A Novel Molecular Method to Express Any Gene of Interest in Brain *in Vivo* with Temporal and Spatial Precision

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The brain, an intricate neuronal nexus, requires the optimum interaction of thousands of biological molecules, many of which have contrasting signalling mechanisms and outputs during ontological development. Specifically, γ-amino butyric acid (GABA) is the most common inhibitory neurotransmitter that hyperpolarizes neurons in mature brains[1]. However, GABA depolarizes and excites neurons in developing brains, and in doing so drives a number of developmental processes that allow proper neural circuit formation[2]. To facilitate the study of developmental molecular profiles, biologists need to express or disrupt gene products during adulthood without affecting their earlier roles during development. Inducible loss of function and gain of function mutations, reverse genetics approaches through which researchers selectively disturb genes with both temporal and spatial precision, enable observation of altered phenotypes. Juxtaposing these mutated phenotypes with their wildtype protein profiles thereby accelerates the deduction of not only gene function during one stage in development but also a simplified approach to understanding protein signalling. Consequently, it is essential to design *in vivo* molecular tools to express a molecule of interest with spatial and temporal precision.

We sought to create *in vivo* molecular tools to block $GABA_A$ transmission with temporal and spatial precision during development. Using *in utero* electroporation, we injected our genes into prenatal mice's cortical neurons. Afterwards, doxycycline, an antibiotic, was used to induce gene expression with the Tet-On system. We designed three plasmids to be electroporated and transcribed in neurons. The first plasmid coded for the protein reverse tetracycline transactivator (rtTA). In the presence of doxycycline, rtTA bound to tetracycline response element (TRE) in our second plasmid, which then initiated transcription of TRE's downstream gene product *ICL* and fluorescent reporter *GFP*[3]. ICL is a protein that blocks $GABA_A$ receptor function by competing with the γ2 subunit of $GABA_A$ receptors thereby preventing receptor anchoring to the

postsynaptic membrane[4]. Our third plasmid expressed fluorescent protein tdTomato, a positive control that allowed visualization of the success and extent of gene expression.

In utero electroporation involved opening the uterus of a pregnant mouse and injecting our DNA mixture into the lateral brain ventricles of P15-16 mouse embryos. Next, we delivered current pulses (42V, 950ms) to the fetuses' heads using electrodes to induce the cortical progenitor neurons to take up the DNA plasmids[5]. Following parturition, doxycycline was administered to the mother allowing the pups to acquire the antibiotic through the mother's milk. Finally, at postnatal day 8, we obtained and sectioned the pups' brains using a cryostat, immunohistochemically stained the sections for GFP and tdTomato to amplify the fluorescent signals, and used a confocal scanning microscope to acquire images of the neurons, in both tdTomato (568nm) and GFP (647nm) channels.

Our results support successful creation of a molecular tool that can temporally express genes in mice brains *in vivo*. We found that there was minimal GFP-ICL expression in the control condition lacking doxycycline induction, and almost all of the neurons expressed tdTomato, a positive control for electroporation (***Figure B***). Furthermore, the majority of neurons expressed both GFP-ICL and tdTomato (***Figure B***) for the doxycycline induction (+DOX) group, backing successful doxycycline induction of GFP-ICL. Additionally, counting the number of cells expressing GFP in –DOX and +DOX conditions showed that +DOX had significantly more GFP expressing cells (***Figure D***, p=0.006). Regarding GFP brightness, the +DOX group had more GFP expression than control conditions (***Figure E***, p=0.18). Moreover, there was a linear positive correlation between GFP and tdTomato expression (***Figure F***), indicating that tdTomato expression can be an accurate predictor of the extent of doxycycline-induced GFP expression.

The purpose of our experiment was to test that doxycycline was able to drive expression of our gene of interest (GOI). We confirmed that negligible amounts of GFP-ICL were expressed for the control condition and doxycycline induction of GOI was successful for the +DOX condition. Our combination of electroporation and plasmid induction techniques is a novel method to express any gene of interest in the brain with temporal precision. Moving forward we will use doxycycline dose-response curve to optimize the amount of induction for the cellular expression level and number of neurons expressing doxycycline. These molecular tools will dissect the role of GABA at developing and mature phases and assess if depolarizing $GABA_A$ transmission restricts glutamatergic synapse formation in developing cortical neurons *in vivo.* We hypothesize that expressing ICL to block $GABA_A$ transmission during development will lead to increased glutamatergic synapse formation, as our lab's previous pharmacological blocking of GABA transmission in *in vitro* organotypic slices showed increased number of dendritic spines. Intellectual disabilities such as Down syndrome have been associated with decreased number of spines[6] while Rett Syndrome is correlated with a diminished spine density[7]. Thus, by elucidating the roles of GABA transmission in synapse formation during development, we aim to open new avenues to restore glutamatergic synapse numbers to normal physiological levels in intellectual disabilities, by potentially increasing the number of spines in Down and Rett syndrome. Amazingly, the practical applications of this molecular tool transcend the scientific field ranging from cancer studies to embryonic development to protein signalling analysis.

Figure Legends

A: Schematic of doxycycline -inducible Tet-On system and electroporation to inject plasmids into prenatal mice. Figure adapted from Salmon[8].

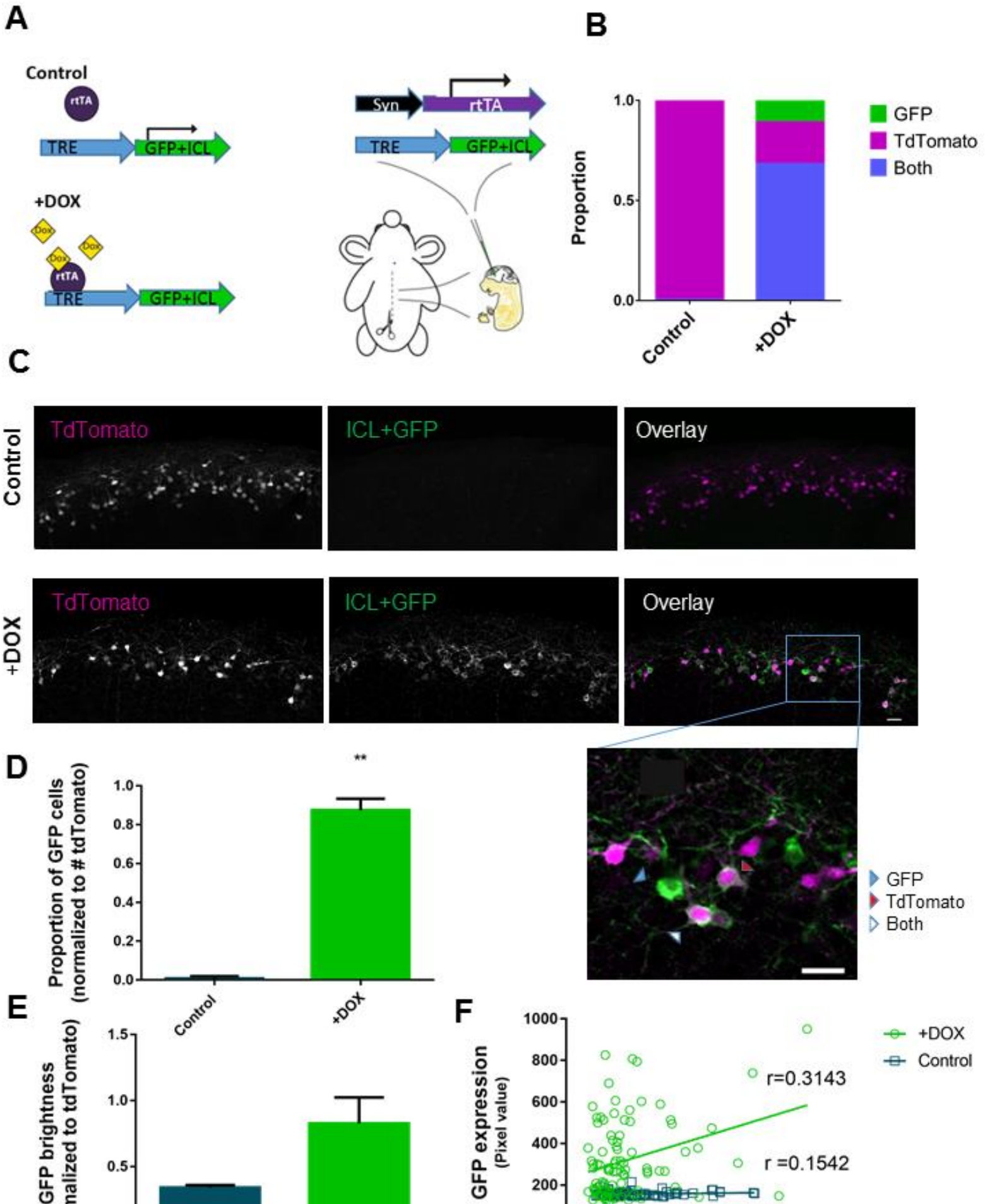
B: Percentage of cells expressing GFP, tdTomato or both relative to the total number of fluorescent cells. Experimental groups were control (-DOX, n=2) and ICL (+DOX, n=4).

C: Control group (-DOX) expresses very little ICL peptide and GFP fluorescent protein; almost all neurons expressing a fluorescent protein are tdTomato neurons. Doxycycline-induced expression (+DOX) of ICL peptide *in vivo;* almost all cortical neurons expressing ICL and GFP also express tdTomato. Scale bar for all figure images is 10µm.

D: Cell counts: There was significant difference (p=0.006) between number of cells that express GFP in the control (n=2, 0.00820±0.00819) and +DOX condition (n=4, 0.874±0.874).

E: GFP channel brightness taken from a region of interest in single cells. Control (-DOX, n=2, 0.3424± 00.01714) and ICL-induced groups (+DOX, n=4, 0.8254±0.1984).

F: Correlation of tdTomato and GFP expression in -DOX (red, n=95, Pearson's r= 0.1542) and +DOX (blue, n=113, r=0.3143).


A
Control
rtTA
TRE
GFP+ICL
+DOX
Dox
rtTA
TRE
GFP+ICL
Syn
rtTA
TRE
GFP+ICL
B
Proportion
1.0
0.5
0.0
Control
+DOX
GFP
TdTomato
Both
C
Control
TdTomato
ICL+GFP
Overlay
+DOX
TdTomato
ICL+GFP
Overlay
GFP
TdTomato
Both
D
Proportion of GFP cells (normalized to # tdTomato)
**
1.0
0.8
0.6
0.4
0.2
0.0
Control
+DOX
E
GFP brightness (normalized to tdTomato)
1.5
1.0
0.5
F
GFP expression (Pixel value)
1000
800
600
400
200
r=0.3143
r =0.1542
+DOX
Control

References

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