilly and McClelland, 1994) and pattern separation (Bakker et al., 2008; Kesner, 2007a;

Leutgeb et al., 2007; McHugh et al., 2007), it is possible that modulating NMDAR-dependent plasticity at this synapse will alter representations of the external environment. If such an alteration led to a distortion of environmental cues, it should be discernable in space-dependent, cognitive tasks. Thus, we examined the performance of the DGNCs-1 mice in two spatial learning and memory paradigms.

We first chose the object recognition task because it provides an elegant method to investigate both spatial and nonspatial one-trial, nonaversive memory. Moreover, object recognition is an ethological paradigm that captures latent learning in the absence of explicit rewards. During training, DGNCs-1 and littermate control subjects spent equivalent time investigating the objects and no differences between genotypes were observed in ambulation, anxiety, grooming, or risk-assessing behaviors (Figure S3). Yet, as previously observed, DGNCs-1 subjects spent more time rearing (Figure S3). In a standard short-term memory paradigm (15 min habituation/training; see Figure S4), both DGNCs-1 and littermate control mice demonstrated a strong preference for the displaced and novel objects, suggesting the change in DG NMDAR-dependent plasticity did not disrupt spatial learning, at least not in this task (Figure 4A).

This raised the exciting possibility that facilitated DG synaptic plasticity could lead to superior neural encoding of space and

There is a strong frequency dependence for the induction of LTP in the DG, whereby tetanic stimulation with lower frequencies of the β and γ range (10–75 Hz) typically fail to elicit LTP (Rick and Milgram, 1996). This high threshold for plasticity is believed to be critical for sparse encoding of the external world and to underlie learning and memory functions of the dentate (Barry et al., 2006; Coulter and Carlson, 2007; Marr, 1971; McNaughton and Morris, 1987). To investigate if not only the level, but also the threshold of LTP, was altered, we delivered a below-threshold stimulus using four tetanic trains at half the previous frequency. Strikingly, DGNCs-1 slices showed strong LTP even with this weak stimulation, while control slices showed very modest short-term potentiation and failed to elicit LTP (Figure 3B). DGNCs-1 slices stimulated with the below-threshold stimulus attained similar levels of LTP as compared to control slices given a strong stimulus (compare white bar in Figure 3A to black bar in Figure 3B). Maximal potentiation was also greater in DGNCs-1 mice following repetitive weak stimulation (Figure 3C). Thus, by overexpressing *Ncs1* in the DG, we created a mouse with a selective enhancement in EC-DG NMDAR-dependent plasticity. Importantly, DGNCs-1 slices and littermate control slices had equivalent LTP in area CA1 (Figure 3D), further demonstrating the regional selectivity of the plasticity enhancement.

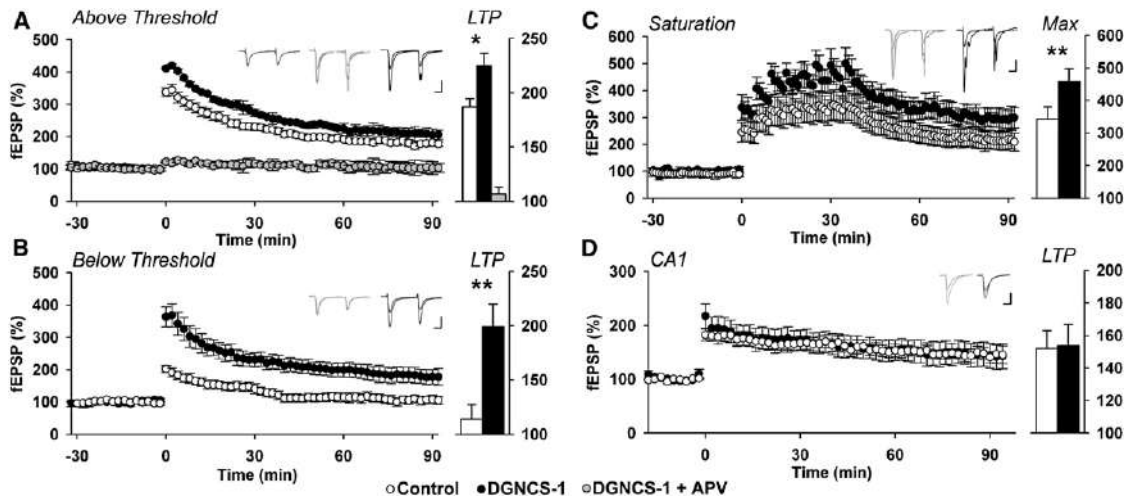


Figure 3. Modest Elevation of NCS-1 Enhances Long-Term Synaptic Plasticity

(A) Modest overexpression of *Ncs1* in the DG enhanced MPP LTP following above-threshold tetanic stimulation (4×100 Hz) and was blocked by the NMDAR antagonist D-APV. (DG NCS-1, $n = 11$ slices from $n = 8$ mice; Control, $n = 20$ slices from $n = 11$ mice; DG NCS-1 + D-APV, $n = 2$ slices from $n = 2$ mice.)

(B) Only slices from DG NCS-1 mice produced robust LTP following below-threshold tetanic stimulation (4×50 Hz). (DG NCS-1, $n = 10$ slices from $n = 9$ mice; Control, $n = 6$ slices from $n = 5$ mice.)

(C) DG NCS-1 slices show higher maximum potentiation following eight sets of weak tetanic stimulation (2×50 Hz each), as compared to controls. (DG NCS-1, $n = 9$ slices from $n = 4$ mice; Control, $n = 3$ slices from $n = 1$ mouse.)

(D) No genotypic difference was observed in LTP along the Schaffer Collateral CA1 synapse. (DG NCS-1, $n = 6$ slices from $n = 3$ mice; Control, $n = 5$ slices from $n = 4$ mice.)

Inset sample traces (scale bar of 0.5 mV and 10 ms) show superimposition of baseline and the periods used to generate bar graphs. Bar graph shows LTP at 60 min and max potentiation for the 5 min period following the final stimulus. * $p < 0.05$, ** $p < 0.01$. Error bars = SEM.

enhance learning. Therefore, we shortened the time given for subjects to become familiar with objects and object locations and again tested short-term memory. Remarkably, only the DG NCS-1 group showed an ability to discriminate between displaced and stationary objects in this harder version of the task (Figure 4B).

It is well established that the DG is involved in short-term memory (Kesner, 2007a), while the involvement of the DG in long-term memory has been less explored. We therefore trained the subjects in a third paradigm for which control animals demonstrate short-term memory (Figure S5), but not long-term memory. Remarkably, DG NCS-1 animals were still able to discriminate between displaced and stationary objects even after a 24 hr interval (Figure 4C). Therefore, DG plasticity may promote the rapid acquisition of both short- and long-term spatial memory.

Ncs1 Overexpression Enhances Morris Water Maze Learning

To investigate if the spatial memory enhancements in the DG NCS-1 mice would translate to another hippocampus-dependent task, we turned to the Morris water maze. In a stringent paradigm using ample distal spatial cues and a large platform in a small pool, subjects were trained with a single trial and then tested for retention of the platform location in a probe (no platform) trial 24 hr later. This procedure was repeated three times using three different platform locations, and the performance of each subject was averaged over all three sessions (see Figure S4 for experimental design). Not surprisingly, control animals failed to demonstrate a preference for the target quad-

rant during the probe trial (Figure 4D). DG NCS-1 animals, however, spent nearly 50% more time searching in the target quadrant than they did in any of the other three quadrants, suggesting they learned and recalled the platform location. Though DG NCS-1 animals did not have a faster latency to the counter location (set as twice the platform area) (Figure 4E), they did pass through it more frequently (Figure 4F) and spent more time searching that area of the maze (Figure 4G).

We also assessed learning and memory in the Morris water maze by training the same subjects with four trials on a single day using a 45 min intertrial interval. In this task, control and DG NCS-1 subjects had similar latencies to the platform by the fourth trial (Figure 4H); however, only DG NCS-1 subjects showed a statistically significant decrease in latency to reach the platform on trial 2 compared to trial 1. When tested in a probe trial 24 hr later, no differences were observed between the genotypes in any of the standard measures of memory performance (Figures 4I–4K). Thus, if control subjects receive sufficient training, they can perform at the level of DG NCS-1 mice.

NCS-1 Controls D2R Surface Expression in Hippocampal Neurons

NCS-1 and D2R associate in vitro and colocalize in monkey and rat striatum (Kabbani et al., 2002), but whether NCS-1 and D2R interact in mouse hippocampus is unknown. Colabeled mouse brain sections for surface D2R under nonpermeabilizing conditions and for NCS-1 after permeabilization showed overlap (Figures 5A–5F). NCS-1 and D2R also coimmunoprecipitated from mouse hippocampal lysates (Figure 5G).

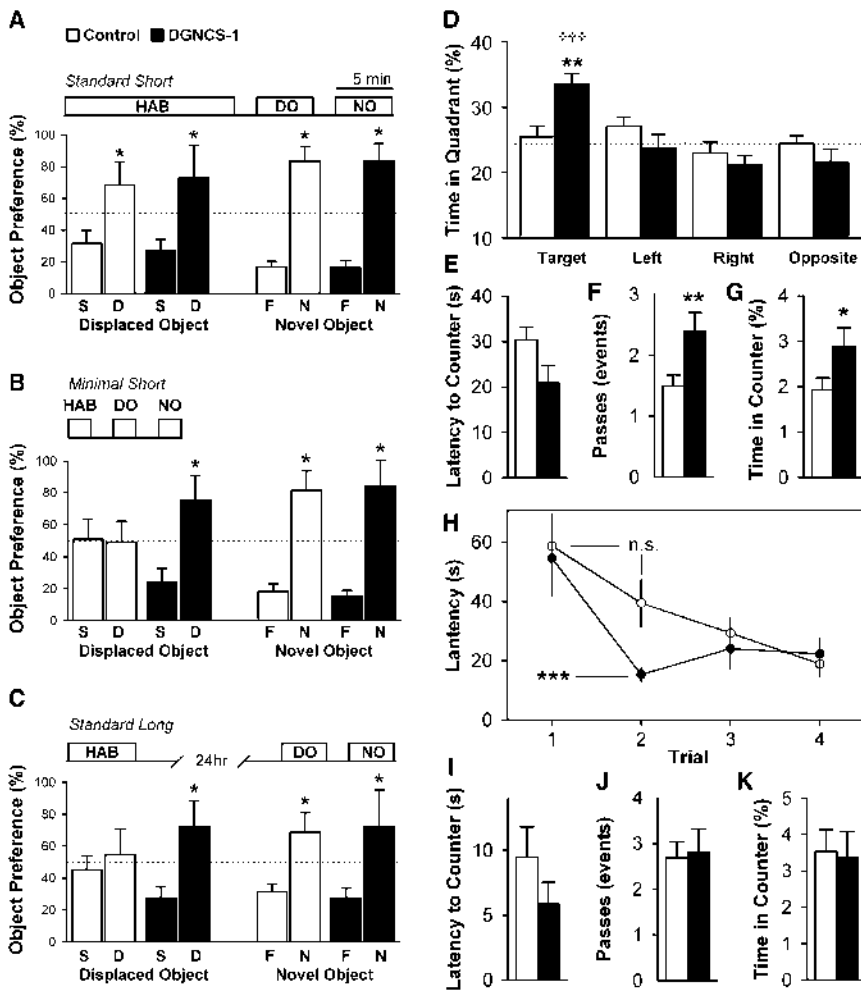


Figure 4. Elevation of DG NCS-1 Enhances Rapid Acquisition of Spatial Memory

(A) In a standard version of the object recognition task, both DGNC-1 and littermate control animals demonstrated a preference for the displaced and novel objects when tested following 2 min intervals (DGNC-1, $n = 8$; Control, $n = 16$). (B) In a minimal habituation/training version of the task, only the DGNC-1 subjects showed discrimination between the displaced and stationary objects (DGNC-1, $n = 9$; Control, $n = 9$). (C) Only DGNC-1 subjects discriminated between displaced and stationary objects when assessed 24 hr after an intermediate level of habituation/training (DGNC-1, $n = 9$; Control, $n = 15$). (D–G) Average of three probe trials, each 24 hr after a single-trial Morris water maze experiment. DGNC-1 mice (D) spent more time in the target quadrant, (E) had a similar latency to the counter, (F) passed more often through the counter, and (G) spent more time in the counter location. (H) Latency to reach the hidden platform for DGNC-1 and littermate control animals for four trials, 45 min apart. Maximal performance was achieved on trial 2 for the DGNC-1 groups and on trial 4 for the control group. (I–K) Probe trial 24 hr after (H). No differences between the groups were found in (I) latency to, (J) passes through, or (K) time spent in the counter location. (DGNC-1, $n = 17$; Control, $n = 33$.) See Figure S4 for detailed schematic of experimental protocols and apparatus. S, stationary object; D, displaced object; F, familiar object; N, novel object; HAB, habituation phase; DO, displaced object phase; NO, novel object phase. n.s. = not significant between trial, *** $p < 0.001$ between trial, ** $p < 0.01$ between genotype, * $p < 0.05$ between genotype. Error bars = SEM.

To investigate the function of NCS-1/D2R interaction, we designed the DNIP, a cell-permeant NCS-1/D2R interfering peptide (Figure 5H), by coupling the HIV-1 TAT protein transduction domain sequence to the minimal region of D2R that binds NCS-1 (Kabbani et al., 2002). Application of the DNIP decreased surface levels of D2R in acute dissociated rat hippocampal cultures (Figure 5I) and in the perforant path of mouse hippocampal slices (Figures S6A–S6C), suggesting endogenous NCS-1 may serve to facilitate D2R surface expression. Indeed, D2R desensitization in HEK293 cells is attenuated by NCS-1 (Kabbani et al., 2002). While whole surface expression of D2Rs in the DG and areas CA1 and CA3 of DGNC-1 brains was comparable to that of littermate control animals (Figures 5Q and 5R), immunofluorescence analysis of D2R surface expression showed an enhancement in surface D2R restricted to the molecular layer of the DG, suggesting that the receptors are properly targeted (Figures 5K–5P).

The DNIP also prevented quinirole from attenuating the forskolin-induced cellular cyclic adenosine monophosphate (cAMP) response (Figure 5J), proving that the active peptide can functionally inhibit D2R. Importantly, the DNIP did not affect surface expression of D1R (Figures S6D–S6F and S7A) or D3R (Figures S6G–S6I). The DNIP also failed to block the D1R-mediated

cAMP response and did not prevent pharmacological antagonism of these receptors (Figure S7B), demonstrating that it is not a nonspecific, global regulator of receptor surface expression.

D2R and G Protein Signaling Mechanisms May Underlie the Enhanced LTP in DGNC-1 Slices

We investigated if the LTP enhancement in the *Ncs1*-overexpressing mice required the interaction of NCS-1 and D2Rs by recording LTP in the presence of the DNIP. The DNIP and srDNIP did not affect basal transmission (Table S1 available online), short-term plasticity (Table S1), or LTP in littermate controls following the below-threshold stimulus (Figure 6A). In DGNC-1 slices, however, the DNIP blocked LTP following the below-threshold stimulus (Figure 6B), suggesting the interaction of NCS-1 and the D2R is critical for facilitation of LTP in DGNC-1 slices, and may also underlie the promotion of specific forms of exploration and enhancements in spatial memory acquisition. The DNIP also attenuated above-threshold LTP in control slices (Figure 2C), demonstrating an endogenous role for the NCS-1/D2R interaction in long-term synaptic plasticity.

To further investigate if D2Rs and downstream signaling pathways of D2Rs underlie enhanced LTP in DGNC-1 slices, we applied L-741,626 at a concentration selective for D2Rs

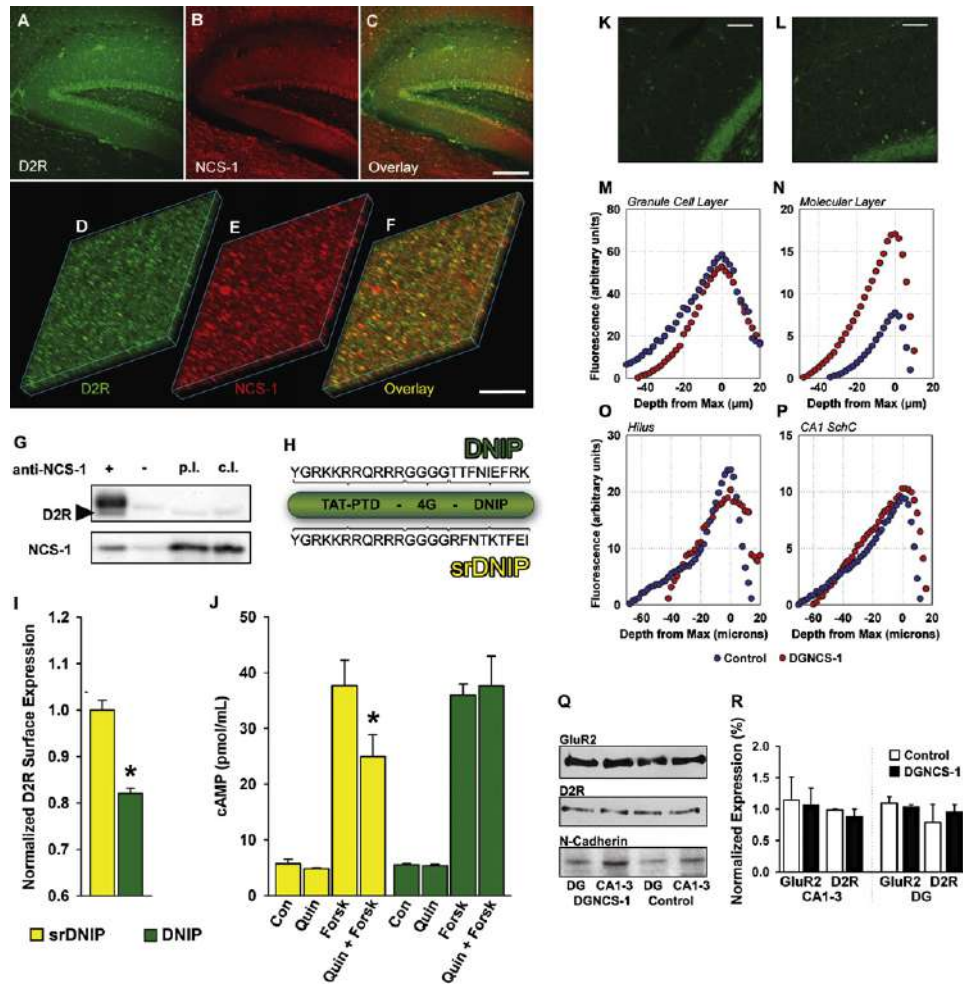


Figure 5. NCS-1 Controls D2R Surface Expression in Mouse Hippocampal Neurons

(A) Confocal image of surface D2R immunostaining in the mouse DG.

(B) Corresponding image of NCS-1 immunostaining from the same section shown in (A).

(C) Overlay of (A) and (B), with codistributed regions appearing in yellow. Scale bar = 240 μ m.

(D–F) Higher-power confocal stacks within MPP of DG molecular layer, shown as isometric projection, for each of D2R, NCS-1, and overlay, respectively. Scale bar = 18 μ m.

(G) Coimmunoprecipitation of NCS-1 and D2R from mouse hippocampal lysates. p.l., pre-cleared lysate; c.l., crude lysate.

(H) DNIP (upper sequence) and srDNIP (lower sequence) fused to TAT.

(I) Colorimetric measurements of D2R surface expression in hippocampal cultures isolated from E17–E19 fetal Wistar rats. Cultures were incubated in either 2 μ M DNIP or srDNIP for 30 min ($n = 3$).

(J) cAMP quantification in hippocampal cultures following 30 min incubation of DNIP or srDNIP with indicated compounds ($n = 3$).

(K and L) Representative surface D2R staining in the dentate of (A) control and (B) DGNCs-1 subjects. Scale bar = 45 μ m.

(M–P) Quantification of confocal z-stack immunofluorescence from DGNCs-1 and control genotypes in (M) DG granule cell layer, (N) DG molecular layer, (O) hilus, and (P) CA1 Schaffer Collateral (SchC) molecular layer ($n = 4$).

(Q and R) Biotinylated surface protein immunoblots of GluR2, D2R, and N-Cadherin as the loading control.

Forsk, forskolin; Quin, quipirole. * $p < 0.05$. Error bars = SEM.

(50 nM). Here we found that the D2R antagonist attenuated and normalized the extent of LTP (Figure 6D), suggesting that D2R is important for DG LTP in wild-type slices and is also critical for the plasticity enhancement in DGNCs-1 slices. The $G_{\beta\gamma}$ blocker galien also attenuated LTP in littermate controls and normalized LTP in DGNCs-1 animals (Figure 6E).

Because mGluR₅-mediated LTD in adjacent brain areas involves NCS-1 (Jo et al., 2008), we also applied the mGluR₅

antagonist MPEP to DGNCs-1 and littermate control slices. Yet we still observed a statistical genotypic difference (Figure 6F), indicating that the mGluR₅ receptor subtype is not involved in this particular form of NCS-1-dependent plasticity. Similarly, inhibitory modulation is not a potential contributor to the enhanced plasticity in DGNCs-1 slices because LTP was prevented by removal of the GABA_A blocker bicuculline from the perfusion aCSF (Figure S8). Moreover, the contribution of

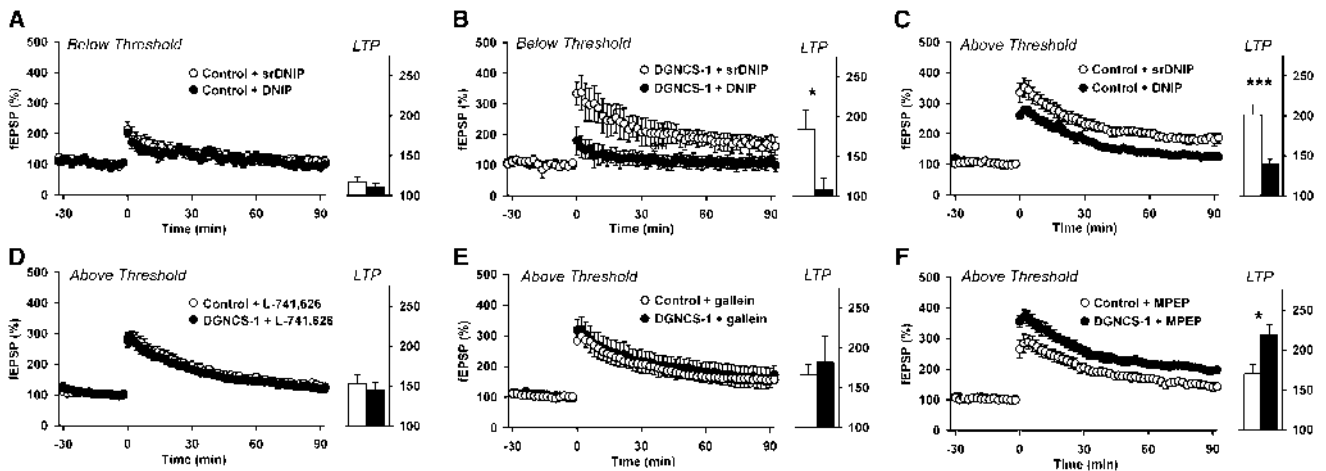


Figure 6. Antagonism of D2R and $G_{\beta\gamma}$ Signaling Normalizes LTP in DG NCS-1 Slices

(A) Neither DNIP nor srDNIP (10 μ M each) had an effect on plasticity following a weak tetanic stimulus (4 \times 50 Hz) in control slices. (DNIP: n = 4 slices from n = 3 mice; srDNIP: n = 3 slices from n = 3 mice.)

(B) LTP (4 \times 50 Hz) in DG NCS-1 slices was attenuated by application of DNIP. (srDNIP: n = 3 slices from n = 3 mice; DNIP: n = 4 slices from n = 4 mice.)

(C) LTP in control slices induced by the above-threshold stimulus (4 \times 100 Hz) was attenuated with DNIP. (srDNIP: n = 4 slices from n = 4 mice; DNIP: n = 7 slices from n = 4 mice.)

(D) LTP (4 \times 100 Hz) in DG NCS-1 slices was normalized to the level of controls with application of 50 nM of the D2R-selective antagonist L-741,626. (Control: n = 11 slices from n = 6 mice; DG NCS-1: n = 6 slices from n = 4 mice.)

(E) LTP (4 \times 100 Hz) in DG NCS-1 slices was normalized to the level of controls with application of 10 μ M of the $G_{\beta\gamma}$ inhibitor gallein. (Control: n = 6 slices from n = 3 mice; DG NCS-1: n = 6 slices from n = 4 mice.)

(F) In the presence of 1 μ M of the mGluR₅ antagonist MPEP, LTP (4 \times 100 Hz) in DG NCS-1 slices was still greater than LTP in littermate control slices. (Control: n = 7 slices from n = 4 mice; DG NCS-1: n = 6 slices from n = 3 mice.)

* $p < 0.05$, *** $p < 0.001$. Error bars = SEM.

NMDAR and AMPAR to baseline field responses was comparable in DG NCS-1 and littermate control slices (Figure S9), and surface expression of GluR2 was comparable between the groups in areas CA1 and CA3 and the DG (Figures 5Q and 5R). The finding that baseline NMDAR transmission is not affected in DG NCS-1 slices demonstrates that although NMDAR-dependent mechanisms are required to initiate LTP in DG NCS-1 slices, baseline NMDAR levels do not mediate the plasticity enhancement.

NCS-1 and D2R Underlie Rearing Behavior and Spatial Memory

We explored if the interaction of NCS-1 and the D2R was important for rearing behavior in DG NCS-1 and littermate subjects by direct infusion of the DNIP to the DG. Representative cannulae tracks and dorsal view of infusion sites are shown in Figure 7B. The DNIP and srDNIP conjugated to fluorescent probes directly infused into the dentate showed a degree of diffusion sufficient to cover approximately one-third of the DG and less than 5% of area CA1 (Figure 7G). High-power 3D confocal z-stack imaging of individual granule neurons demonstrated that the peptides penetrate into cells (Figure 7H). DG NCS-1 and control subjects infused 3–5 hr prior to examination demonstrated equivalent rearing behavior in a safe environment (Figure 7A). There were no differences in any of the other standard measures (not shown). Because the DNIP normalized rearing behavior in this type of environment, NCS-1 interaction with the D2R is likely important for the promotion of exploratory rearing in DG NCS-1 subjects.

Infusion of the DNIP also blocked both the short- and long-term spatial memory enhancements (Figures 7C and 7D), but the same dose did not affect the performance of littermate control animals in the standard version of the task (Figure 7E). To investigate if endogenous levels of NCS-1 and D2R cooperate to mediate the rapid acquisition of spatial memory, we infused a higher (5x) dose of the peptide into the DG of control mice and again examined displaced object learning and memory. Here, the DNIP prevented spatial memory formation (Figure 7E), indicating that the NCS-1/D2R interaction is required in this task. Blockade of D2Rs by bilateral infusion of L-741,626 into the DG also prevented displaced object discrimination in littermate control animals on dox (Figure 7F), demonstrating the importance of D2R in spatial memory acquisition. Importantly, novel object recognition (which involves other brain areas) was unaffected by either the DNIP or L-741,626, suggesting that the relationship between NCS-1 and the D2R in the DG is not critical for enhancing single-modality learning and memory (see Figure 8A).

DISCUSSION

To understand the molecular underpinnings of exploration and its link to learning and memory, we must understand the molecular underpinnings of plasticity in relevant brain regions. Here we identify the importance of the DG in a specific form of exploration. We also demonstrate a potential link between DG NMDAR-dependent LTP and exploratory rearing behavior in a mouse line that demonstrates enhanced rapid acquisition of

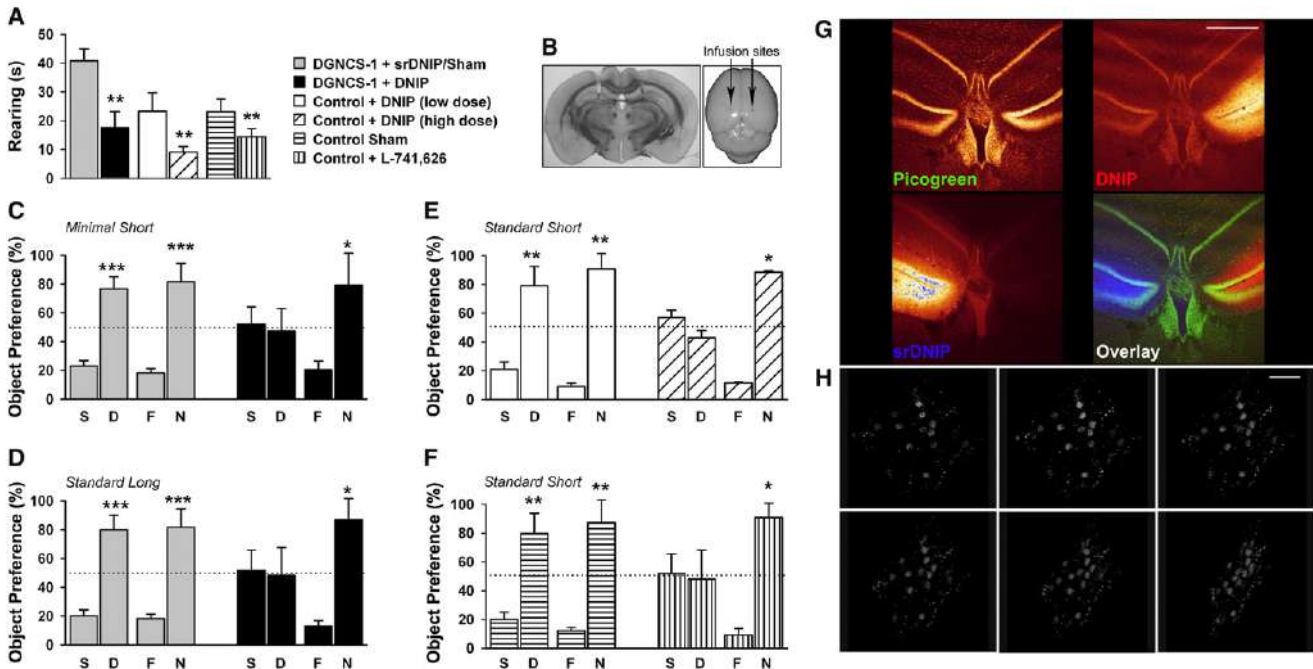


Figure 7. D2R is Important for Exploratory Rearing and Spatial Memory in DG NCS-1 and Control Subjects

(A) Preinfusion of a low dose of DNIP directly into the DG of cannulized subjects attenuated curiosity-driven rearing behavior compared to sham-treated or srDNIP-infused DGNCs-1 subjects. Only a high dose (5x) of DNIP reduced rearing behavior in control subjects. L-741,626 reduced rearing behavior in control animals. (DGNCs-1 + srDNIP/sham: n = 9; DGNCs-1 + DNIP: n = 7; Control + DNIP_{low dose}: n = 7; Control + DNIP_{high dose}: n = 2; Control sham: n = 5; Control + L-741,626: n = 4.)

(B) Representative coronal section and dorsal view of a cannulized brain after removal of cannulae, showing infusion tracks and surgical placement.

(C and D) The DNIP blocked both the (C) minimal short-term and (D) long-term spatial memory (displaced object discrimination) enhancement in DGNCs-1. Nonspatial memory (novel object discrimination) remained intact (n = 7). srDNIP-infused (n = 3) or sham-treated DGNCs-1 subjects (n = 6) demonstrated rearing similar to untreated DGNCs-1 subjects.

(E) The same dose of peptide had no effect on littermate controls in the standard version of the short-term memory task (n = 7). A higher (5x) dose of DNIP abolished spatial learning in control mice while preserving novel object recognition (n = 2).

(F) L-741,626 selectively abolished displaced object discrimination when infused bilaterally in the DG of control subjects (n = 4). Sham-treated controls still demonstrated discrimination (n = 5).

(G) Fluorescently conjugated srDNIP (blue) and DNIP (red) infused into the dentate 3 hr prior to sacrifice for processing and imaging. Picogreen was applied to label nuclei. Overlay is shown in lower right panel. Scale bar = 500 μ m.

(H) High-power 3D z-stack confocal images displayed at various rotations of the srDNIP in neurons of the DG. Scale bar = 35 μ m.

S, stationary object; D, displaced object; F, familiar object; N, novel object. *p < 0.05, **p < 0.01, ***p < 0.001. Error bars = SEM.

spatial memory. We identify the calcium sensor NCS-1 as a regulator of D2R surface expression in the DG molecular layer and employ two forms of D2R antagonism (the DNIP and L-741,626) that suggest that D2Rs underlie the promotion of exploratory behavior, LTP, and memory in the DGNCs-1 mouse line.

Selection of Model

NCS-1 was a promising protein to study because of its expressional regulation during in vivo LTP (Genin et al., 2001) and involvement in learning and memory in *C. elegans* (Gomez et al., 2001). We selected an inducible transgenic system to genetically perturb *Ncs1* expression in adult mice because NCS-1 is critical in the development of the nervous system and muscle (Coukell et al., 2004; Hui et al., 2007). Constitutive overexpression, or null or conditional mutations, may have caused developmental abnormalities, obscuring interpretation of the data. It was also our aim to isolate and study a subregion of

the hippocampus in order to better understand its function. As such, we unveil the importance of the DG in exploratory behavior and rapid memory acquisition.

The Role of the DG in Rapid Acquisition of New Spatial Memory

Sparse encoding is a central feature for pattern separation processes in the DG (Bakker et al., 2008; Kesner, 2007a; Leutgeb et al., 2007; McHugh et al., 2007) and is believed to be critical for accurate spatial mapping during learning and memory (Coulter and Carlson, 2007; Marr, 1971). Sparse encoding is only possible because DG granule neurons form synaptic contacts with a relatively modest number of CA3 pyramidal neurons. Thus, it is important that the number of DG neurons stimulated during encoding of the environment does not change such that inputs into CA3 become nonspecific or inadequately orthogonalized, a situation that could result in catastrophic

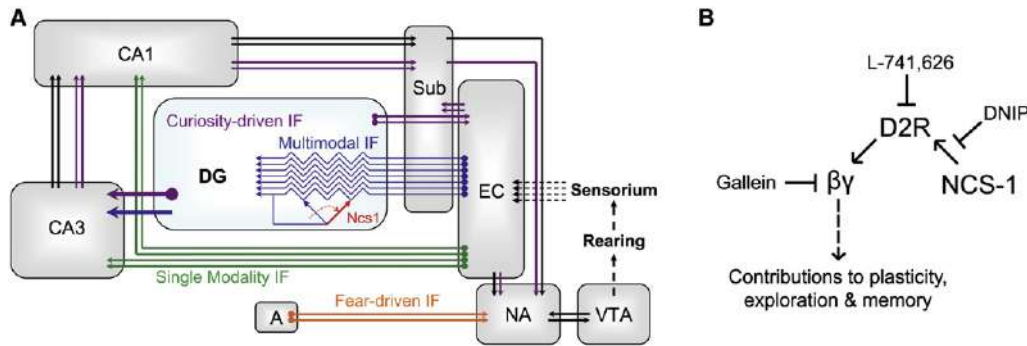


Figure 8. Models for DG Regulation of Curiosity and Multimodal Memory Formation and NCS-1/D2R Regulation of Synaptic Plasticity

(A) Multimodal information from the sensorium is integrated in the EC and projected (blue arrows) to the DG where pattern separation occurs. Here NCS-1 (red arrow) lowers the LTP induction threshold, as depicted by the variable resistor (jagged blue arrows), and enhances EC-DG LTP to promote rapid acquisition of space-dependent memory. Single-modality information processing (green arrows) may occur via direct projections from the EC to areas CA1 or CA3. Circuitry responsible for exploratory or curiosity-driven behavior (purple arrows) arises in the DG and projects through the EC and/or hippocampal trisynaptic loop to the nucleus accumbens. Bidirectional signaling between the nucleus accumbens and ventral tegmental area results in rearing behavior. More rearing, in turn, contributes to a richer sensorium, potentially completing a positive-feedback loop. Circuitry responsible for fear-driven rearing (orange arrows) arises in the amygdala, a system not affected in our model. IF, information; DG, dentate gyrus; CA, cornu ammonis; EC, entorhinal cortex; Sub, subiculum; NA, nucleus accumbens; A, amygdala; VTA, ventral tegmental area. Arrow thickness approximates relative strength of synaptic contact. Arrow number approximates relative amount of innervating projections.

(B) NCS-1 drives D2R surface expression, a process blocked by DNIP. Activation of D2R (blocked by L-741,626) stimulates $G_{\beta\gamma}$ downstream signaling mechanisms (blocked by gallein) that contribute to plasticity, exploration, and memory.

interference (Hetherington, 1990). A lack of memory deficits in the DGNCS-1 animals suggests sparse encoding in the DG can be preserved when the threshold for plasticity is reduced and the ceiling of plasticity is increased.

The Role of the DG in Spatial Novelty versus Object Novelty

DGNCS-1 mice display enhanced displaced object recognition, but normal novel object recognition. This might suggest NCS-1 and D2Rs function specifically in the DG to mediate multimodal information storage (Figure 8A). One major difference between novel object discrimination and displaced object discrimination is the number of sensory cues required to differentiate old from new (i.e., produce a mismatch). To recognize a novel spatial change, the many distal cues of the surrounding environment must be used, requiring integration of multiple sources of sensory information (multimodal input). On the other hand, to recognize a new object, any single cue (shade, shape, texture, etc.) can be employed to generate a mismatch and signal novelty. Since DG LTP is enhanced in these animals, and could underlie the enhancements in memory (though we do not attempt to prove this directly), it may be reasonable to also predict DG LTP does not underlie novel object recognition. Instead, other regions of the hippocampus are likely more important. Direct perforant path projections from the EC to areas CA3 or CA1 of the hippocampus may sufficiently encode single-modality, nonspatial information without undergoing conjunctive encoding or pattern separation processes via the DG (see model in Figure 8A).

The Role of the DG in Exploration and the Link to Memory

An isolated enhancement in exploratory rearing by selective alteration of the DG is exciting because while the medial hypo-

thalamus, amygdala, and nucleus accumbens are all known to be critical for defensive rearing behavior (Lever et al., 2006; Sandner et al., 1987; Silveira and Graeff, 1992), anatomical regions specifically responsible for rearing in safe conditions have proved more difficult to identify (Lever et al., 2006).

Since novelty detection is required for novelty exploration and the DG is important for novelty detection (Kesner et al., 2004), it is ideally suited to play a role in generating novelty exploration. Indeed, the DG sends projections to the nucleus accumbens, a region responsible for initiating rearing (Lever et al., 2006), via two major paths. The first is through the trisynaptic hippocampal loop and the other is through the EC and subiculum. Either, or both, of these pathways could be critical to initiating exploration. We observe promotion of a specific form of exploratory behavior in a mouse line with selective manipulation to the DG, but other subregions of the hippocampus may also be important, particularly if signaling from the DG to the nucleus accumbens is relayed—and therefore potentially modified—through the remainder of the hippocampal circuit.

Though facilitated DG NMDAR-dependent plasticity in DGNCS-1 animals may underlie the enhanced learning and memory in this mouse line, it is worth entertaining the hypothesis that increased curiosity may also contribute. Rearing in novel environments allows animals to make use of a superior vantage point, including longer sight and alternative air currents (Barry et al., 2006; Hetherington, 1990; Lever et al., 2006). A mutant model of fragile X syndrome demonstrates reduced rearing (Mineur et al., 2002), and dopamine dysfunction has recently been implicated to underlie fragile X syndrome (Weinshenker and Warren, 2008). DGNCS-1 subjects behave as if they incorporate a richer sensorium, which could be due to increased rearing, facilitated plasticity, or both. Though we do not test this directly, we agree with the prediction that a richer sensorium

can ultimately translate into a more detailed environmental map (Barry et al., 2006), which in turn could enhance spatial memory acquisition and the potential for novelty detection. In Figure 8A, we provide a model for how NCS-1 and “curiosity” originating in the DG might help establish spatial maps and memory.

Several studies have found stereotypy and increased locomotion in rodent models of schizophrenia, such as following MK801 administration (Tiedtke et al., 1990). However, hyperactivity is more frequently found for horizontal locomotion (Clapcote et al., 2007; Kellendonk et al., 2006). Moreover, because hyperactivity persists in brightly lit environments typical for locomotion experimentation, increased rearing in the DGNCS-1 animals does not likely signify a psychiatric endophenotype. We feel this result supports the selectivity of induced *Ncs1* expression since NCS-1 and D2Rs are both implicated in schizophrenia as well as other psychiatric disorders.

Clinical Considerations

It is important to identify promising molecular targets that could be used to enhance cognition in human diseases. We feel NCS-1 and the D2R are such targets. Moreover, while in this study we focused on the DG, these findings have broad implications for other diseases of the nervous system since NCS-1 and the D2R are coexpressed in many cell types and are implicated in mental illnesses, including schizophrenia, bipolar disorder, and addiction (Berke and Hyman, 2000; Joyce et al., 1993; Kabbani and Levenson, 2006; Koh et al., 2003; Saab and Roder, 2007; Souza et al., 2006).

There is good reason to predict that the DNIP would most readily reduce D2R surface expression in areas of the brain that show high levels of D2R expression, such as the striatum. Because the striatum is implicated in neuroendophenotypes of psychiatric disorders, including schizophrenia (Kellendonk et al., 2006) and addiction (Gerdeeman et al., 2003), it would be interesting to test the effects of the DNIP in relevant rodent models.

In summary, we show that NCS-1 and D2R combine to regulate exploratory behavior, NMDAR-dependent perforant path plasticity, and rapid acquisition of short- and long-term spatial memory. These results offer insights into behavioral, cellular, and molecular mechanisms governing the origin of exploration and the formation of memory, and underscore the importance of their relationship.

EXPERIMENTAL PROCEDURES

For full experimental procedures, see the accompanying Supplemental Data.

Generation of *Camk2a-rtTA2* and *tetO-ncs* Transgenic Mice

The DG-restricted *rtTA2-M2* line (designated “*Tg(Camk2a-rtTA2-901)SMαn*” on the Mouse Genome Informatics database [MGI], <http://www.informatics.jax.org/>) was created by random integration of the *Camk2a-rtTA2* transgene as previously described (Michalon et al., 2005). The *tetO-ncs* transgenic line was created in pure inbred C57BL/6 oocytes at the Princess Margaret Hospital Transgenic facility, Toronto (designated “*Tg(tetO-Ncs1)JRod*” on the MGI database).

Dox Administration

Mice were fed fresh dox food daily at time of lights out (7:00 p.m.). Three to five hours prior, food was prepared from powdered Purina Mouse Chow, water,

and dox (Doxycycline/Doxycycline Hyclate, obtained from the Mount Sinai Hospital pharmacy in Toronto).

Vibratome Sections and LacZ

LacZ staining was carried out as previously described (Michalon et al., 2005).

RNA Isolation, cDNA Synthesis, and PCR

RNA was extracted with Trizol Reagent (Invitrogen) as described by the manufacturer. cDNA was synthesized with MonsterScript 1st – Strand cDNA Synthesis Kit (Epicenter Biotechnologies; MS041050) and random nonomers according to the manufacturers’ instructions.

Protein Isolation, Westerns, and Quantification

DG subregions were punched out from hippocampal slices (Figure S10). The remainder of each slice was taken as the CA1 and CA3 enriched fraction. The ends of the hippocampus excluding the slices were used as the hippocampal fraction.

Immunohistochemistry

Frozen 10 μM sections were fixed for 10 min in 4% PFA, stained overnight in primary antibody, and reacted with the appropriate peroxidase secondary. In all figures, black = less staining.

Electrophysiology

Experiments were conducted essentially as previously described (Henderson et al., 2001).

Behavior

All experiments were approved by the local committee on animal care and conformed to the national guidelines (CCAC; <http://www.ccac.ca>). Experiments were performed on DGNCS-1 and littermate control animals on dox. Experimenters were blind to the genotypes of the subjects.

Safe and Fearful Novel Environment Behavioral Examination

An identical Plexiglas arena (inner dimensions: 42 cm³) and objects were used for the safe (~50 lux) and fearful (~400 lux) environments. See Figure S4 for a schematic diagram of the test.

New Frontier Exploration Task

Mice were allowed to climb from their home cage onto any of four platforms, each elevated 15 cm above the floor and connected to novel environments, dubbed “frontiers” (18 cm × 30 cm). See Figure S4 for a schematic diagram of the test.

Hole Board Test

Behavioral observations were made for 5 min in a dimly lit circular environment ($r = 25$ cm, 40 cm high) containing four circular holes ($r = 1$ cm) elevated 8 mm above a clean surface.

Light/Dark Box

Mice were placed in a closed black Plexiglas chamber (20 cm³) for 2 min, after which a trap door was raised 4 cm to allow entry into a brightly lit transparent Plexiglas chamber (20 cm³). The location of the animal was then recorded for a further 2 min.

Object Recognition

Experiments were conducted in a square Plexiglas environment as depicted in Figure S4, using objects upon which the mice were unable to climb. All protocols followed the same sequence of five sections: (1) habituation, (2) interval, (3) displaced object discrimination, (4) interval, and (5) novel object discrimination.

Morris Water Maze

A schematic of apparatus and procedure is shown in Figure S4. Acquisition and probe trials were a maximum of 180 s and 60 s, respectively. The minimal procedure was repeated three times and results are presented as the average performance from all three sessions.

Immunofluorescence in DGNCS-1 Slices

Three-hundred-micrometer slices were immersed in 4% PFA in PBS for 5 min to provide light fixation without disrupting plasma membranes. Further processing was carried out at 4°C in PBS plus 2% BSA.

Immunofluorescence in DNIP-Treated Slices

Slices were incubated for 1 hr in an oxygenated aCSF bath containing 10 μ M of either the active or scrambled DNIP prior to brief fixation and immunostaining.

Coimmunoprecipitation

Using protein A Sepharose CL-4B, coimmunoprecipitation was continued according to the manufacturers' instructions.

Peptide Synthesis

Peptides were prepared by Dr. Wang at the Advanced Protein Technology Centre at the Hospital for Sick Children, Toronto. The nine amino acids of the active DNIP sequence (Kabbani et al., 2002) were pseudorandomized to generate the scrambled DNIP. Blasts confirmed no strong homology to the mouse proteome.

Primary Cultures of Hippocampal Neurons

Cultures were prepared from hippocampii dissected from E17–E19 fetal Wistar rats and incubated at 37°C in a 5% CO₂ incubator in Neurobasal/B27 medium for 10–14 days before use.

Cell-ELISA Assays

Cell-ELISA assays (colorimetric assays) were performed essentially as previously described (Lee et al., 2002; Man et al., 2000). Cell surface expression is presented as the ratio of colorimetric readings under nonpermeabilized conditions to those under permeabilized conditions.

cAMP Assays

After 30 min treatment of neurons with 10 μ M forskolin, 10 μ M quinpirole, 1 μ M SCH23390, or a combination as appropriate, cAMP assays were performed as directed by the manufacturer (R&D systems).

Cannulization and Infusions

Stainless steel guide cannulae were positioned at coordinates -2.0 mm from bregma, ± 1.5 mm from midline, and -1.2 mm from the dura. DNIP peptide (low dose, 25 ng/ μ l) was infused 3–5 hr before behavioral analysis 0.5 mm below the termination of the guide cannulae at 0.1 μ l/min for 5 min.

Statistical Analysis

We used two-way ANOVA Tukey HSD for comparisons of equal n or unequal n where required.

SUPPLEMENTAL DATA

Supplemental data for this article include Supplemental Experimental Procedures, ten figures, and one table and can be found at [http://www.cell.com/neuron/supplemental/S0896-6273\(09\)00626-6](http://www.cell.com/neuron/supplemental/S0896-6273(09)00626-6).

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