Supplementary Information

Nitroreductase-mediated cell ablation uncovers the pivotal role of ependymoglial cells in entire cortex regeneration in axolotls

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Supplementary Table 1 The success rate of spinal cord transplantation surgery in axolotls

Supplementary Table 2 The success rate of brain transplantation surgery in axolotls

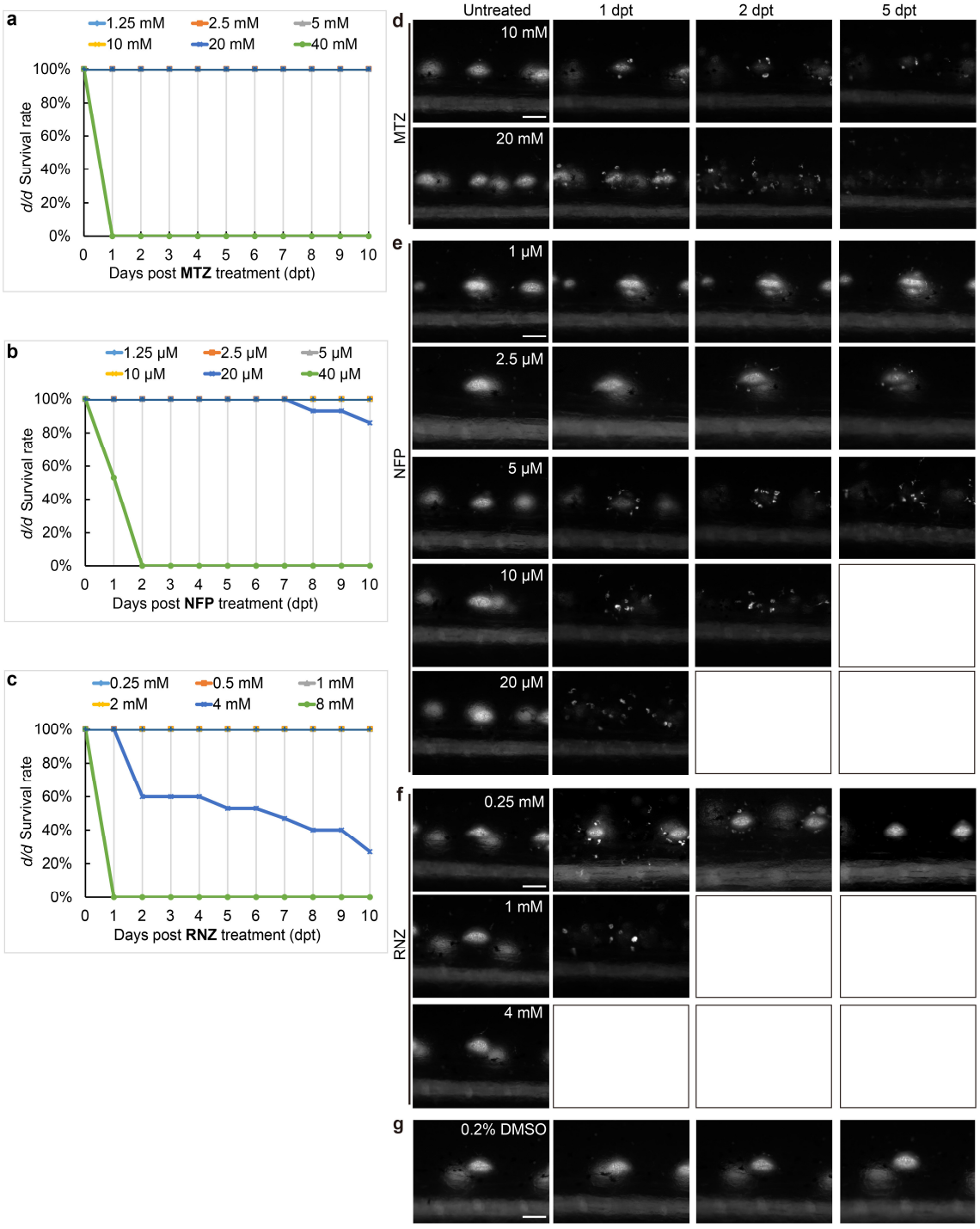
Supplementary Table 3 Primers used in this study

Supplementary Video 1 *NeuroD6:CherryNTR2.0* predatory behavior at 10-day post-MTZ treatment (uploaded separately)

Supplementary Video 2 *NeuroD6:CherryNTR2.0* predatory behavior at 10-day post-DMSO treatment (uploaded separately)

Supplementary Video 3 *NeuroD6:CherryNTR2.0* predatory behavior at 50-day post-MTZ treatment (uploaded separately)

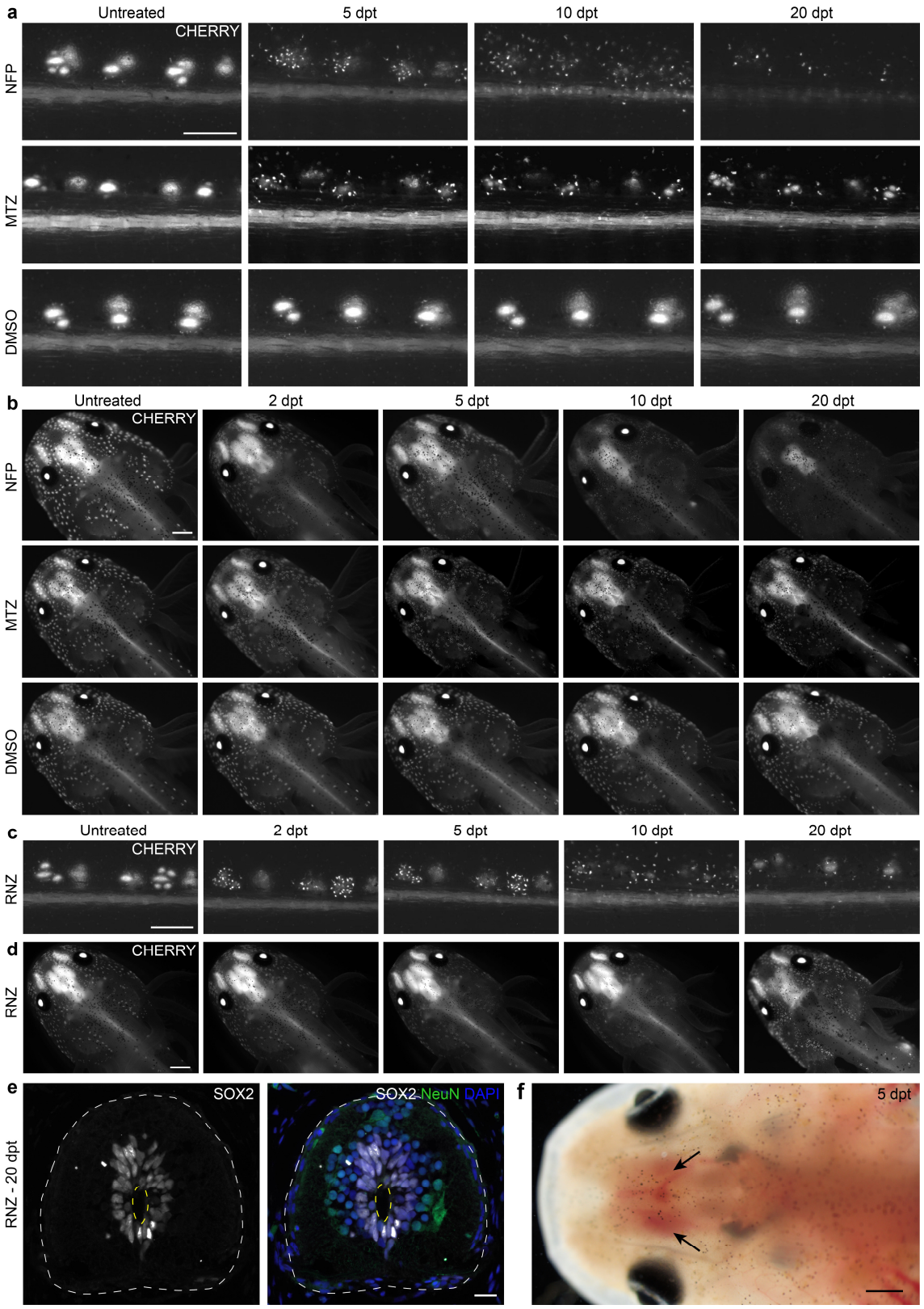
Supplementary Video 4 *NeuroD6:CherryNTR2.0* predatory behavior at 50-day post-DMSO treatment (uploaded separately)



Supplementary Fig. 1 Evaluation of nonspecific toxicity and working concentration of different prodrugs

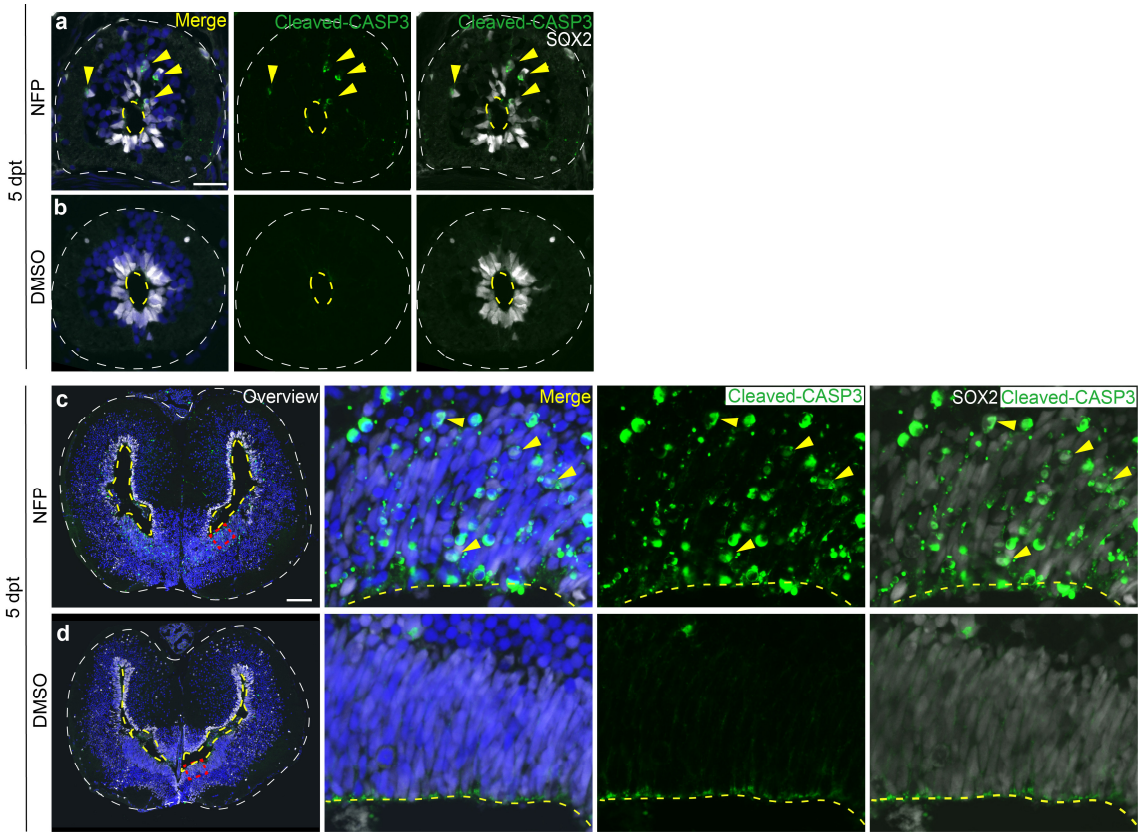
a-c Survival of *d/d* axolotl larvae following a 24-hour incubation with varying concentrations of MTZ (**a**, 1.25-40 mM), NFP (**b**, 1.25-40 µM) and RNZ (**c**, 0.25-8 mM) (n = 15 larvae per condition). Survival was monitored within 10 days post-treatment. **d-g** Representative time-series images of CHERRY^{+} neuromast ablation in *Sox2:Cherry-*

NTR axolotls treated with different concentrations of MTZ (**d**, 10, and 20 mM), NFP (**e**, 1, 2.5, 5, 10, and 20 μM), RNZ (**f**, 0.25, 1, and 4 mM), and DMSO as control (**g**, 0.2%) (n = 3 larvae per condition). Empty frames indicate tracked axolotls sacrificed at the timepoint. Abbreviation: dpt, days post-treatment. Scale bars: 500 μm in **d**, **e**, **f**, **g**.



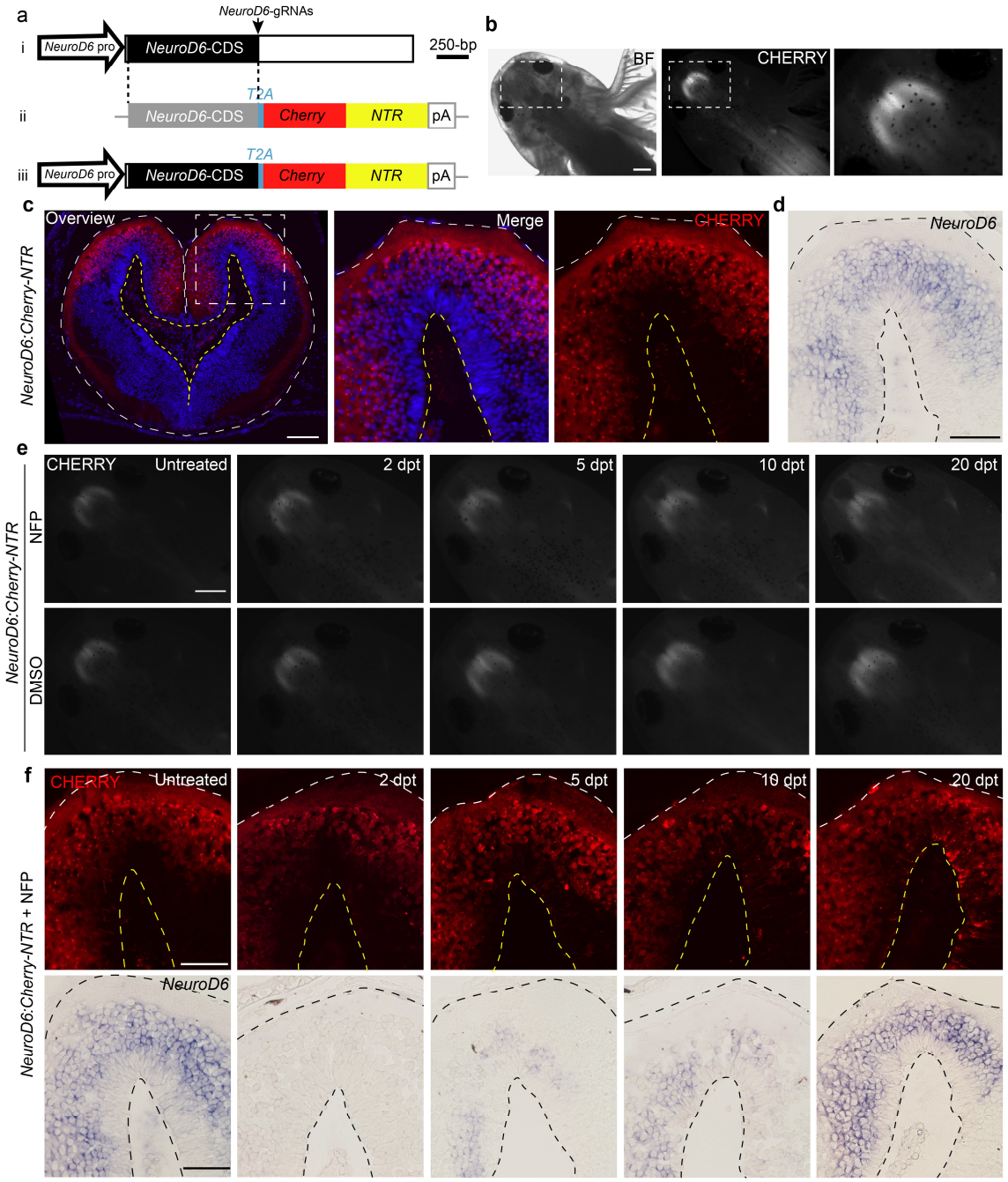
Supplementary Fig. 2 Evaluation of efficacy of prodrugs for targeted cell ablation in *Sox2:Cherry-NTR* axolotls

a, **b** Representative time-series CHERRY fluorescence images from tail (**a**) and head (**b**) of *Sox2:Cherry-NTR* axolotls treated with NFP, MTZ or DMSO. NFP-treated axolotls show fluorescence reduction in brain (**b**, top panel), spinal cord and neuromasts (**a**, top panel). MTZ-treated axolotls display scattered fluorescence in neuromasts (**a**, middle panel) while no significant change in brain (**b**, middle panel) and spinal cord. DMSO-treated axolotls have no fluorescence change (**a**, **b**, bottom panel). **c**, **d** Representative time-series CHERRY fluorescence images of tail (**c**) and head (**d**) images of *Sox2:Cherry-NTR* axolotls treated with RNZ are shown. RNZ-treated axolotls display scattered CHERRY fluorescence in neuromasts (**c**) while no significant change in spinal cord (**d**). **e** Representative immunofluorescence images of spinal cord sections from RNZ-treated *Sox2:Cherry-NTR* animals at 20 dpt, showing SOX2 (white), NEUN (green), combined with DAPI (blue). RNZ-treated animals retained $SOX2^{+}$ cells and maintained spinal cord architecture. The yellow dashed lines outline the lumen surface of spinal cord. The white dashed lines outline the shape of spinal cord. **f** RNZ-treated animals showed cerebral hemorrhage (adverse effect). The black arrows indicate the area of cerebral hemorrhage. Abbreviation: dpt, days post-treatment. Scale bars: 500 μm in **a**, **b**, **c**, **d**; 50 μm in **e**; 2 mm in **f**.



Supplementary Fig. 3 NTR/NFP ablates targeted cells through apoptotic pathway in axolotls

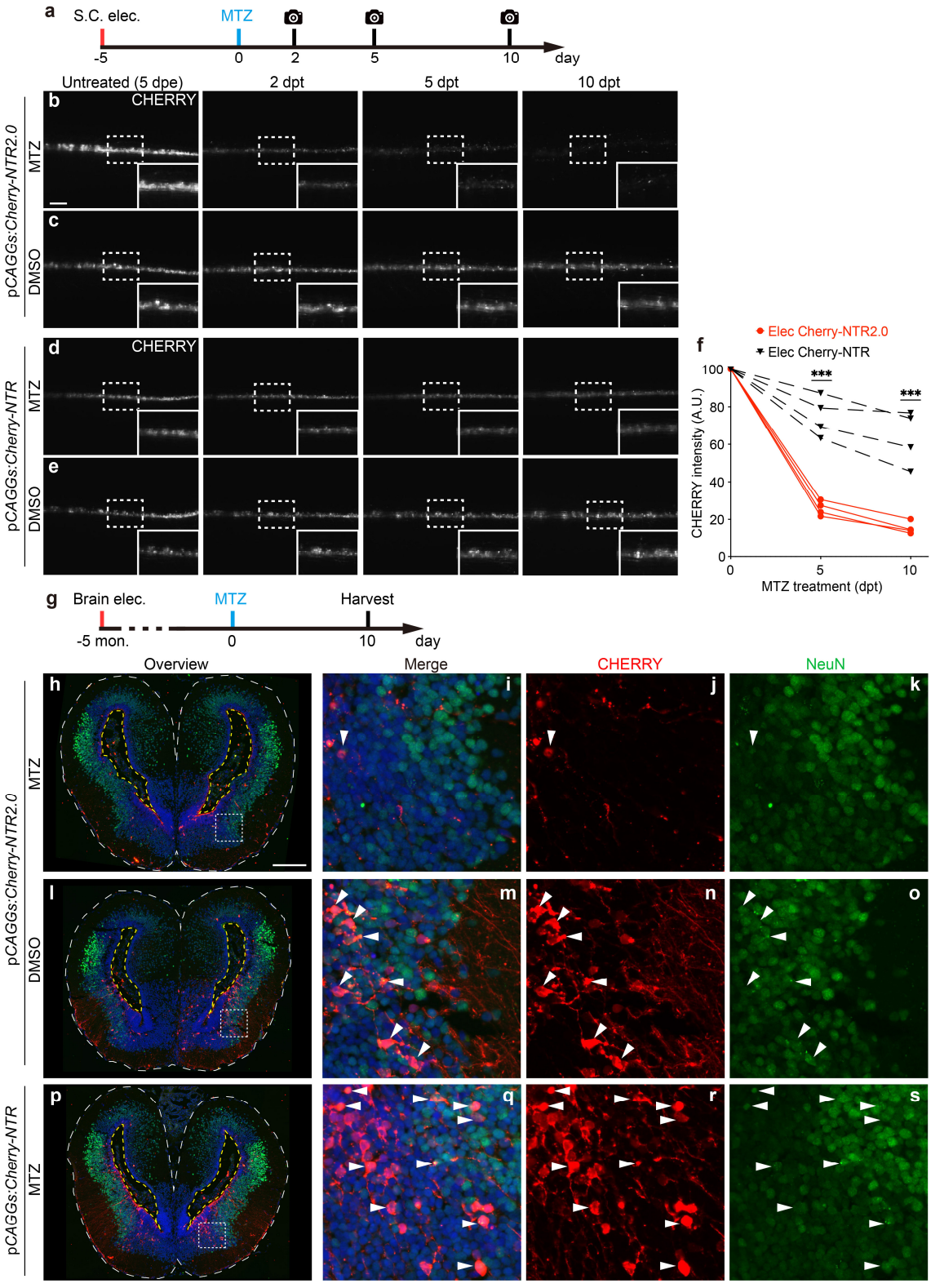
a, **b** Representative immunofluorescence images of spinal cord sections of *Sox2:Cherry-NTR* animals treated with NFP (**a**) or DMSO (**b**) at 5 dpt, showing SOX2 (white), cleaved Caspase-3 (Cleaved CASP3, green), combined with DAPI (blue). **c**, **d** Representative immunofluorescence images of brain sections of *Sox2:Cherry-NTR* animals treated with NFP (**c**) or DMSO (**d**) at 5 dpt, showing SOX2 (white), cleaved Caspase-3 (green), combined with DAPI (blue). The yellow arrowheads indicate the double-positive cells of cleaved Caspase-3 and SOX2. The red dashed boxes outline the higher magnification area of brain. The yellow dashed lines outline the lumen surface of spinal cord and brain. The white dashed lines outline the shape of spinal cord and brain. Abbreviation: dpt, days post-treatment. Scale bars: 50 μm in **a**, **b**; 200 μm in **c**, **d**.



Supplementary Fig. 4 Generation and characterization of *NeuroD6:CherryNTR* knock-in axolotls

a-d Generation and characterization of *NeuroD6:Cherry-NTR* knock-in axolotls. (**a**) Knock-in strategy for generating *NeuroD6:Cherry-NTR* axolotls. (i) Wild-type axolotl *NeuroD6* gene structures. Solid rectangles represent coding sequences of the exons; empty rectangles indicate untranslated regions. (ii) The targeting construct, p*GEMT-NeuroD6*-CDS-*T2A-Cherry-NTR*-pA, contains the *NeuroD6*-CDS (without stop codon) follow by *T2A*, the *Cherry-NTR* fusion sequence, and a polyadenylation signal (pA). (iii)

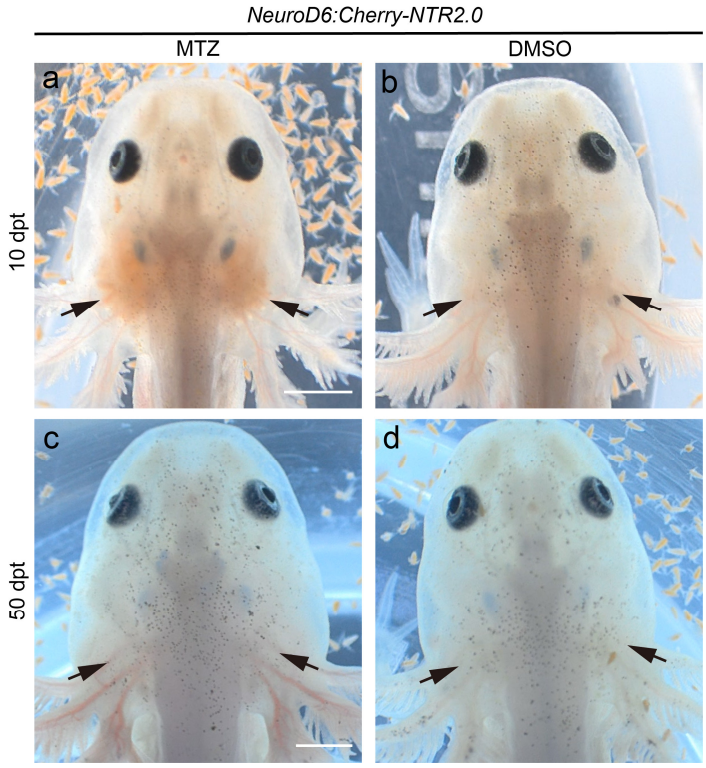
The *NeuroD6* alleles after knockin of the *T2A-Cherry-NTR* cassettes. (**b**) Bright field and CHERRY fluorescent images of *NeuroD6:Cherry-NTR* knock-in axolotls, showing CHERRY expression in the brain. (**c, d**) Representative immunofluorescence and ISH images show the consistency of CHERRY (**c**) and *NeuroD6* (**d**) expression on adjacent telencephalon sections. **e** Representative time-series live images of CHERRY fluorescence following NFP (upper panel) or DMSO (lower panel) treatment in *NeuroD6:Cherry-NTR*. CHERRY fluorescence exhibited no changes in both NFP group and DMSO group. **f** Representative time-series immunofluorescence and ISH images of *NeuroD6:Cherry-NTR* telencephalon, show persistent CHERRY fluorescence (red, upper panel) despite a transient *NeuroD6* mRNA decline at 2 days post-treatment. The white dashed box outlines the higher magnification area. The yellow or black dashed lines outline the lumen surface of brain. The white or black dashed lines outline the shape of brain. Abbreviations: dpt, days post-treatment; ISH, in situ hybridization. Scale bars: 1 mm in **b** and **e**; 200 μm in **c**;100 μm in **d** and **f**.



Supplementary Fig. 5 Comparison of cell-ablation efficacy between NTR2.0 and NTR following electroporation

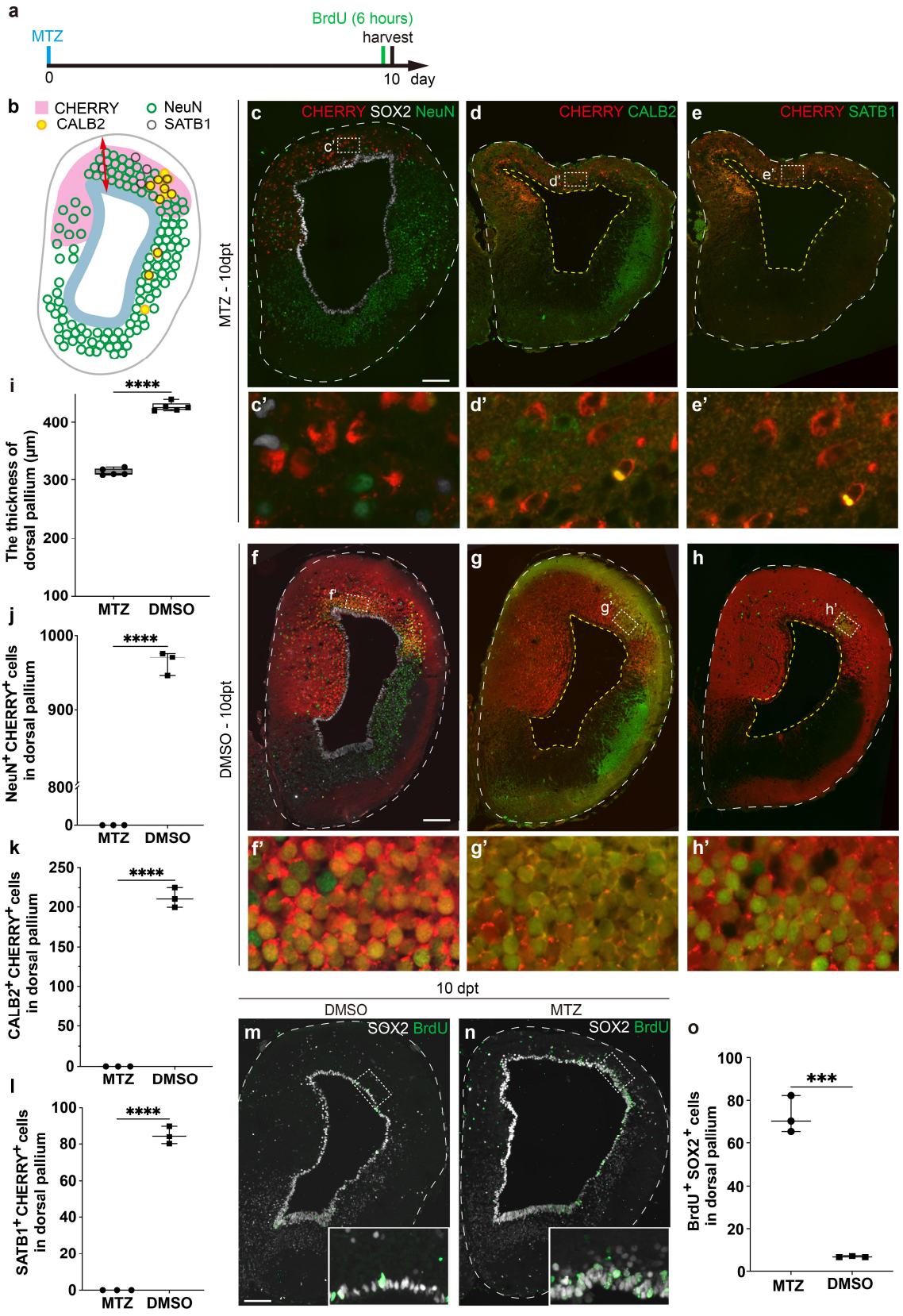
a The timeline of the experimental design including spinal cord electroporation, MTZ administration, and subsequent monitoring of CHERRY fluorescence. **b-e** Representative

time-series live CHERRY fluorescence images of the spinal cord electroporated with p*CAGGs:Cherry-NTR2.0* (**b** and **c**) or p*CAGGs:Cherry-NTR* (**d** and **e**), followed by treatment with 1 mM MTZ (**b** and **d**) or 0.2% DMSO (**c** and **e**). The white dashed boxes are shown at a higher magnification in the insets. **f** Quantification of CHERRY fluorescence intensity of electroporated axolotls with p*CAGGs:Cherry-NTR2.0* or p*CAGGs:Cherry-NTR* before and after treatment with MTZ (n = 4 each). The 0 dpt timepoint serves as the baseline (100%). Data were analyzed by unpair two-tailed Student's *t*-test and represented as mean ± SEM. ****$p<0.0001$. Source data are provided as a Source Data file. **g** The timeline of the experimental design including brain electroporation, MTZ administration, and subsequent monitoring of CHERRY fluorescence. **h-s** Representative immunofluorescence images of telencephalon electroporated with p*CAGGs:Cherry-NTR2.0* (**h**-**o**) or p*CAGGs:Cherry-NTR* (**p-s**), and followed by treatment with 5 mM MTZ or 0.2% DMSO, stained for CHERRY (red) and NeuN (green), combined with DAPI (blue). MTZ-treated telencephalon electroporated with p*CAGGs:Cherry-NTR2.0* show the elimination of (**h**, higher magnification in **i**-**k**) $CHERRY^+/NEUN^+$ neurons by 10 dpt compared to DMSO-treated telencephalon (**i**, higher magnification in **m**-**o**) and MTZ-treated telencephalon electroporated with p*CAGGs:Cherry-NTR* (**p**, higher magnification in **q**-**s**). The white arrowhead indicated the $CHERRY^+/NEUN^+$ neurons. The white dashed boxes outline the higher magnification area. The yellow dashed lines outline the lumen surface of brain. The white dashed lines outline the shape of brain. Abbreviations: dpt, days post-treatment; dpe, days post-electroporation; S.C., spinal cord; elec., electroporation. Scale bars: 500 μm in **b**-**e** and **h**, **l**, **p**.



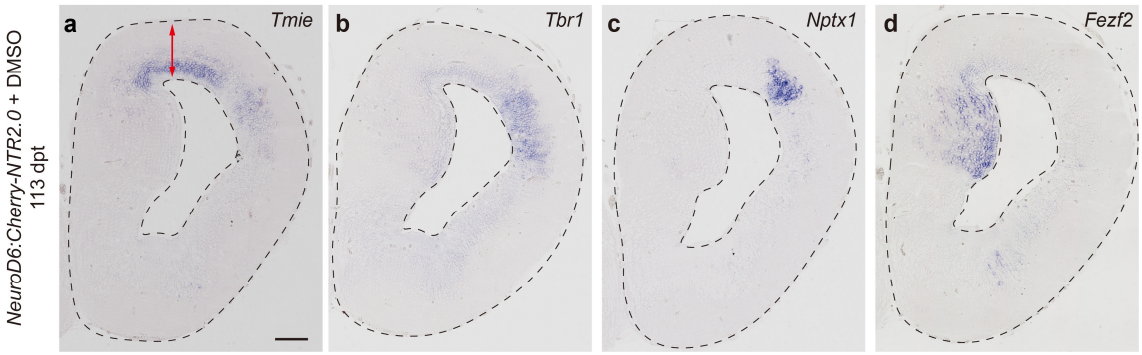
Supplementary Fig. 6 Pallial neuron ablation-induced swallowing dysfunction and recovery following neuronal regeneration in *NeuroD6:Cherry-NTR2.0* axolotls

a-d Captured images from videos of predatory behavior in *NeuroD6:Cherry-NTR2.0* axolotls treated with MTZ or DMSO, demonstrate swallowing impairment following cortical neuron ablation and recovery after neuronal regeneration. At 10 dpt, MTZ-treated axolotls can prey on artemia but retain them in the oral cavity (**a**), indicate impaired swallow function; while DMSO-treated control (**b**) exhibit normal ingestion. At 50 dpt, both MTZ-treated (**c**) and control (**d**) axolotls exhibit normal ingestion after prey on artemia, indicated MTZ-treated axolotls restored swallowing function after neuronal regeneration. The black arrows indicate the oral cavity. Abbreviation: dpt, days post-treatment. Scale bars: 2mm in **a-c**.



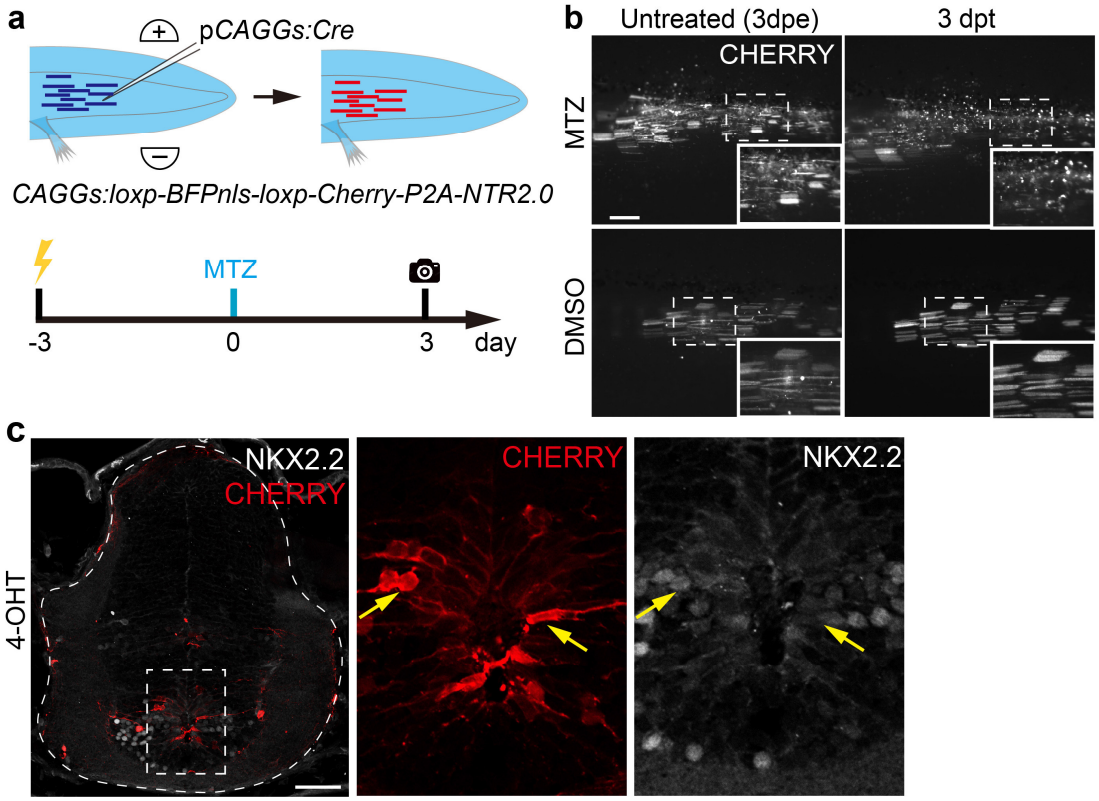
Supplementary Fig. 7 NTR2.0/MTZ system achieves efficient neuronal ablation in adult axolotl brain

a The timeline of the experimental design including MTZ administration, BrdU administration (6 h before harvest), and samples harvest for analysis. **b** Scheme of axolotl telencephalon depicting the spatial distribution of $CHERRY^+$ cells and neuronal subtypes (CALB2, SATB1, NeuN) in *NeuroD6:Cherry-NTR2.0* axolotls. The red double-headed arrow indicates the measurement location for dorsal pallium thickness. **c**-**h** Representative immunofluorescence images of axolotl telencephalon in *NeuroD6:Cherry-NTR2.0* axolotls with MTZ or DMSO treatment at 10 dpt. Compared to control animals (**f**, higher magnification in **f'**), MTZ-treated animals exhibited a marked reduction in $CHERRY^+$ cells (**c**, higher magnification in **c'**), while $SOX2^+$ EGCs (white, **c**) remain primarily distributed in the ventricular zone (VZ). In contrast to DMSO-treated controls (**f**, **f'**), MTZ treatment abolished co-expression of the pan-neuronal marker NeuN with $CHERRY^+$ cells (**c**, **c'**). Similarly, restricted-area neuron markers CALB2 (**d**, higher magnification in **d'**) and SATB1 (**e**, higher magnification in **e'**) showed loss of co-expression in MTZ-treated animals compared to controls (**g**, **h** and higher magnification **g'**, **h'**). The white dashed boxes are shown at a higher magnification. **i** Quantification of the dorsal pallium thickness in the MTZ and DMSO groups at 10 dpt (n = 5 each). **j**-**l** Quantification of $CHERRY^+$ $NeuN^+$ (**j**), $CHERRY^+$ $CALB2^+$ (**k**), $CHERRY^+$ $SATB1^+$(**l**) in the MTZ and DMSO groups at 10 dpt (n = 3 each). **m**, **n** Immunofluorescence staining of SOX2 (white) and BrdU (green) in the telencephalon of MTZ and DMSO treated *NeuroD6:Cherry-NTR2.0* axolotls at 10 dpt. BrdU was intraperitoneally injected 6 hours prior to sampling. The white dashed boxes are show in high magnification in insets. **o** Quantification of $SOX2^+$/$BrdU^+$ double-positive cells in the MTZ and DMSO treated groups at 10 dpt (n = 3 each). For quantification, five adjacent 16-μm-thick sections from equivalent positions of telencephalic regions were analyzed in MTZ or DMSO treated animals. Each dot represents an individual animal's mean value. Data were analyzed by unpair two-tailed Student's *t*-test and represented as mean ± SEM. *** $p < 0.001$, ****$p<0.0001$. Source data are provided as a Source Data file. The yellow dashed lines outline the lumen surface of brain. The white dashed lines outline the shape of brain. Abbreviation: dpt, days post-treatment. Scale bars: 200 μm.



Supplementary Fig. 8 Expression of pallium-located neuronal marker genes in *NeuroD6:Cherry-NTR2.0* axolotls at 113 days post-DMSO treatment

a-d Representative in situ hybridization images for telencephalon in *NeuroD6:Cherry-NTR2.0* at 113 days post-DMSO treatment, including pallium-located neuron subtype markers *Tmie* (**a**), *Tbr1* (**b**), *Nptx1* (**c**), *Fezf2* (**d**). The red double-headed arrow indicates the measurement location for dorsal pallium thickness. The black dashed lines outline the shape of brain. Scale bar: 200 μm.



Supplementary Fig. 9 Evaluation of the Cre-inducible NTR2.0 transgenic axolotl via Cre plasmid electroporation and inducible Cre transgenic animals

a Scheme and timeline of muscle electroporation followed by MTZ treatment. The p*CAGGs:Cre* plasmid was transfected to muscle in order to induce Cre-mediated recombination in *CAGGs:loxP-BFPnls-loxP-Cherry-P2A-NTR2.0* axolotls. **b** Live images demonstrated robust CHERRY expression in electroporated muscle at 3 days post-electroporation, followed by a significant dispersion in CHERRY signal after 3 days of 2mM MTZ treatment (upper panel), while 0.2% DMSO-treated remain unaffected (lower panel). The white dashed boxes are show in high magnification in insets. **c** Immunohistochemical analysis of midbrain sections from *CAGGs:loxP-BFPnls-loxP-Cherry-P2A-NTR2.0*/*Nkx2.2:memCherry-T2A-Cre-ER*^{T2} double transgenic axolotls treated with tamoxifen for eight days, staining with CHERRY (red) and NKX2.2 (white). Cytosolic CHERRY signals are observed in a subpopulation of NKX2.2^{+} cells, indicating successful conversion and expression of NTR2.0 in these cells. The yellow arrow indicates Cytosolic CHERRY^{+}/NKX2.2^{+} cells. The white dashed boxes outline the higher magnification area. The white dashed line outlines the shape of midbrain. Abbreviations: dpt, days post-treatment; dpe, days post-electroporation. Scale bars: 200 μm in **b**; 100 μm in **c**.

Supplementary Table 1:

The success rate of spinal cord transplantation surgery in axolotls.

Total Operated Animals	Successful Cases	Unsuccessful Cases	Success Rate
30	28	2	93.3% (28/30)

Note: The success of spinal cord transplantation was defined by normal morphology in the regenerated tail segment, in which presented the donor-derived CHERRY positive spinal cord.

Supplementary Table 2:

The success rate of brain transplantation surgery in axolotls.

Total Operated Animals	Successful Cases	Unsuccessful Cases	Success Rate (%)
36	6	30	16.6% (6/36)

Note: The success of telencephalon transplantation was defined by normal morphology in the integrated telencephalon, in which presented the donor-derived CHERRY positive telencephalon.

Supplementary Table 3:

Primers used in this study

Primer name	Sequence (5'-3')	Purpose
CAGGs_Cherry-Fw	GTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCA AGGAGTTCATGC	To amplify CherryNTR sequence, insert into CAGGs backbone.
BamHI_NTR_R	TCGACTGCAGAATTCggatccTTACACTTCGGTTAAG	
CAGGs_Cherry-Fw	GTGAGCAAGGGCGAGGAGGATAACATGGCCATCATCA AGGAGTTCATGC	To generate fused CherryNTR2.0 sequence, insert into CAGGs backbone.
fuse_NTR2.0_Cherry-Rev	ggcagcttgaacaatagtcatCTTGTACAGCTCGTCCATGCC	
fuse_CherryNTR2.0-Fw	GGCATGGACGAGCTGTACAAGatgactattgttcaagctgcc	
BamHI_NTR2.0-Rev	TCGACTGCAGAATTCggatccttagatttcggtaaaaacagtctctgctgacc	
CAGGs_Cre-Fw	AGCAAGGGCGAGGAGGATAACATGTCCAATTTACTGA CCGTACACCAA	To amplify Cre sequence, insert into CAGGs backbone.
pA_EcoRI_Cre-Rev	GCGATGACTCGACTGCAGAATTCggatccttaATCGCCATCT TCCAGCA	
loxp_XmaI_Cherry-Fw	TCTAAGCTTCTGCAGCCCGGGGCCGCCATGGTGAGCAA GGGCGAGGA	To amplify loxp-Cherry-P2A-NTR sequence, insert into CAGGs backbone.
PolyA_ClaI_NTR2.0-Rev	TGAGGAGTGAATTAATCGATttagatttcggtaaaaacagtctctg	
T2A_Cherry-Fw	GACGTGGAGGAGAATCCCGGCCCTATGGTGAGCAAGG GCGAGGAGGATA	To amplify CherryNTR sequence, insert into enzyme restricted pGEMT backbone.
pA_BamHI_NTR-Rev	TGACTCGACTGCAGAATTCGGATCCTTACACTTCGGTT AAGGTGATGTT	
T2A_Cherry-Fw	GACGTGGAGGAGAATCCCGGCCCTATGGTGAGCAAGG GCGAGGAGGATA	To amplify CherryNTR2.0 sequence, insert into enzyme restricted pGEMT backbone.
pA_BamHI_NTR2.0-Rev	CGACTGCAGAATTCGGATCCttagatttcggtaaaaacagtctctgctgac c	
MluI-NeuroD6 CDS-Fw	TAATACGACTCACTATAGACGCGTATGTTAACTCTTCC CTTTG	To amplify NeuroD6 full CDS, insert into enzyme restricted pGEMT backbone.
Sph1-NeuroD6 CDS-Rev	TTAGAAGACTTCCTCTGCCCTCGCATGCATTATGAAAA ACTGCATTTAAT	
Sox2_Fw	TGAACCAAGAGGGAACACGTACCTC	For genotyping Sox2 and NeuroD6 transgenic animals.
Neurod6_Fw	tgctgtccatcggttgtgttgatc	
Cherry (649-622)_Rev	CCACGATGGTGTAGTCCTCGTTGTGGG	
Cherry (192-213)_Fw	CATCCTGTCCCCTCAGTTCATG	
polyA_Rev	GCCCTCCCATATGTCCTTCCGAGTG	
Tbr1_antisense-Fw	GCGCACCCTTCTACCAGCTCTCC	To generate Tbr1 antisense ISH probe.
T7+Tbr1-Rev	TTGAAATTAATACGACTCACTATAGGGGTGGCATGTG GTACACTGCGATCCG	
Fezf2_antisense-Fw	CCGTGCAGAGCCTGGGCTACGAG	To generate Fezf2 antisense ISH probe.
T7+Fezf2-Rev	TTGAAATTAATACGACTCACTATAGGGTGTAGCCGGCG TGGATGCGGATG	
NeuroD6_antisense-Fw	GCGAGAGATTTGCGCAGAATTGTG	To generate NeuroD6 antisense ISH probe.
T7+Neurod6-Rev	TTGAAATTAATACGACTCACTATAGGGCGCCTGTTCAT TTGGCACAGC	
Tmie_antisense-Fw	GCAACAACAGAGACCCCTAAAAAGAAAC	To generate Tmie antisense ISH probe.
T7+Tmie-Rev	TTGAAATTAATACGACTCACTATAGGGCTAGGTGCCTT GGAGAGCCATCT	
Nptx1_antisense-Fw	CTCCAGAACCATGCTATGAAGTC	To generate Nptx1 antisense ISH probe.
T7+Nptx1-Rev	TTGAAATTAATACGACTCACTATAGGGTAAACTGAAC AATTCATGTCATTTGAGCG	