Supplementary Data

Mouse breeding

Both *Emx1-Cre* and *Dlx6a-Cre* mouse lines were backcrossed with Black Swiss mice for more than 6 generations before being bred with *Sp4* heterozygous mice on the same Black Swiss background. To generate F1 mice for genetic rescue studies, *Sp4* heterozygous mice on 129S background were bred with *Sp4* and *Emx1-Cre* double heterozygous mice on the Black Swiss background. All F1 *Sp4* heterozygous mice were sacrificed. Four genotypes were retained for further studies. Using the same breeding strategy, we generated 4 genotypes of mice with or without the *Dlx6a-Cre* transgene.

Supplemental Figure 1. Generation of *Sp4* rescue mice for molecular and behavioral analyses. (a) *Sp4* heterozygous mice ($Sp4^{+/Hypo}$; $Emx1^{+/+}$) on the 129S background were bred with *Sp4* and *Emx1-Cre* double heterozygous mice ($Sp4^{+/Hypo}$; $Emx1^{+/+(Cre)}$) on the Black Swiss background to generate F1 mice. Four genotypes were selected for all subsequent molecular and behavioral studies. **(b)** *Sp4* heterozygous mice ($Sp4^{+/Hypo}$) on the 129S background were bred with *Sp4* and *Dlx6a-Cre* double heterozygous mice ($Sp4^{+/Hypo}$; *Tg(Dlx6a-Cre)*) on the Black Swiss background to generate F1 mice. Four genotypes were selected for all further studies.

Conditioned fear test

Apparatus: The procedure used in the fear conditioning experiment was adapted from previous studies (Toth et al. 2014) using automated fear conditioning chambers. In the *Emx1-Cre* rescue experiment, 4 San Diego Freeze Monitor chambers were used (San Diego, CA), which uses sixteen infrared photobeams surrounding the chamber to continuously detect the mouse's movements. The latencies and numbers of beam interruptions were recorded and used for determination of conditioned fear (freezing) response. Freezing was determined using

an automated method that relied upon the latency to break the infrared beams in 5 sec epochs. A freezing bout was counted if it took 1 sec or more to break a beam after the start of each 5 sec recording epoch. This algorithm shows a high correlation with observer-based fear ratings (Gresack et al. 2010; Valentinuzzi et al. 1998). The *Dlx6a-Cre* rescue experiment was conducted using the same parameters (see below) but in 4 Med Associates Fear conditioning chambers for mice (St. Albans, VT). These chambers use a video tracker system with an algorithm for freezing counts based on pixel change per video frame. We used a motion index of change in less than 18 pixels across 30 frames (1 sec) as the cut off for scoring freeze time, which has been found to best correlate with observer based freeze scoring (Anagnostaras et al. 2010).

Testing Parameters: The testing parameters are as previously described (Toth et al. 2014). After a 60-min habituation period in an adjacent room, mice were placed in the conditioning chamber. After an acclimation period (2 min), mice were presented with a tone (CS: 75 dB, 4 kHz) for 20 sec that co-terminated with a foot shock (US: 1 sec, 0.5 mA). A total of three tone-shock pairings were presented with an inter-trial interval of 40 sec. Freezing was measured during tone presentations and for 40 sec after shock. Mice were replaced in their home cage 40sec after the final shock. The chambers were cleaned with water after each session. Twenty-four hours later, subjects were re-exposed to the conditioning chamber to assess context-dependent fear retention. The habituation period and features of the chamber were identical to those used during conditioning. Mice were then placed in the chambers and tested for freezing for 2 min during which time no shocks or tones were presented and freezing was scored. Twenty-four hours after the context fear-retention test, mice were tested for CS-induced fear retention. The context of the chambers was altered across several dimensions (tactile, odor, visual) for this test in order to minimize generalization from the conditioning context. After a 5 min acclimation period, during which time no tones were presented (“pre-tone”), 5 tones were presented for 20 sec with an inter-trial interval of 5 sec to test recall of cued

fear. Freezing was scored during and after each tone presentation. Baseline freezing during the acclimation period prior to the tone presentation was also assessed. Subjects were returned to their home cage immediately after termination of the last tone.

Results: In the *Emx1-Cre* rescue cohort there was a significant reduction in acquisition of fear conditioning in *Sp4* hypomorphs, with hypomorphs showing significantly reduced freezing by the 2nd and 3rd tone-CS presentation compared to control mice [Sp4 X CS trial interaction: $F(2,158)=9.6$, $p<0.0001$; Main effect of Sp4 at the 1st, 2nd and 3rd tone-CS respectively: $F(1,79)=1.7, 22.7, 7.521$, $p>0.05$, $p<0.0001$, $p<0.01$]. *Sp4* hypomorphs continued to show reductions in freezing at the contextual and cued fear recall tests on Day 2 and Day 3 [Day 2 Main effect of Sp4: $F(1,79)=41$, $p<0.0001$; Day 3 Main effect of Sp4: $F(1,79)=19.5$, $p<0.0001$.]. The *Emx1-Cre* gene did not interact with *Sp4* genotype in any of the tests. In the *Dlx6a-Cre* rescue cohort, *Sp4* hypomorphs also showed a significant reduction in fear learning [Sp4 X CS trial interaction: $F(2,170)=4.55$, $p=0.014$; Main effect of *Sp4* at the 3rd tone-CS: $F(1,85)=6.3$, $p=0.014$] that did not interact with the *Dlx6a-Cre* gene. There were no main effects or interactions between any of the groups on Day 2 or Day 3.

Discussion: The primary finding of these supplementary studies is that *Sp4* hypomorphic mice exhibit significant reductions in acquisition of pavlovian fear conditioning (Day 1) which were not reversed by restoration of *Sp4* gene expression in forebrain excitatory or GABAergic neurons (Fig. S2a+d). Reduced contextual and cued fear recall in *Sp4* hypomorphic mice was also not affected by the presence of the *Emx1-Cre* gene (Fig. S2b+c). Surprisingly, we did not detect reduced context or cued fear recall in the *Sp4* hypomorphs in the *Dlx6a-Cre* rescue cohort, likely due to the relatively low freezing in control animals in the context and cued fear recall tests (Fig. S2e+f). This low freezing response at recall may have been due to differences in the two fear conditioning chambers used across the two experimental cohorts. Generation of genetic

rescue mice suitable for behavioral analyses needs large breeding cohorts, which is expensive and time-consuming. In the future, studies using different conditioning parameters in the Med Associates boxes to induce stronger fear conditioning in controls will be needed to confirm if the fear conditioning deficits in *Sp4* hypomorphic mice can be reversed by the *Dlx6a-Cre* gene.

Supplemental Figure 2. *Sp4* gene reduction results in reduced fear learning that is not reversed by restoration of *Sp4* in either exciatory (via *Emx1-Cre*) or GABAergic (via *Dlx6a-Cre*) neurons. a+d: Acquisition of conditioned fear to tone conditioned-stimulus. **p<0.01 vs. control mice, *post hoc* simple comparisons. **b+e:** Two minute context retention test. **c+f:** Cued fear retention test (block of 5 20-s tone cues). **p<0.01 vs. control mice, Main effect of *Sp4* gene, see supplementary results for details. All data are expressed as mean±SEM percentage of time freezing.

References

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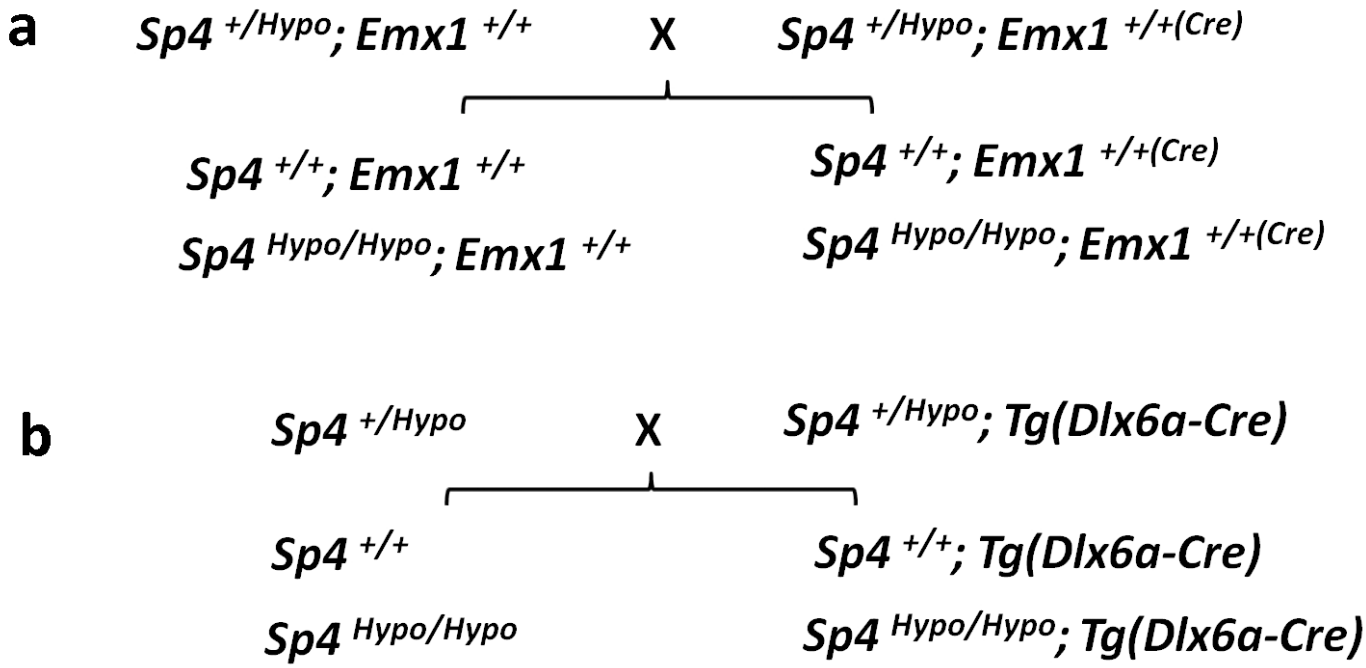
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Sp4 +/Hypo; Emx1 +/+
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Sp4 Hypo/Hypo; Emx1 +/+(Cre)
b
Sp4 +/Hypo
X
Sp4 +/Hypo; Tg(Dlx6a-Cre)
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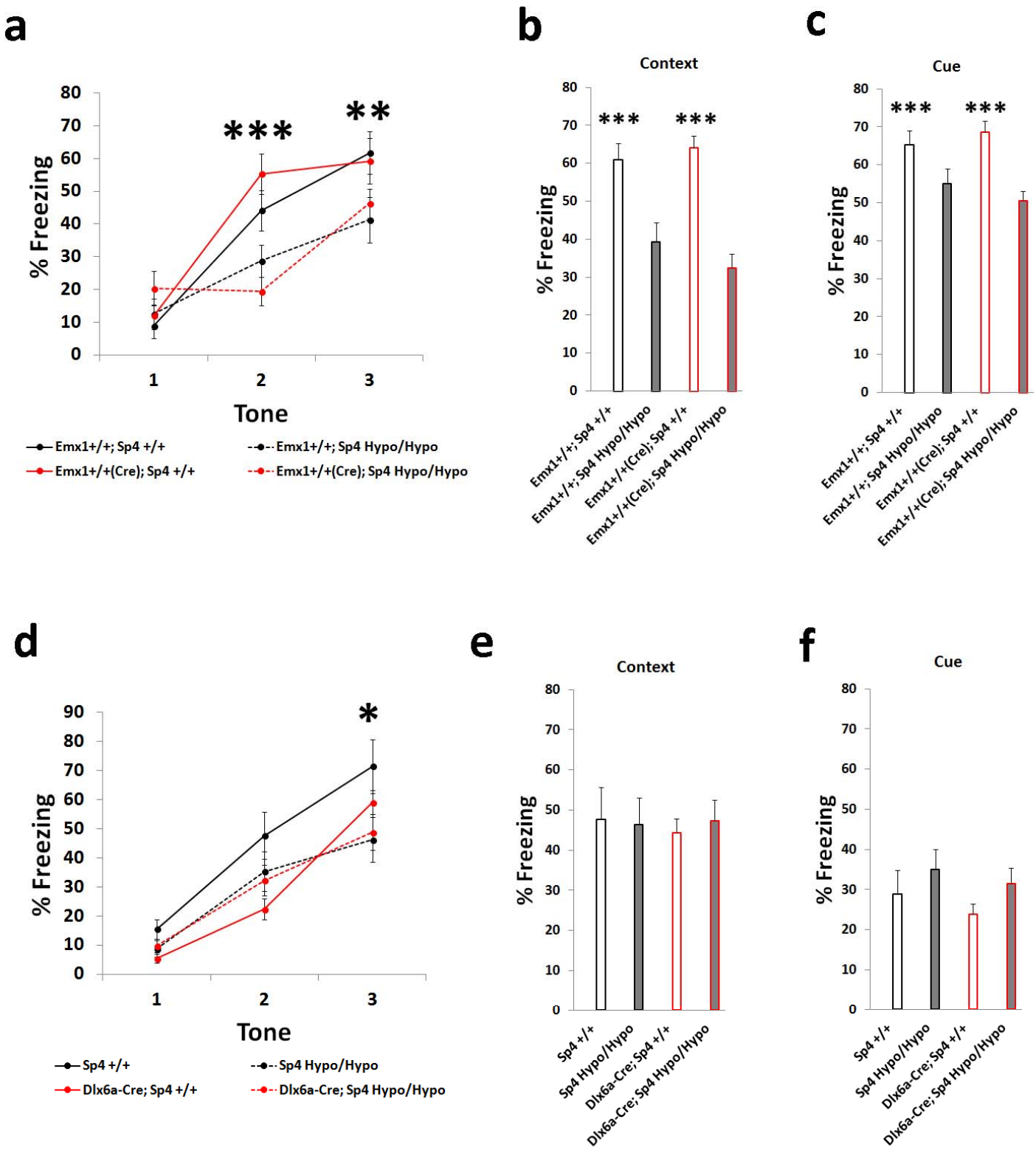
Sp4 Hypo/Hypo
Sp4 Hypo/Hypo; Tg(Dlx6a-Cre)

Figure 1S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Sp4 Hypo/Hypo
Dlx6a-Cre; Sp4 +/+
Dlx6a-Cre; Sp4 Hypo/Hypo

Figure 2S