# SUPPLEMENTARY MATERIALS

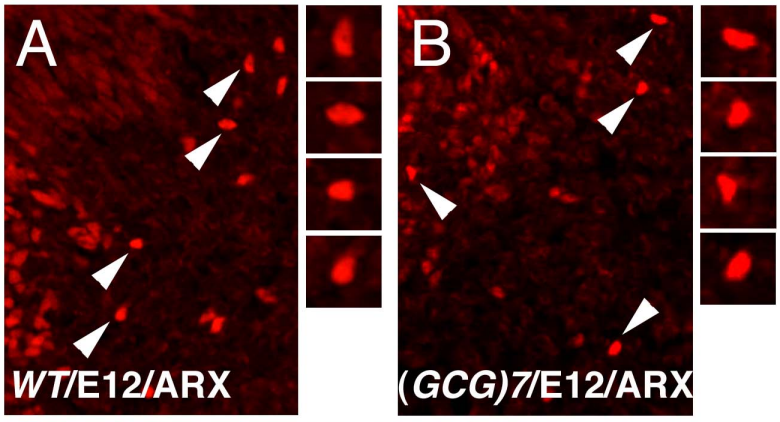
## Figure and Table Legends

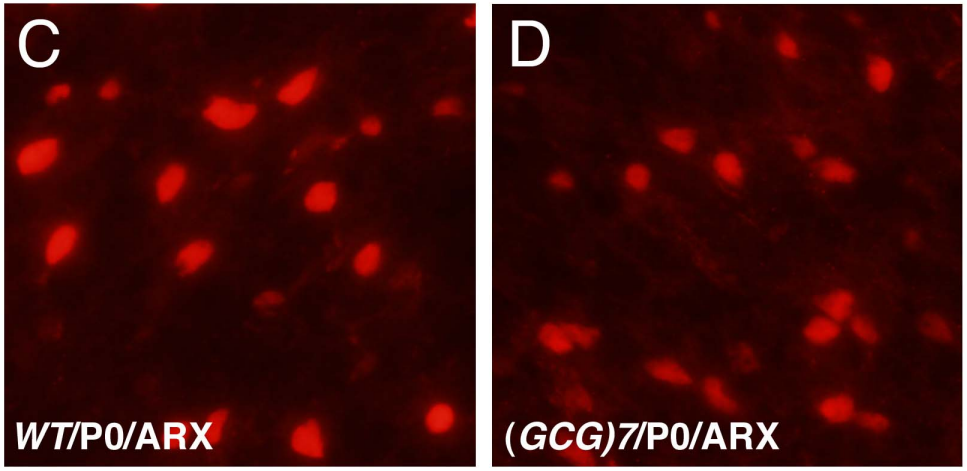
### Supplementary Figure S1

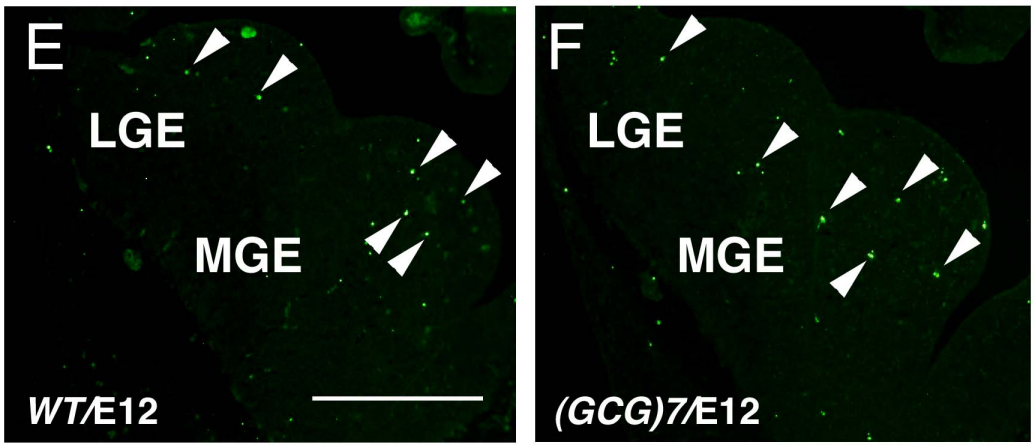
**No intranuclear inclusions and increased apoptosis in *Arx*$^{(GCG)/Y}$ mice**

**A-D**, No specific formation of intranuclear inclusions (apparition of nuclear inclusions) was seen in the migratory ARX$^+$ cells from ganglionic eminence at E12 (A, B) and in cortical ARX$^+$ cells at P0 (C, D) of *Arx*$^{X/Y}$ (A, C) and *Arx*$^{(GCG)7/Y}$ (B, D) mice. ARX$^+$ nuclear images with white arrowheads in (A) and (B) are magnified. **E-F**, Average apoptotic cell number per arbitrary area of genglionic eminence of *Arx*$^{X/Y}$ and *Arx*$^{(GCG)7/Y}$ embryos at E12 was 16.5±1.5 and 16.9±2.3, respectively (n=5, $p>0.5$. Some apoptotic cells are marked with white arrowheads) and thus no increase in the number of apoptotic cells was detected in the ganglionic eminence of *Arx*$^{(GCG)7/Y}$ embryos as compared to *Arx*$^{X/Y}$ embryos. For the apoptotic assays, an ApopTag Plus Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon) was used according to the manufacturer's instructions. Scale bar: E, F, 500 μm.

# Supplementary Fig. S1



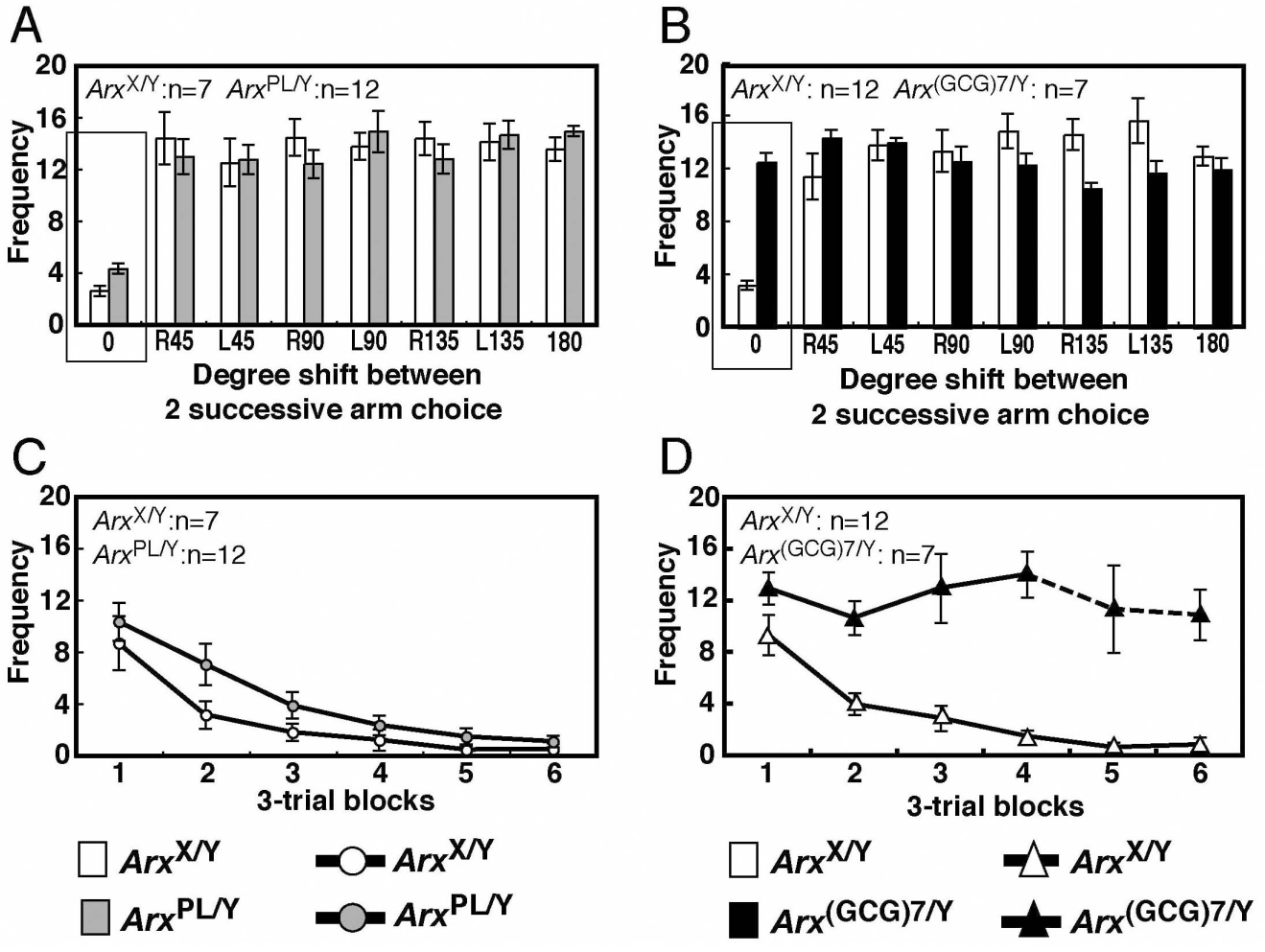



**Supplementary Figure S2**

**Non-spatial egocentric strategy in the win-shift task**

Analysis of the shifting manner in the win-shift task for *Arx*$^{PL/Y}$ and *Arx*$^{(GCG)7/Y}$ mice. Every arm choice in all trials was classified by relative direction (L or R) and degree shift (0, 45, 90, 135 or 180) to the previous arm choice, and the frequency of each class was calculated among *Arx*$^{PL/Y}$ (A) and *Arx*$^{(GCG)7/Y}$ mice (B). Multiple comparisons with Scheffe's F test demonstrated a significantly equal distribution from 45 to 180° for the *Arx*$^{PL/Y}$, *Arx*$^{(GCG)7/Y}$ or *Arx*$^{X/Y}$ mice ($P>0.05$). *Arx*$^{PL/Y}$ mice clearly avoided revisiting arms, as their frequency of 0° was significantly less than any other angle for each mouse ($P<0.05$) (A). *Arx*$^{(GCG)7/Y}$ mice, however, showed no significant difference between the frequency of 0° and any other angle ($P>0.05$) (B). Throughout the training, the frequency of 0° showed no significant reduction ($P>0.05$) (C). These results indicate that *Arx*$^{(GCG)7/Y}$ mice chose an arm at random during the training. Moreover, when divided into averaged groups of three trials, the frequency of 0° shown in the square in (A) and (B) showed a significant reduction ($P<0.05$) in the *Arx*$^{PL/Y}$ mice (C) but no significant change ($P>0.05$) in the *Arx*$^{(GCG)7/Y}$ mice (D).

# Supplementary Fig. S2



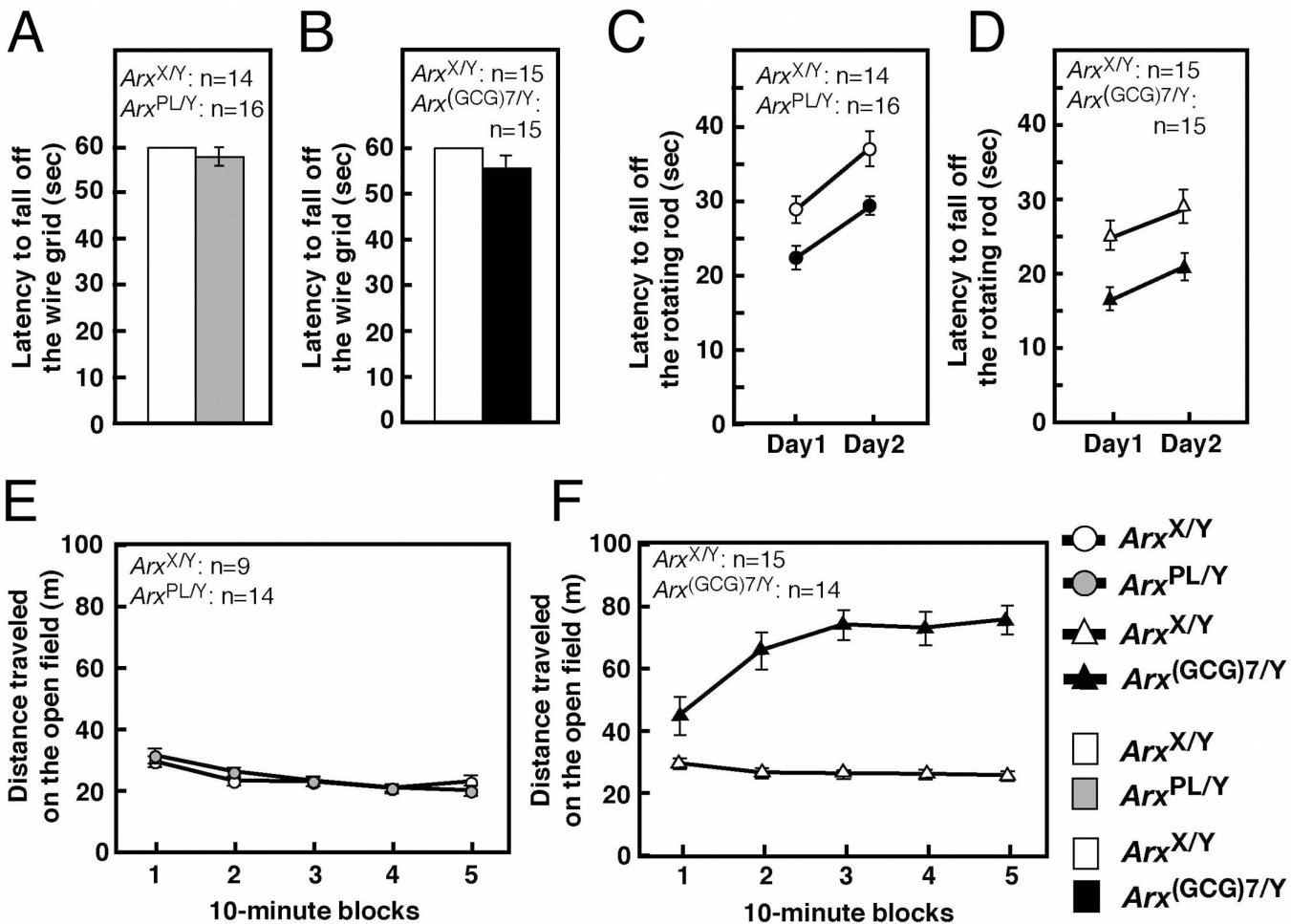
**Supplementary Figure S3**

**Impaired motor coordination in both *Arx*$^{PL/Y}$ and *Arx*$^{(GCG)/Y}$ mice**

**A**, **B**, Neuromuscular strength was tested by the wire-hanging test. Neither *Arx*$^{PL/Y}$ nor *Arx*$^{(GCG)7/Y}$ mice showed significant differences in latency to fall off the wire grid (P= 0.301 and P=0.083, respectively; Mann-Whitney U-test). **C**, **D**, Using the rotarod to evaluate motor coordination and/or balance, the latency to fall off the rotating drum was significantly shorter in both mutants (*Arx*$^{PL/Y}$ mice: genotype effect, $F[1,28]=11.369$, $P<0.01$; genotype x day interaction, $F[1,28]=0.1459$, $P=0.705$; *Arx*$^{(GCG)7/Y}$ mice: genotype effect, $F[1,28]=10.879$, $P<0.01$; genotype x day interaction, $F[1,28]=0.0447$, $P=0.834$). **E**, **F**, For spontaneous locomotor activity in an open field arena, *Arx*$^{PL/Y}$ mice showed no difference in the distance traveled (genotype effect, $F[1,21]=0.0949$, $P=0.761$; genotype x time interaction, $F[4,84]=1.057$, $P=0.383$) (E). In contrast, *Arx*$^{(GCG)7/Y}$ mice exhibited a clear and significant increase in the distance traveled (genotype effect, $F[1,27]=75.724$, $P<0.0001$; genotype x time interaction, $F[4,108]=18.029$, $P<0.0001$) (F). These results revealed that the two mutants possessed similar motor coordination deficits without neuromuscular weakness and that the *Arx*$^{(GCG)7/Y}$ mice were hyperactive during spontaneous locomotion.

Methods: *Wire-hanging test*: The wire-hanging test was performed as described previously (Karl et al., 2003). The latency to fall off was measured with a 60-sec cut-off time. *Rotarod*: An accelerating rotarod (O'Hara & Co., Tokyo, Japan) was used. The mouse was placed on the rotating drum, and the latency of its fall was measured. The rotation speed increased from 10 rpm to 35 rpm over a 50-sec period, and the top speed was maintained until the mouse fell off, with a 60-sec cut-off time. The mouse was given three trials a day at 30-min intervals for two days. The average of three trials per day was used for statistical analysis. *Open field*: The mouse was placed at the corner of an open field arena (50 x 50 cm, O'Hara & Co.) and allowed to explore the field freely for 50 min. Distance traveled, time spent in the center, and several moving indices were analyzed with Image J OF software.

# Supplemetary Fig. S3



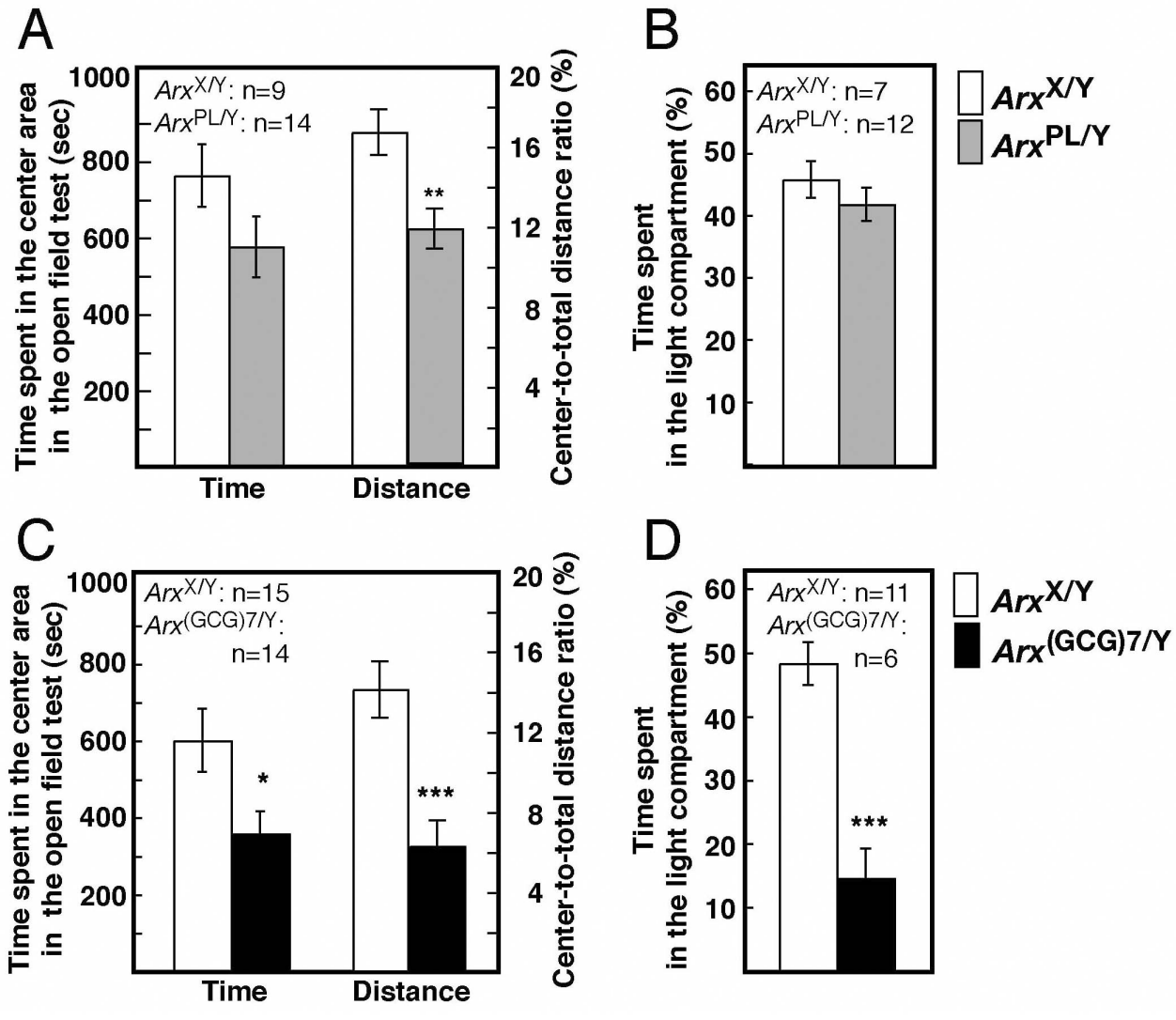
**Supplementary Figure S4**

**Anxiety in both *Arx*$^{PL/Y}$ and *Arx*$^{(GCG)/Y}$ mice**

Anxiety was evaluated using the conflict between exploration in a novel environment and the aversion to open and/or bright spaces that exists in mice. In the *Arx*$^{PL/Y}$ mice in the open field test, there was no significant difference in the time spent in the open center area (P=0.818), but there was a lower ratio of the distance traveled in the center area compared with the total distance traveled (P<0.01) (A). In the light-dark transition test, the *Arx*$^{PL/Y}$ mice spent no significant time in the light compartment (B). These results indicate that the *Arx*$^{PL/Y}$ mice have little, if any, increase in anxiety-like behavior. On the other hand, the *Arx*$^{(GCG)7/Y}$ mice spent less time in the open center area (P=0.025) and showed a lower distance ratio (P<0.001) in the center area (C). In the light-dark transition test, the *Arx*$^{(GCG)7/Y}$ mice showed a significant reduction in the percentage of time spent in the light compartment (P<0.0001) (D). The *Arx*$^{(GCG)7/Y}$ mice consistently showed an aversion to open or light environments, which shows that they have significantly increased anxiety-like behavior. Incidentally, in the light-dark transition test, there was no significant difference in the distance traveled by the two strains of mice (data not shown). Significant differences from *Arx*$^{X/Y}$ mice are represented as * for P<0.05, ** for P<0.01 and *** for P<0.001.

Method: *Light-dark transition*: The light-dark transition test was performed as described previously (Miyakawa et al., 2001), using the testing apparatus (O'Hara & Co.). The mouse was given a 10-min period to explore the apparatus freely. The time spent in the illuminated compartment was analyzed with Image J LD software.

# Supplemetanry Fig. S4

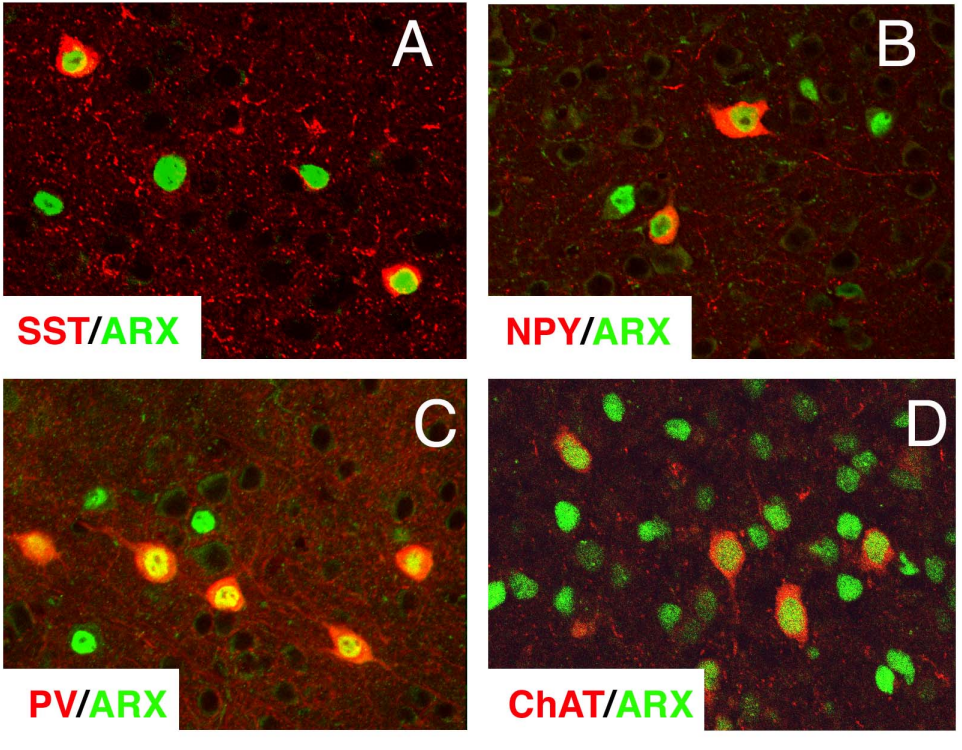


**Supplementary Figure S5**

**Expression of SST, NPY, PV and ChAT in Arx$^+$ neurons**

Subtype markers (red) of GABAergic interneuons including somatostatin (SST, A), neuropeptide Y (NPY, B), and parvalbumin (PV, C) are expressed in Arx$^+$ cells (green) in the cortex at P1m. ChAT$^+$-expression (red) is also seen in the ARX$^+$ cells (green) in the basal nuclei at P1m (D).

# Supplementary Fig. S5

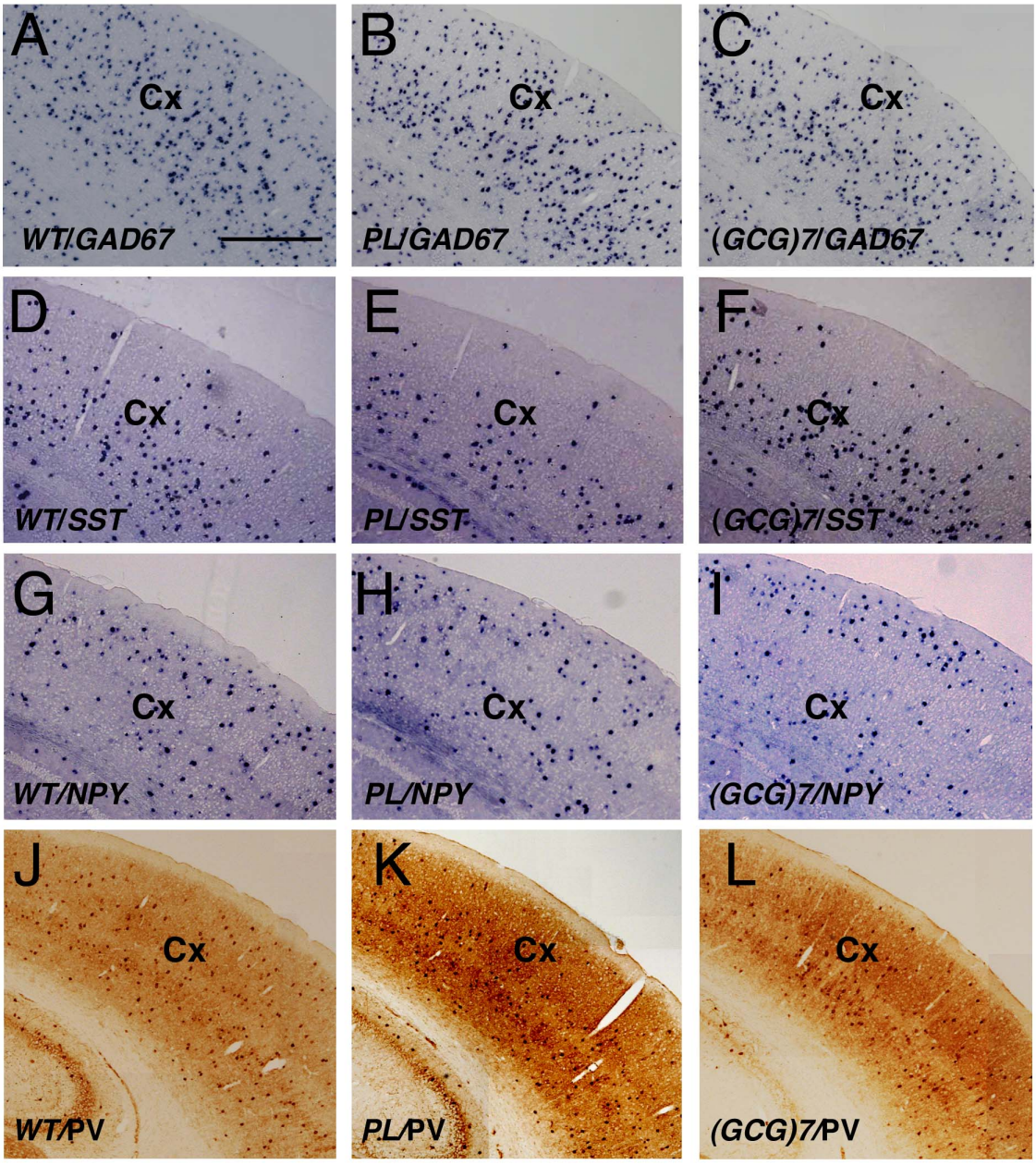


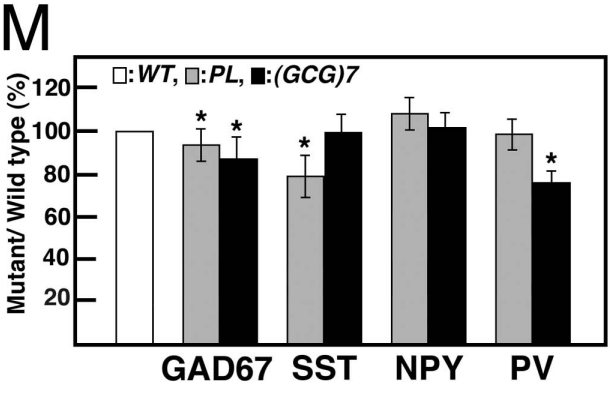
**Supplementary Figure S6**

**Slight reduction of GABAergic interneurons in the cortex of *Arx*$^{PL/Y}$ and *Arx*$^{(GCG)/Y}$ mice**

GAD67$^{+}$ neurons showed slight reduction in the somatosensory cortex of *Arx*$^{PL/Y}$ or *Arx*$^{(GCG)7/Y}$ mice (92.7±7.8%, and 87.5±10.4%, P<0.02, n=3, respectively. B, C, M). SST$^{+}$ interneurons were reduced in *Arx*$^{PL/Y}$ mice (74.1±4.0%, p<0.01, n=3), while the SST$^{+}$ interneurons of *Arx*$^{(GCG)7/Y}$ mice maintained levels similar to those in the *Arx*$^{X/Y}$ mice (E, F, M). NPY$^{+}$ interneurons of both mutants maintained levels similar to those in the *Arx*$^{X/Y}$ mice (H, I, M). On the other hand, PV$^{+}$ interneurons were reduced in *Arx*$^{(GCG)7/Y}$ mice (81.1±5.1%, p<0.02, n=3), while PV$^{+}$ interneurons in *Arx*$^{PL/Y}$ mice maintained levels similar to those in the *Arx*$^{X/Y}$ mice (K, L, M). These observations suggest that only a portion of the cortical GABAergic interneuron subtypes were slightly affected by the mutation. Furthermore, GABAergic interneurons in the hippocampus showed the same tendency as cortical GABAergic interneurons (data not shown). Scale bars: A-L, 500 μm.

# Supplementary Fig. S6



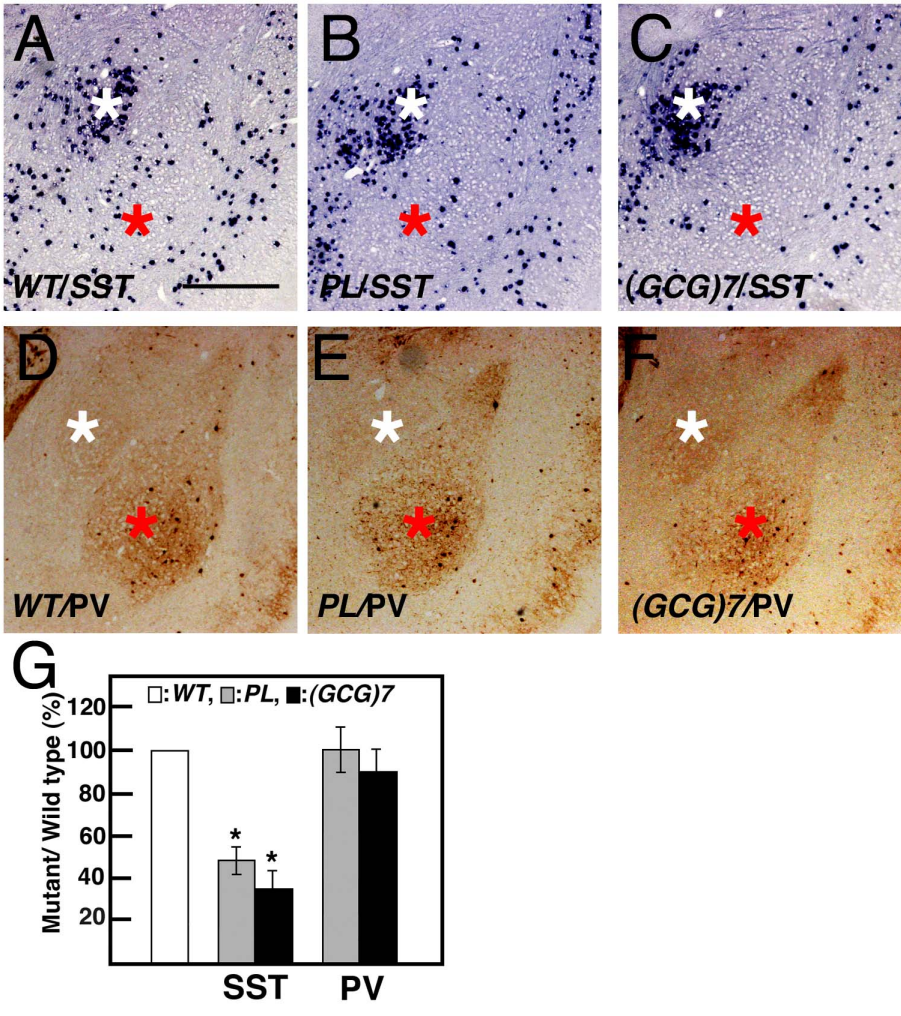



**Supplementary Figure S7**

**Reduction of GABAergic interneurons in basolateral amygdala in *Arx*$^{PL/Y}$ and *Arx*$^{(GCG)/Y}$ mice**

Arx is expressed in the developing caudal ganglionic eminence in addition to the MGE and LGE and participates in GABAergic interneuron development in the amygdala (Poirier et al., 2004). The number of SST$^+$ interneurons in the basolateral amygdala of *Arx*$^{PL/Y}$ and *Arx*$^{(GCG)7/Y}$ mice was significantly reduced, to 48.3±6.2% and 31.9±8.7% ($p<0.001$, n=3), respectively (red stars in A-C, G), of the number in *Arx*$^{X/Y}$ mice, while the number of PV$^+$ interneurons was not decreased (red stars in D-F, G). Furthermore, no reduction in SST$^+$ interneurons was detected in the central amygdala (white stars in A-f); thus, the mutant phenotypes of SST$^+$, but not PV$^+$, interneurons in the basolateral amygdala appeared similar to the striatum. Scale bars: A-F, 500 μm.

# Supplementary Fig. S7



**Supplementary Table S1**

**Correlation between learning and locomotor activity or anxiety-like behavior in *Arx*$^{(GCG)7/Y}$ mice**

Learning activities tend to be affected by increased locomotor activity (Miyakawa et al., 2001), so we investigated whether the learning activities of *Arx*$^{(GCG)7/Y}$ mice with high locomotor activities (**Supplementary Fig. S3F**) were affected by their locomotor activities. The *Arx*$^{(GCG)7/Y}$ mice examined with the passive avoidance appeared to have increased locomotor activity in the open field. The latency for entry after shocks in the passive avoidance test was not correlated with the locomotor activity in the open field (A1). In the mice examined either in the two tasks of the radial maze, in which the open field test had not been conducted (as described in Materials and Methods section), the *speed* (distance traveled divided by the time spent to complete a trial) and the *total number of lit- and unlit arm choices* during the habituation phase were used as a locomotor activity in the win-shift task and in the win-shift task, respectively. These two locomotor indices were confirmed to increase in *Arx*$^{(GCG)7/Y}$ mice but not in *Arx*$^{PL/Y}$ mice (A2, A3), implying increased locomotor activity in the two tasks of the radial maze. Correlation between learning and locomotor activities of *Arx*$^{(GCG)7/Y}$ mice was estimated using the two indices. For the win-shift task, there were no significant correlations between mean accuracy and mean speed, although the mean speed was confirmed to increase only in *Arx*$^{(GCG)7/Y}$ mice (A2). In the win-stay task, there were also no significant correlations between the mean unlit-to-lit ratio over the training phase and the total arm choices over the habituation phase, although the *Arx*$^{(GCG)7/Y}$ mice had a significantly larger number of total arm choices (A3). These results indicated that increased locomotor activity was not crucial in the various learning deficits in *Arx*$^{(GCG)7/Y}$ mice.

The *Arx*$^{(GCG)7/Y}$ mice showed a stronger tendency to avoid entering a lit arm as shown by the reduction of the ratio of lit-arm choices to unlit-arm choices over the habituation phase (B). No significant correlation was found, however, between the mean unlit-to-lit ratio over the training phase and the aversion to lit arms in the habituation phase (B) and thus the statistical estimation indicated that win-stay performance was not due to aversion to the lit arms.

**(A) Correlation between learning indices and locomotor activity**

| | *Arx*$^{X/Y}$ | *Arx*$^{(GCG)7/Y}$ | *Arx*$^{X/Y}$ | *Arx*$^{PL/Y}$ |
|---|---|---|---|---|
| **(1) The passive avoidance test** | | | | |
| Locomotor activity: Total distance traveled in the open field (cm) | 13174 (±555) | 33178 *** (±2273) | 11741 (±718) | 11991 (±451) |
| Learning index: Latency for entry after shocks (sec) | 260 (±21) | 67 *** (±25) | 271 (±17) | 179 * (±26) |
| Correlation (Speaman's correlation coefficient by rank test) | rs=0.20 p=0.44 | rs=-0.13 p=0.63 | - | - |
| **(2) The win-shift task of the radial maze** | | | | |
| Locomotor activity: Mean speed over training phase (cm/sec) | 8.41 (±0.25) | 10.37 *** (±0.32) | 8.28 (±0.36) | 7.36 (±0.39) |
| Learning index: Mean accuracy over training (%) | 66.1 (±1.8) | 41.7 *** (±1.6) | 72.6 (±2.1) | 64.2 ** (±1.4) |
| Correlation (Peason's correlation coefficient test) | r=0.57 p=0.052 | r=0.019 p=0.96 | - | - |
| **(3) The win-stay task of the radial maze** | | | | |
| Locomotor activity: The total number of arm choices over the habituation phase | 127.9 (±9.7) | 200.0 ** (±14.7) | 110.1 (±5.4) | 117.8 (±7.1) |
| Learning index: Mean ratio of unlit choices to lit choices over the training phase | 1.44 (±0.07) | 1.99 *** (±0.05) | 1.07 (±0.10) | 0.96 (±0.06) |
| Correlation (Peason's correlation coefficient test) | r=-0.44 p=0.33 | r=0.31 p=0.69 | - | - |

**(B) Correlation between learning index and aversion to lit arms in the win-stay task of the radial maze**

| | *Arx*$^{X/Y}$ | *Arx*$^{(GCG)7/Y}$ | *Arx*$^{X/Y}$ | *Arx*$^{PL/Y}$ |
|---|---|---|---|---|
| Aversion to lit arms: Mean ratio of lit choice to unlit choice over the habituation phase (%) | 0.78 (±0.03) | 0.51 (±0.03) *** | 0.86 (±0.08) | 1.03 (±0.08) |
| Learning index: Mean ratio of unlit choices to lit choices over the training phase | 1.44 (±0.07) | 1.99 *** (±0.05) | 1.07 (±0.10) | 0.96 (±0.06) |
| Correlation (Peason's correlation coefficient test) | r=-0.73 p=0.059 | r=0.34 p=0.66 | - | - |