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**CHARACTERIZATION OF A NOVEL NEUROTRANSMITTER PHENOTYPIC
PLASTICITY IN THE CENTRAL NERVOUS SYSTEM**

A Thesis in

Biology

by

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ABSTRACT

Neurotransmitter phenotype for a given neuron is determined not only by intrinsic but also extrinsic factors during development. While intrinsic factors might be responsible for the majority of neurotransmitter phenotype, the role of extrinsic factors has been explored but the underlying molecular mechanism remains largely unknown. Here, we report an inducible plasticity of inhibitory neurotransmitter phenotype after alteration of postsynaptic receptors together with cell adhesion molecules. In embryonic hypothalamic cultures, inhibitory neurotransmission is mediated by GABAergic neurons only. Although no glycinergic currents can be detected in pure neuronal cultures, functional glycinergic synapses are inducible in cocultured HEK 293T cells expressing glycine receptors (GlyRs) and a cell adhesion molecule neuroligin-2 (NL-2). In fact, mixed GABAergic and glycinergic synapses are formed on HEK 293T cells that co-express NL-2, GlyRs and GABA_A-Rs. Importantly, hypothalamic neurons do express high level of GlyRs in addition to GABA_A-Rs. Ectopic expression of NL-2 or GlyRs alone in hypothalamic neurons cannot change the GABAergic transmitter phenotype. However, coexpression of NL-2 and GlyRs in hypothalamic neurons induces robust functional glycinergic synapses in hypothalamic cultures. Moreover, both α -homomeric and $\alpha\beta$ -heteromeric GlyRs can be clustered at the postsynaptic sites of induced synapses. Our data reveal that molecular manipulation of postsynaptic receptor organization, not receptor expression, is critical in altering the neurotransmission phenotype. Such inducible plasticity in central neurons also suggests that brain function is much flexible in adapting to extrinsic signals.

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INTRODUCTION

Inducible changes in the neurotransmission phenotype have been demonstrated during development, under pathological conditions and as a response to alterations in the activity (Spitzer et al., 2004; Trudeau and Gutierrez, 2007). Not only intrinsic, but also extrinsic factors have been implicated in the determination of the neurotransmitter phenotype. In developing neurons of *Xenopus laevis* spinal cord, excitatory and inhibitory neurotransmission phenotypes are homeostatically regulated by distinct patterns of spontaneous Ca^{2+} spike activity. In this model, suppression of activity leads to an enhanced expression of excitatory neurotransmitter markers while reducing inhibitory markers, and the reverse occurs when activity is increased (Borodinsky et al., 2004). The inducible changes in the neurotransmitter phenotype is then followed by the expression of appropriate receptors on the apposing postsynaptic sites (Borodinsky and Spitzer, 2007). The glutamatergic granule cells of adult rat hippocampus also display neurotransmitter plasticity after epileptic activity (Gutierrez et al., 2003). GABAergic phenotype is shown to reappear, possibly to maintain the balance between excitatory and inhibitory neurotransmission (Gutierrez et al., 2003; Gomez-Lira et al., 2005). A developmental switch has been recently demonstrated in rat lateral superior olive neurons from predominantly GABAergic to predominantly glycinergic phenotype over the first two postnatal weeks, in which presynaptic neurotransmitter content governs the postsynaptic receptor expression (Nabekura et al., 2004). While intrinsic factors might be largely responsible for the neurotransmitter phenotype,

the role of extrinsic factors has been investigated in recent studies, the underlying molecular mechanism is still poorly understood.

Glycine and γ -aminobutyric acid (GABA) are the main fast inhibitory neurotransmitters in the mammalian central nervous system (CNS). They activate two distinct types of ligand-gated chloride channels, glycine receptors (GlyRs) (Legendre, 2001), and GABA_A receptors (GABA_ARs) (Luscher and Keller, 2004), respectively. Most, if not all, inhibitory synapses in the forebrain are thought to be GABAergic, while both GABA and glycine mediate the inhibitory neurotransmission in the spinal cord (Takahashi and Momiyama, 1991; Oleskevich et al., 1999; Gao et al., 2001), brain stem (Stevens et al., 1996; Muller et al., 2006), cerebellum (Dieudonne, 1995), and retina (Han et al., 1997; Protti et al., 1997). Some inhibitory interneurons in spinal cord, brain stem and cerebellum can even co-release GABA and glycine from a single presynaptic terminal resulting in mixed glycinergic / GABAergic synaptic events (Jonas et al., 1998; O'Brien and Berger, 1999; Nabekura et al., 2004; Dugue et al., 2005). Networks of interneurons that use GABA and/or glycine as their neurotransmitter have critical functions in regulating the sensory and motor signals that control movement, vision, and audition as well as inflammatory pain sensitization (Lynch, 2004; Betz and Laube, 2006). For efficient modification of excitation by inhibition, GABA_ARs and GlyRs act separately or together to allow rapid and synapse specific adaptation in the network.

The GlyRs are members of the pentameric superfamily of ligand-gated ion channels. To date, four ligand binding α subunits (α 1- α 4) and one structural β

subunit have been described (Lynch, 2004; Betz and Laube, 2006). The $\alpha 2$ subunit has been identified as an embryonic form of GlyRs in the spinal cord and brain stem where it forms homopentamers that are mainly found extrasynaptically (Becker et al., 1992; Takahashi, 2005). Expression of GlyR $\alpha 2$ subunit decreases during early postnatal maturation, whereas expression of $\alpha 1$ and $\alpha 3$ subunits increases (Sato et al., 1992; Takahashi et al., 1992). However, $\alpha 2$ transcripts and functional GlyRs are also found in adult hippocampus, cerebral cortex and thalamus (Malosio et al., 1991; Flint et al., 1998; Ghavanini et al., 2005; Zhang et al., 2008). GlyRs in the adult brain are mainly composed of $\alpha 1$ and β subunits that are localized at synaptic sites, and have a proposed stoichiometry of $2\alpha:3\beta$ (Meyer et al., 1995; Grudzinska et al., 2005). The GlyR $\alpha 1$ mRNA is expressed in adult spinal cord, brain stem, and cerebellum but also in hypothalamus, thalamus, and the colliculi (Malosio et al., 1991). The β subunit is widely expressed both in adult and embryonic brain, although it cannot form functional receptors alone (Fujita et al., 1991; Malosio et al., 1991).

Although glycinergic synapses are thought to be excluded from the higher brain regions, accumulating evidence suggests that functional GlyRs, as well as glycine containing cell bodies and fibers are widely distributed in the CNS (Malosio et al., 1991; Rampon et al., 1996). Moreover, similar concentrations of GABA and glycine are found in the synaptosomes isolated from brain stem, cerebellum, cortex, and hippocampus (Engblom et al., 1996). Despite the presence of both pre- and postsynaptic machinery necessary for glycinergic inhibitory synaptic transmission in cortical and subcortical areas, application of

GABA_AR antagonists completely blocks all fast synaptic inhibition (Randle et al., 1986; Wuarin and Dudek, 1993; Mody et al., 1994; Mori et al., 2002). It is suggested that the non-synaptic GlyRs are involved in cortical development (Flint et al., 1998), suppressing hyperexcitability in hippocampus (Chattipakorn and McMahon, 2003; Zhang et al., 2008), and osmoregulation in hypothalamus (Hussy et al., 2001). Although these studies provide important non-synaptic functions, the function of the glycinergic synapse components in forebrain inhibitory neurotransmission remains largely unknown.

Here, we provide evidence for a novel inducible plasticity in cultured hypothalamic neurons and demonstrate a phenotypic switch from purely GABAergic transmission to one mediated by both GABA and glycine. We show that co-expressing GlyR subunits with neuroligin-2 (NL-2), a postsynaptic cell adhesion molecule, induce glycinergic synapse formation in HEK 293T cells when cocultured with embryonic hypothalamic neurons. Moreover, mixed GABAergic and glycinergic synapses are formed on HEK cells that co-express NL-2, GlyRs and GABA_A-Rs. In pure hypothalamic cultures, glycinergic synapses are induced upon co-expression of GlyRs and NL-2 but not GlyRs or NL-2 alone. Functional analysis of the glycinergic synapses indicates that both α -homomeric and $\alpha\beta$ -heteromeric GlyRs can be clustered at the postsynaptic sites. The data we present here suggests that the postsynaptic expression of functional receptors and cell adhesion molecules play a crucial role in determining neurotransmitter phenotype and provides a novel mechanism for manipulating synaptic activity.

EXPERIMENTAL METHODS

1. Cell Culture and transfection

Hypothalamic cultures were prepared from E18 Sprague – Dawley rat embryos as described previously (Protti et al., 1997). In short, the medial hypothalamus was dissected, cut into small cubes, and digested in 0.05% trypsin/EDTA solution (pH 7.2) and 25 U/ml DNaseI for 30 min at 37⁰C. After enzyme treatment, tissue blocks were triturated gently by using a fire-polished Pasteur pipette and the dissociated neurons were then plated on poly-D-lysine coated coverslips (0.1 mM, 4000-8000 cells/cm²) covered by a monolayer of astrocytes. The culture medium contained 500 ml of MEM (Invitrogen, Eugene, OR), 5% FBS (HyClone, Logan, UT), 10 ml of B-27 supplement (Invitrogen), 100 mg NaHCO₃, 20 mM D-glucose, 0.5 mM L-glutamine, and 25 U/ml penicillin/streptomycin.

Neurons were transfected with a modified Ca²⁺-phosphate transfection protocol developed in our lab (Jiang and Chen, 2006). DNA/Ca²⁺-phosphate precipitate was prepared by using the Clontech CalPhosTM Mammalian Transfection Kit (BD Bioscience, Palo Alto, CA). Cells were incubated in the presence of the precipitate for 30 min in 5% CO₂ culture incubator. After incubation, the precipitate was removed by washing coverslips with transfection medium pre-equilibrated in 10% CO₂. Neurons were utilized after 24-48 hrs of transfection.

Human Embryonic kidney (HEK) 293T cells were maintained in DMEM supplemented with 10% FBS, and 25 unit/ml penicillin/streptomycin. HEK 293T cells were transfected with the same method described above and cocultured with hypothalamic neurons as described previously (Dong et al., 2007). In general, 24 hours after transfection, cells were mechanically dissociated by fire-polished Pasteur pipette and plated on top of 5-7 DIV hypothalamic cultures. The cocultures were utilized in electrophysiological recordings after 2-3 days of coculture. Each experiment was repeated in at least two different batches of cultures.

2. Plasmid Constructs

The glycine receptor subunits ^{myc} α 1 and ^{myc} α 2 plasmids were gifts of Dr. A. Triller (Laboratoire de Biologie Cellulaire de la Synapse, France) (Meier et al., 2000). The rat α 1 and α 2 subunits were modified by insertion of the myc epitope by site-directed mutagenesis. Myc epitope-tagged forms of the α 1 and α 2 were cloned into pEGFP-N1 eukaryotic expression vector with a CMV promoter. The Glycine β subunit was kindly provided by Dr. X. Zhang (Institute of Neurosciences, Chinese Academy of Sciences, China) (Zhang et al., 2008). The human β subunit was cloned into pBK-CMV vector at EcoRI site. The GABA_A receptor subunits α 2, β 3, myc- γ 2 were gifts from Dr. B. Luscher (Pennsylvania State University) (Aldred et al., 2005). The rat α 2 and β 3 subunit cDNAs were cloned in pBCBam backbone. The mouse myc 9E10 epitope-tagged γ 2 cDNA was cloned in pEGFP-N, substituting the ^{myc} γ 2 cDNA from EGFP. The myc epitope was inserted between amino acids

four and five of the mature $\gamma 2$ polypeptide. The murine HA-tagged NL-2A expression vectors (pNiceNLG-2) were obtained from Dr. P. Scheiffele (Columbia University) (Chih et al., 2005).

3. Electrophysiology

Whole-cell recordings were performed in voltage clamp mode by using Multiclamp 700A amplifier (Molecular Devices) (Deng and Chen, 2003). Patch pipettes were pulled from borosilicate glass and fire polished to resistance of 3-7 M Ω . The recording chamber was continuously perfused with a bath solution containing 128 mM NaCl, 30 mM Glucose, 25 mM HEPES, 5 mM KCl, 2 mM CaCl₂, and 1 mM MgCl₂ (pH 7.3, adjusted with NaOH, ~320 Osm). The pipette solution contained 135 mM KCl, 10 mM HEPES, 2 mM EGTA, 10 mM Tris-phosphocreatine, 4 mM MgATP, 0.5 mM Na₂GTP (pH 7.3, adjusted with KOH, ~300 Osm). Data were acquired by using the pCLAMP 9 software (Molecular Devices), sampled at 2-10 kHz and filtered at 1-2 kHz, and analyzed with Clampfit 9.0 (Axon Instruments). Pressure application of agonists was made by utilizing Picospritzer III (Parker Instruments). Miniature and spontaneous events were analyzed by MiniAnalysis software (Syntosoft). Only single spontaneous events were counted into analysis. Analyzed data were expressed as mean value \pm standard error of the mean, and the student's *t* test was used for statistical analysis. N represents number of the cells recorded.

4. Drugs

GABA, glycine, strychnine hydrochloride (STR), tetrodotoxin (TTX), picrotoxin (PTX) and flunitrazepam were purchased from Sigma (St. Louis, MO). Bicuculline

(BIC) and 6-cyano-7-nitroquinoxaline-2,3 (1H,4H)-dione (CNQX) were obtained from Tocris (Ellisville, MO). CNQX, PTX, and flunitrazepam were initially dissolved as concentrated stock solutions in dimethyl sulfoxide (DMSO) and diluted to appropriate concentrations in the bath solution. The final DMSO concentration was lower than 0.1%. Other drugs were first dissolved in ion-free water and freshly diluted to the final concentrations in bath solution before experiments.

RESULTS

1. Cultured hypothalamic neurons do not contain glycinergic synapses but they express functional glycine receptors

To characterize the inhibitory synaptic transmission in cultured hypothalamic neurons, whole cell patch-clamp recordings were performed in the presence of glutamate receptor antagonist CNQX (10 μ M). Spontaneous inhibitory postsynaptic currents (sIPSCs) occurred at a rate of 2.01 ± 0.12 Hz, and the average amplitude was 51.4 ± 7.0 pA ($n = 13$). Consistent with the previous reports (Dong et al., 2007), inhibitory synaptic currents were completely abolished by GABA_A receptor antagonist bicuculline (BIC, 20 μ M) in all 13 neurons recorded (Fig. 1A), suggesting that there are no glycinergic synapses in cultured hypothalamic neurons. However, bath application of glycine (100 μ M) induced a significant whole cell current, and the current density (55.2 ± 4.2 pA/pF, $n=15$) was similar to that induced by GABA (100 μ M) (64.4 ± 3.7 pA/pF, $n=15$; $p>0.1$), indicating that similar amounts of functional GlyRs and GABA_A-Rs are expressed on the neuronal membrane (Figs. 1B and C). Additionally, glycine-evoked whole-cell currents were blocked by strychnine (STR, 1 μ M), a specific glycine receptor antagonist, suggesting that these currents are indeed mediated by glycine receptors. To examine the subunit composition of the GlyRs in the cultured neurons, we utilized the pharmacological advantage conferred by picrotoxin, which blocks α subunit-containing homomeric GlyRs more effectively than heteromeric $\alpha\beta$ receptors (Pribilla et al., 1992; Yoon et al., 1993).

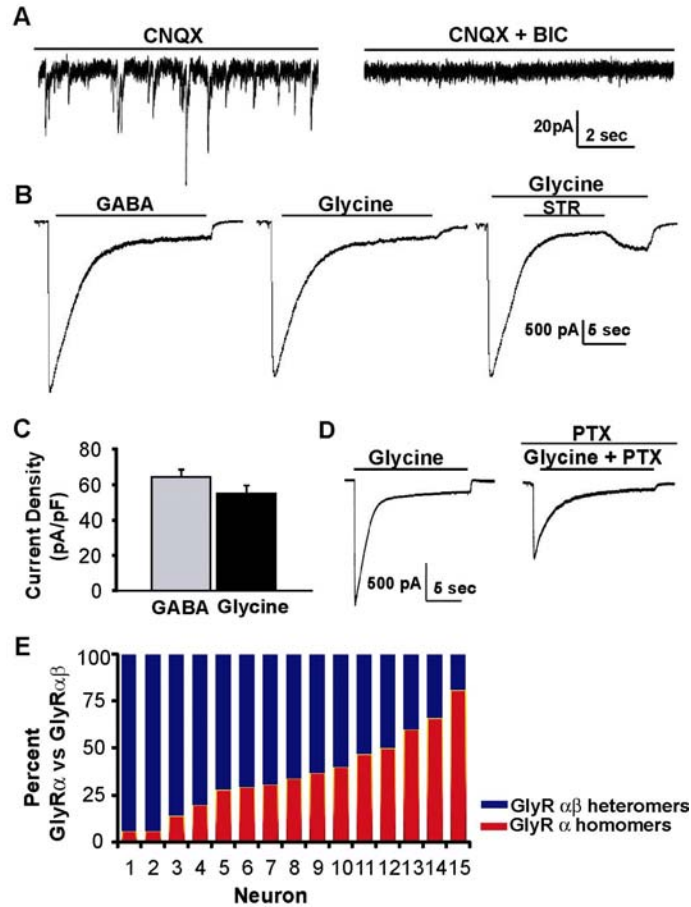


Figure 1. Cultured hypothalamic neurons do not contain glycinergic synapses but they express functional GlyR homomers and heteromers. (A) A typical recording trace showing sIPSCs in the presence of glutamate receptor antagonist CNQX (10 μ M) that were completely abolished by bath application of BIC (20 μ M) and CNQX (n=13). Traces recorded at -70 mV holding potential. **(B)** Sample traces showing currents induced by application of GABA (100 μ M, left trace), and glycine (100 μ M, middle trace). The glycine current was blocked by STR (1 mM), the glycine receptor antagonist (right trace) **(C)** The GABA-induced whole-cell current density in cultured hypothalamic neurons was comparable to glycine-induced whole-cell current density (n=15; $p > 0.1$, Student's *t* test). **(D)** Traces recorded from a cell showing currents induced by glycine (100 μ M), in the absence (left trace) or presence (right trace) of PTX (100 μ M). **(E)** Bar graph showing the percentage of PTX inhibition in 15 of the neurons recorded. Red bars show the percentage of the current inhibited by PTX, representing the proportion of GlyR α homomers. The blue bars represent the percentage of currents that escaped inhibition, indicating the proportion of GlyR $\alpha\beta$ heteromers.

Bath application of glycine (100 μ M) together with 100 μ M picrotoxin (PTX), a concentration commonly used to identify glycine receptor subunit composition (Mori et al., 2002; Zhang et al., 2008), incompletely blocked the glycine-induced currents in 15 neurons tested (Fig. 1D). Furthermore, PTX inhibition varied among neurons from as small as 6.3% inhibition of the glycine-induced current (neuron 1 in Fig. 1E) to 81.7 % inhibition (neuron 15 in Fig. 1E). This data indicates differential expression levels of both homomeric α subunit-containing receptors and heteromeric $\alpha\beta$ containing glycine receptors in cultured hypothalamic neurons.

2. Glycine receptor subunits and Neuroligin-2 induce glycinergic synapse formation on HEK cells when co-cultured with hypothalamic neurons.

Consistent with the previous studies, cultured hypothalamic neurons in our cultures contain significant amounts of glycine receptors, but they do not form or maintain glycinergic synapses. We therefore sought to determine the molecules involved in glycinergic synapse formation. One candidate molecule is neuroligin-2 (NL-2) because it is so far the only synaptic cell adhesion molecule that specifically localizes to inhibitory synapses (Graf et al., 2004; Chih et al., 2005; Varoqueaux et al., 2006), while the other members of the neuroligin family, NL-1 and NL3 are mostly found at excitatory synapses (Varoqueaux et al., 2006). Particularly, trans-synaptic interactions between postsynaptic NL-2 and presynaptic β -neurexin have been suggested to promote the recruitment of pre- and postsynaptic molecules to form functional GABAergic synapses (Graf et al.,

2004; Levinson et al., 2005; Levinson and El-Husseini, 2005). In a recent study, our lab have demonstrated that functional GABAergic synapses can be reconstituted by co-expression of NL-2 with GABA_A-R in HEK 293T cells that are cocultured with neurons (Dong et al., 2007). We utilized a similar *in vitro* heterococulture assay to explore glycinergic synapse formation. We co-transfected HEK 293T cells with NL-2 and GlyR subunits α 1, α 2 or α 1 β , all of which are shown to form functional receptors that concentrate at glycinergic synapses (Bechade et al., 1996; Handford et al., 1996), and then cocultured these cells with hypothalamic neurons. To ensure the formation of $\alpha\beta$ heteromers instead of α 1 homomers, α 1 and β subunits were transfected at a ratio of 1:4 (Pribilla et al., 1992; Zhang et al., 2008). GFP was also co-transfected to identify the transfected cells. After 2–3 days of coculture, synaptic responses were monitored by whole-cell patch-clamp recordings on HEK 293 T cells. Surprisingly, co-expressing NL-2 with glycine receptor subunits induced robust spontaneous synaptic-like currents in transfected HEK 293T cells (Figs. 2A-C). These spontaneous events were completely blocked by GlyR antagonist STR (0.5 μ M), but were less sensitive or completely insensitive to GABA_A-R antagonist BIC (20 μ M), indicating that they are glycinergic inhibitory postsynaptic currents (IPSC_{Gly}). All cells co-expressing NL-2 with either GlyR α 1 alone (n=15) or together with GlyR α 1 β (n=10) displayed sIPSC_{Gly}, whereas 7 out of 15 cells expressing GlyR α 2 exhibited sIPSC_{Gly}. Peak-scaled IPSCs shows that the synaptic currents

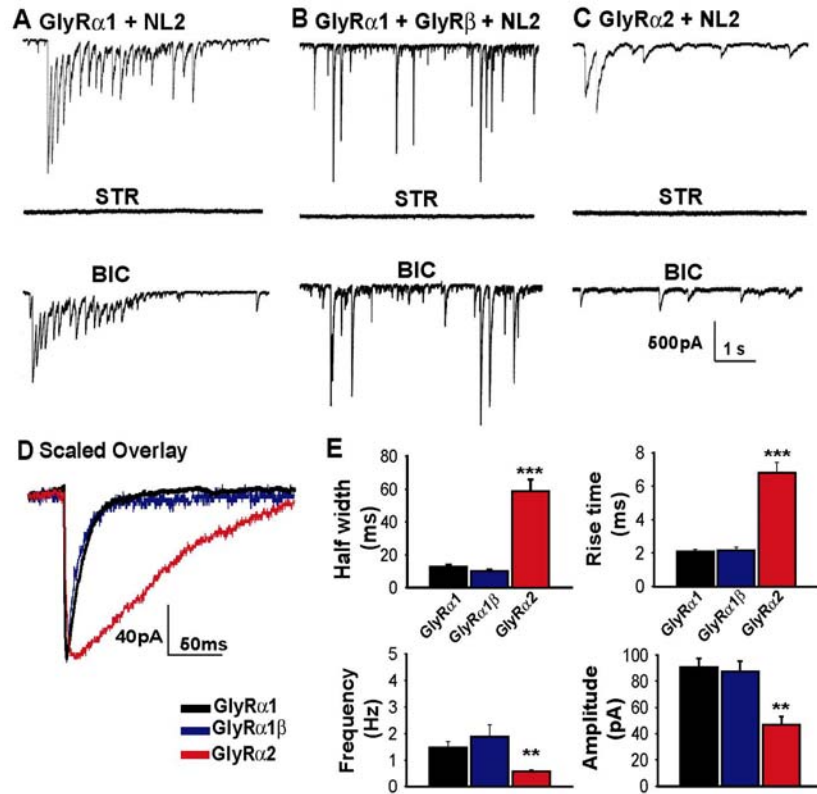


Figure 2. Glycine receptor subunits and Neuroigin-2 induce glycinergic synapse formation on HEK cells when co-cultured with hypothalamic neurons. Representative traces showing sIPSCs detected in HEK 293T cells co-transfected with NL-2 and (A) GlyR α 1, (B) GlyR α 1 and GlyR β , and (C) GlyR α 2. All cells co-expressing NL2 with GlyR α 1 (n = 15), or GlyR α 1 and GlyR β (n = 10) showed synaptic currents, while 7 in 15 cells co-expressing GlyR α 2 with NL-2 showed spontaneous synaptic currents. sIPSCs were completely blocked by STR (0.5 μ M, middle traces), but not abolished by BIC (20 μ M, bottom traces). Scale bar applies to A, B, and C. (D) Three types of averaged sIPSCs were scaled to the same amplitude and superimposed (α 1-IPSC_{Gly}, black, n = 54; α 1 β -IPSC_{Gly}, blue, n = 68; α 2-IPSC_{Gly}, red, n = 35). (E) Comparison of mean half width (ms), rise (ms), frequency (Hz), and amplitude (pA) of the three types of IPSC_{Gly} (mean \pm s.e.m, n_{GlyR α 1} = 15, n_{GlyR α 1 β} = 10, n_{GlyR α 2} = 7). Note that all four paradigms for α 2-IPSC_{Gly} were significantly different than those for α 1-IPSC_{Gly}, and α 1 β -IPSC_{Gly}.

mediated by $\alpha 1$ homomers and $\alpha 1\beta$ heteromers displayed faster decay and rising phases than that by $\alpha 2$ homomers (Fig. 2D). This was consistent with the previously reported receptor kinetics of glycine receptors in neurons (Takahashi et al., 1992; Singer et al., 1998; Singer and Berger, 1999; Legendre, 2001). In our preparations, the majority of the glycinergic events displayed monoexponential decay kinetics, but a subpopulation decayed with a bi-exponential phase. We therefore utilized the half width (ms) for kinetic characterization of the receptors expressed at the synapses. Specifically, $\alpha 1$ -IPSC_{Gly} decayed with a mean half width of 12.9 ± 1.2 ms (n=15) and $\alpha 1\beta$ -IPSC_{Gly} decayed with a half width of 10.2 ± 0.9 ms (n = 10), whereas the mean half width of $\alpha 2$ -IPSC_{Gly} was 59.9 ± 7.0 ms (n = 7) (Fig. 2E). The half width of the $\alpha 1$ -IPSC_{Gly} was not significantly different from that of $\alpha 1\beta$ -IPSC_{Gly} ($p > 0.4$; Student's *t* test), however the half width of $\alpha 2$ -IPSC_{Gly} was significantly slower than both the $\alpha 1$ -IPSC_{Gly} and $\alpha 1\beta$ -IPSC_{Gly} ($p < 0.001$). The mean rise time (10-90%) $\alpha 1$ -IPSC_{Gly} (2.09 ± 0.15 ms, n = 15) was similar to the rise time of $\alpha 1\beta$ -IPSC_{Gly} (2.15 ± 0.17 ms, n = 10; $p > 0.7$), but the $\alpha 2$ -IPSC_{Gly} (6.80 ± 0.63 ms, n = 7) rose significantly slower than both the $\alpha 1$ -IPSC_{Gly} and $\alpha 1\beta$ -IPSC_{Gly} ($p < 0.001$). GlyR $\alpha 1$ mediated events occurred at a rate of 1.47 ± 0.2 Hz and the mean current amplitude was 90 ± 6 pA, which was similar to the frequency and amplitude of sIPSCs in HEK 293T cells coexpressing GlyR $\alpha 1\beta$ and NL-2 (2.08 ± 0.44 Hz and 87 ± 7 pA respectively; $p > 0.5$). However, the mean frequency and amplitude of $\alpha 2$ -IPSC_{Gly} (0.55 ± 0.2 Hz and 47 ± 18 pA) were significantly smaller than those of $\alpha 1$ -IPSC_{Gly} and $\alpha 1\beta$ -IPSC_{Gly} ($p < 0.02$) (Fig. 2.E), which might suggest that NL-2 is more potent in clustering GlyR $\alpha 1$ homomers

and $\alpha 1\beta$ heteromers in postsynaptic terminals than GlyR $\alpha 2$ homomers. Moreover, short pulses (5 ms) of glycine (50 μ M) were pressure-ejected to the cells in the presence of BIC (40 μ M), generating large currents in all five of the HEK 293 T cells co-transfected with GlyR $\alpha 1$ and NL-2 (2256 ± 731 pA) (Fig 3.A, C). However, 5 ms pressure application of GABA (1 mM), in the presence of BIC (40 μ M) induced no current in all 4 cells tested and when the local GABA application was prolonged to 100 ms, only one cell displayed a whole cell current of 98.8 pA (Fig 3.B). This indicates that functional glycine receptors are expressed on the surface of the HEK cell membranes, and are specifically activated by glycine rather than GABA released from presynaptic nerve terminals.

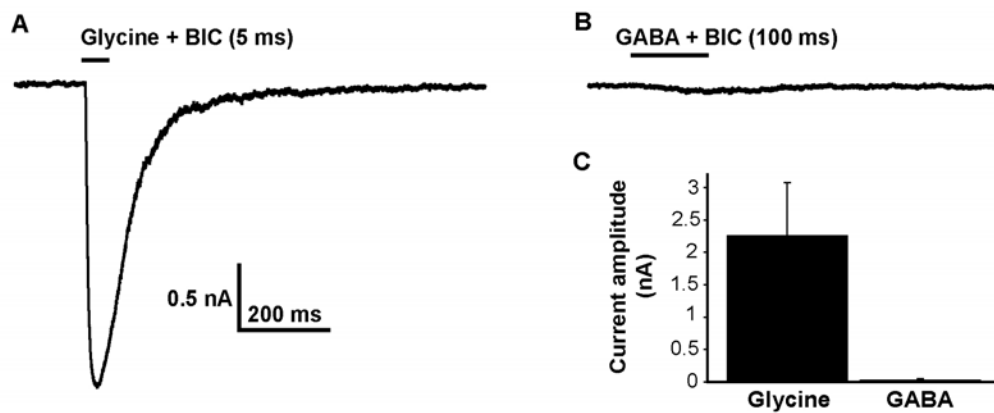


Figure 3. GABA is only a weak agonist for glycine receptors expressed on HEK 293T cells. (A) A typical trace showing the current activated when short pulses (5 ms) of glycine (50 μ M) and BIC (40 μ M) were pressure-applied to the cells in the presence of BIC (40 μ M). HEK 293 T cells were co-transfected with GlyR $\alpha 1$ and NL-2. (B) In only one out of four cells pressure application of GABA (1 mM) and BIC (40 μ M) activated a current. (C) Comparison of the mean currents activated by glycine ($n = 5$, mean \pm s.e.m) and GABA ($n = 4$) in the presence of BIC (40 μ M).

These results indicate that NL-2 can organize glycinergic postsynaptic apparatus in non-neuronal cells. More interestingly, given that pure hypothalamic neurons do not contain glycinergic synapses, our hetero-coculture results suggest a phenotypic change in neurotransmission, from GABAergic to glycinergic, when postsynaptic target is altered.

3. Mixed synapses are formed onto HEK cells co-expressing GlyR α 1 subunit and GABA_A-R subunits together with NL-2

Previous studies have shown that GABA_A-R and GlyR are expressed at the same synapse, which results in a mixed GABAergic/glycinergic neurotransmission (Jonas et al., 1998; O'Brien and Berger, 1999, , 2001). Since NL-2 and GABA_A-R receptors can also reconstitute functional GABAergic postsynaptic machinery in HEK 293T cells cocultured with hypothalamic neurons, we decided to test whether mixed synapses are formed onto HEK 293T cells expressing NL-2 with GlyR α 1 and GABA_A-R. Spontaneous synaptic currents recorded from transfected HEK 293T cells in normal bath solution displayed two populations of sIPSCs (Fig. 4.A). Pharmacological isolation further revealed the presence of both GABAergic and glycinergic synaptic currents (Fig. 4.B). Addition of the glycine receptor antagonist STR (0.5 μ M) blocked fast decaying events (n = 6) (Fig. 4.B). The remaining sIPSCs were eliminated by the application of BIC (20 μ M, data not shown). After washout of STR, application of BIC (20 μ M) to the same cell selectively eliminated the slower decaying spontaneous currents and the residual glycinergic

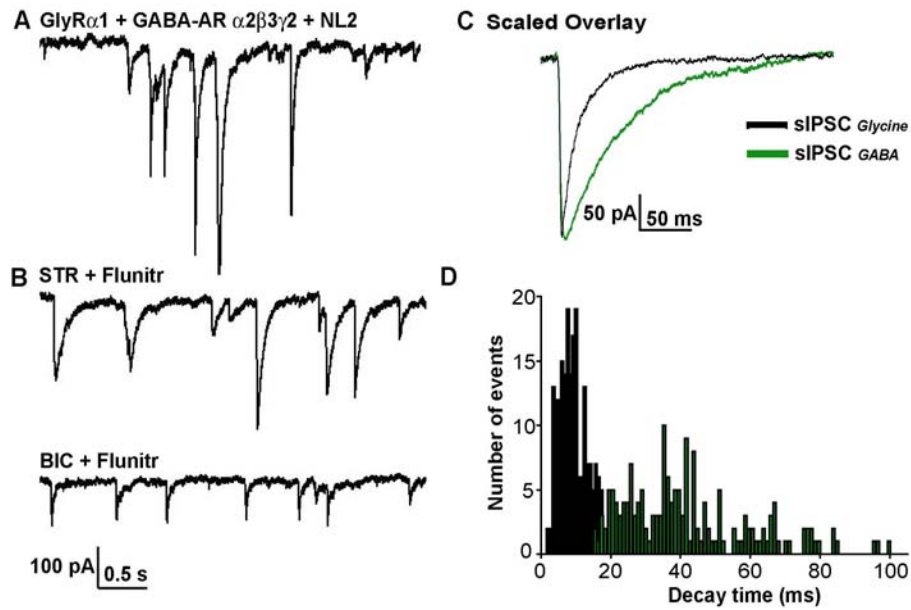


Figure 4. Mixed synapses are formed onto HEK cells co-expressing GlyR subunit and GABA_AR subunits together with NL-2. (A) A typical example showing sIPSCs recorded from a HEK 293 T cell co-transfected with NL-2, GlyR α 1, and GABA_AR, and co-cultured with hypothalamic neurons for 2-3 days. Recordings were performed at -70 mV. (B) Pharmacological isolation of sIPSCs in the presence of flunitrazepam (2 μ M). On the same cell, addition of STR (0.5 μ M) blocked faster decaying glycinergic sIPSCs; while addition of BIC (20 μ M) blocked slower decaying GABAergic sIPSCs (n = 6). (C) Scaled overlay of averaged sIPSCs recorded in the presence of BIC (20 μ M, sIPSC_{Gly}, black, n = 52) and in the presence of STR (0.5 μ M, sIPSC_{GABA}, green, n = 38). (D) Distribution of the decay kinetics of glycinergic (black bars, BIC, n = 211) and GABAergic (green bars, STR, n = 183) sIPSCs yields two separate populations of synaptic currents. Recordings were pooled from two transfected HEK 293T cells. Bin size, 1 ms.

sIPSC were completely blocked by STR. To enhance the difference in decay kinetics between the two components, we included the benzodiazepine flunitrazepam (Jonas et al., 1998). Flunitrazepam (2 μ M), selectively prolonged the decay time of GABA_AR mediated sIPSCs (on average 1.85 ± 0.27 fold increase, $n = 8$), but did not significantly alter the kinetics of GlyR mediated sIPSCs (on average, by a factor of 1.07 ± 0.18 change, $n = 6$). In the presence of flunitrazepam and either STR or BIC, the GABAergic sIPSCs decayed with an average half width of 46.6 ± 3.8 ($n = 8$), while the glycinergic sIPSCs had a half width of 12.3 ± 1.45 ms ($n = 6$, mean \pm s.e.m.). Scaled overlay of the averaged sIPSCs demonstrate that GABAergic IPSCs display longer decay time than that of glycinergic IPSCs (Fig. 4.C), which is consistent with the previously reported studies (Jonas et al., 1998; O'Brien and Berger, 1999; Nabekura et al., 2004; Dugue et al., 2005). The mean rise time (10%-90%) of the IPSC_{GABA} was 5.07 ± 0.43 ms, while rise time of sIPSC_{Gly} was 2.78 ± 0.33 ms. The distributions of decay times (peak to 34%) for glycinergic and GABAergic sIPSCs, recorded in the presence of flunitrazepam also show that the GABA_AR and GlyR mediated events constitute significantly different populations of events on the same HEK 293T cell (Fig. 4.D). To investigate the possibility of GABA and glycine co-release from the same vesicle, we have also performed recordings in the presence of TTX, which blocks action potential dependent synaptic activity. We observed single quantal glycinergic and GABAergic events on the same HEK cell both in the presence or absence of BIC or STR, including some events that displayed bi-exponential decay kinetics. However, the synaptic responses were significantly reduced after application of TTX (0.5 μ M), suggesting that spontaneous

events detected in the artificial synapses depend upon the neuronal activity of the network formed in the culture. Therefore, our results are not yet clear to conclude whether GABA and glycine are co-released from a single vesicle in the synapses formed between HEK 293T cells and neurons. Nonetheless, these results strongly suggest that NL-2 can cluster both GABA_A and glycine receptors on the postsynaptic sites, reconstituting mixed synapses on HEK 293T cells that display distinct properties.

4. Overexpressing NL-2 with GlyR α 1, but not NL-2 or GlyR α 1 alone in hypothalamic neurons induce glycinergic synaptic currents

The experiments so far implicate an inducible switch in the neurotransmitter phenotype in artificial synapses formed between hypothalamic neurons and non-neuronal HEK 293T cells. To determine whether a similar phenotypic change can also occur in inter-neuronal synapses, we explored the effects of GlyRs and NL-2 on glycinergic synapse formation in cultured hypothalamic neurons. We transfected neurons with GlyR α 1, NL-2, or GlyR α 1 together with NL-2 at 5-7 DIV and recorded sIPSCs at 8-12 DIV. Glutamate receptor antagonist CNQX (20 μ M) was always present in the solutions. Addition of BIC (40 μ M) eliminated all the sIPSCs in neurons expressing GlyR α 1 alone (n = 8) or NL-2 alone (n = 10) (Fig. 5.A, B, upper traces). Application of STR (0.5 μ M) did not affect the amplitude or frequency of GABAergic events in these neurons (lower traces); however, an increase in the frequency of sIPSCs in NL-2 transfected neurons was observed (data not shown). Because the hypothalamic neurons already express high amounts of functional

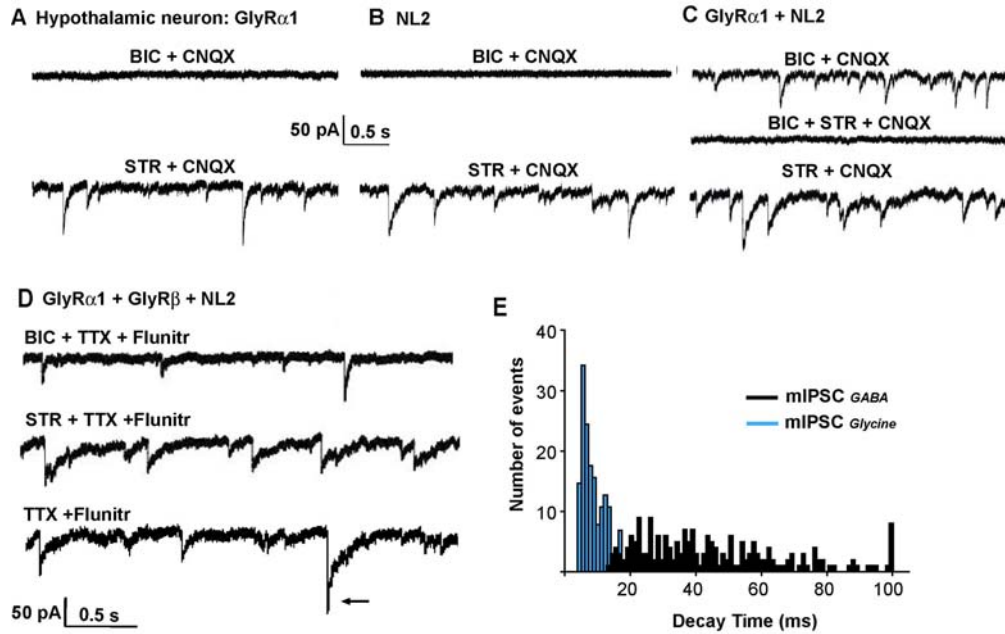


Figure 5. Overexpressing NL-2 with GlyR α 1, but not NL-2 or GlyR α 1 alone in hypothalamic neurons induce glycinergic synaptic currents. Typical traces of sIPSCs from cultured hypothalamic neurons transfected with **(A)** GlyR α 1, **(B)** NL-2, and **(C)** GlyR α 1 together with NL-2. CNQX (20 μ M) was present in all solutions. Application of BIC (40 μ M) completely abolished sIPSC in all of the neurons expressing GlyR α 1 (n = 8) and NL-2 (n = 10) (A, B, upper traces), bottom traces show GABAergic sIPSCs in the presence of STR (0.5 μ M, in conjunction with CNQX). BIC did not abolish sIPSCs in all 11 of the neurons expressing NL-2 together with GlyR α 1(C, upper). These events were completely blocked by STR (middle). Bottom trace shows the GABAergic sIPSCs from the same cell. **(D)** Pharmacological isolation of mIPSCs from neurons co-expressing NL-2, GlyR α 1 and GlyR β . Flunitrazepam (2 μ M) was present in all solutions. On the same cell, addition of BIC (40 μ M) blocked slower decaying GABAergic mIPSCs, upper trace; while, addition of STR (0.5 μ M) blocked faster decaying glycinergic mIPSCs, middle trace (n = 6). When STR and BIC were excluded, neurons displayed a third type of event with both fast and slow decaying phases (bottom trace, dual component event is shown with an arrow). **(E)** Distribution of the decay kinetics of glycinergic (blue bars, BIC, 40 μ M, n = 180) and GABAergic (black bars, STR, 0.5 μ M, n = 210) mIPSCs. Recordings were pooled from 2 transfected neurons. Bin size, 1 ms.

glycine receptors on the neuronal membranes (Fig. 1.C), which are suggested to localize at non-synaptic regions (Hussy et al., 2001), it was expected that increasing the expression of GlyR did not induce formation of glycinergic synapses. Moreover, although NL-2 overexpression increased the frequency of spontaneous events, possibly by generating new synapses (Chih et al., 2005), these newly formed synapses did not induce glycinergic presynaptic terminals. However, in neurons overexpressing GlyR α 1 and NL-2, application of BIC (40 μ M) did not eliminate fast decaying responses that were completely blocked by STR (0.5 μ M), showing that these responses were glycinergic (Fig. 5.C). The spontaneous glycinergic events occurred at a frequency of 0.51 ± 0.07 Hz and the amplitude was 47.8 ± 5.5 pA, while the GABAergic sIPSC frequency and amplitude recorded from the same cell was 1.71 ± 0.31 Hz and 48.3 ± 4.8 pA, respectively (n = 11).

We then analyzed the nature of miniature IPSCs in the presence of TTX (0.5 μ M) and CNQX (20 μ M) in neurons overexpressing NL-2 together with GlyR α 1 and β subunits. Flunitrazepam (2 μ M) was also included to enhance the decay kinetics between GABAergic and glycinergic responses. In the presence of BIC (40 μ M), glycinergic mIPSCs were detected with a fast decaying kinetics (mean half width 9.78 ± 1.22 ms, rise time 2.18 ± 0.17 ms, n = 6) (Fig. 5.D, upper). The events occurred at a rate of 0.31 ± 0.04 Hz and the current amplitude was 18.9 ± 0.79 pA (mean \pm s.e.m). On the same cell, GABAergic mIPSCs were observed by application of STR (0.5 μ M), which had a slower decay kinetics (mean half width 51.9 ± 3.4 ms, rise time 3.06 ± 0.2 ms) (Fig. 5.D, middle). Neither GABAergic mIPSC frequency nor mean current amplitude was significantly different from that of

glycine mIPSCs (frequency 0.79 ± 0.17 Hz, amplitude 26.35 ± 2.4 pA, $p > 0.01$, $n = 6$). In the absence of both STR and BIC, we observed events with three types of decay kinetics: fast decaying glycinergic events, slow decaying GABAergic events, and dual component events that have both fast decaying component and slow decaying component (Fig. 5.D, bottom, a dual component event is indicated with an arrow). Events that exhibit two components have been suggested to indicate the co-release of GABA and glycine from the same presynaptic vesicle (Jonas et al., 1998; O'Brien and Berger, 1999; Nabekura et al., 2004). The distribution of 34% to decay times in the presence of STR ($0.5 \mu\text{M}$, mIPSC_{GABA}, black, $n = 210$), or BIC ($40 \mu\text{M}$, mIPSC_{Glycine}, blue, $n = 180$) indicate two separate populations of events (Fig. 5.D). Our observations show that increasing the expression of NL-2 and GlyR, but not NL-2 or GlyR expression alone, can induce glycinergic synapses in hypothalamic neurons, similar to the glycinergic synapses formed between HEK 293T cells and neurons. This indicates that NL-2 might function as a central organizer in clustering glycine receptors at the postsynaptic sites opposing to presynaptic terminals.

5. Postsynaptic characterization of newly formed glycinergic synapses

Formation of postsynaptic machinery for glycinergic inhibitory neurotransmission is thought to require gephyrin accumulation at postsynaptic sites, which directly interacts with the GlyR β subunit (Kirsch et al., 1993; Kirsch et al., 1995; Meyer et al., 1995; Feng et al., 1998). Thus, hetero-oligomeric α/β GlyRs are thought to be the dominant form of synaptic receptors. However, in spinal cord neurons GlyR $\alpha 1$

and $\alpha 2$ subunit mRNAs are found in postsynaptic specializations of dendrites, whereas GlyR β and gephyrin mRNAs are mostly localized in the soma (Racca et al., 1997). Additionally, homo-oligomeric $\alpha 1$ GlyRs are functionally identified in the synapses of zebrafish Mauthner cell (Legendre, 1997). To determine the molecular mechanisms underlying the novel phenotypic switch in cultured neurons, we investigated the postsynaptic composition of newly formed synapses by analyzing the effects of picrotoxin on GlyR mediated synaptic currents in transfected HEK 293T cells and neurons. Picrotoxin (PTX, 100 μ M) blocked the majority of the synaptic events recorded from HEK 293T cells co-transfected with GlyR $\alpha 1$ and NL-2, and cocultured with hypothalamic neurons (on average $94 \pm 3\%$ inhibition, $n = 6$) (Fig. 6.A left). As expected, PTX inhibition was significantly smaller in HEK 293T cells expressing NL-2 together with GlyR $\alpha 1$ and β ($15.5 \pm 3.7\%$ inhibition, $n = 8$) (Fig. 6.A, right) compared to $\alpha 1$ -IPSC_{Gly} ($p < 0.001$) (Fig. 6.B). This indicates that in HEK 293T cells, both GlyR $\alpha 1$ homomers and GlyR $\alpha 1\beta$ heteromers are effectively clustered at postsynaptic sites of the hemi-synapses. Application of PTX in conjunction with BIC (40 μ M) and CNQX (20 μ M) in neurons co-expressing NL-2 with GlyR $\alpha 1$ blocked $83.9 \pm 9\%$ of glycinergic IPSCs ($n = 11$) (Fig. 6.C, left). This suggests that the majority of the synaptic currents are mediated by exogenous $\alpha 1$ homomers, however, a subpopulation of the synaptic GlyRs also contain $\alpha 1\beta$ heterooligomers. A likely explanation is that the endogenous GlyR β subunit has been incorporated with the GlyR $\alpha 1$ subunit.

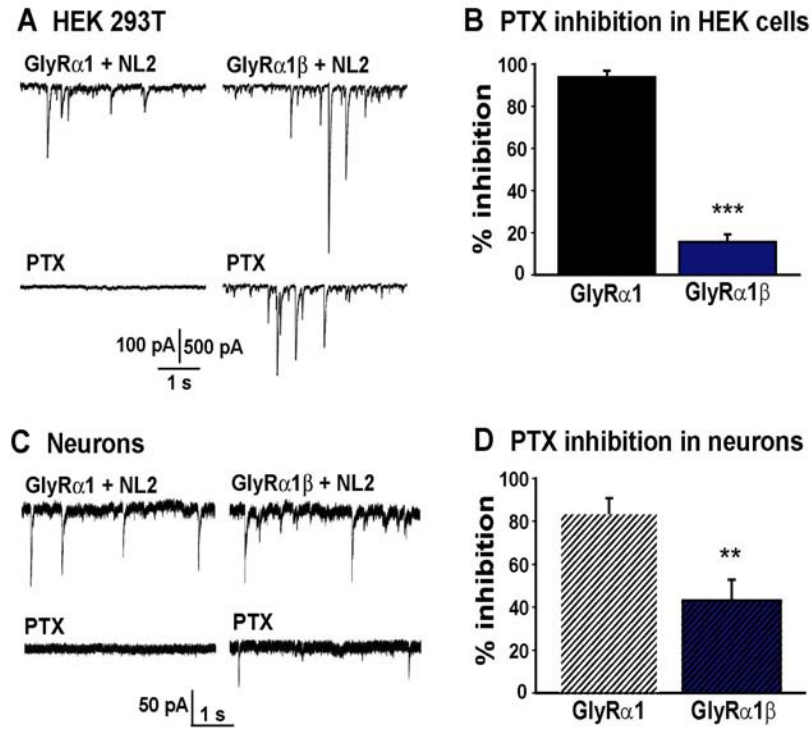


Figure 6. Postsynaptic characterization of newly formed glycinergic synapses. (A) Glycinergic sIPSCs recorded from HEK 293T cells expressing NL-2 with GlyR α 1 (left) or GlyR α 1 and GlyR β (right). Transfected HEK cells were cocultured with 5-7 DIV hypothalamic neurons for 2-3 days. Typical traces recorded before (upper) and during (bottom) 100 μ M picrotoxin (PTX) application. **(B)** PTX blocked $94 \pm 3\%$ of α 1-IPSC_{Gly} ($n = 6$), and $15.5 \pm 3.7\%$ of α 1 β -IPSC_{Gly} ($n = 8$). **(C)** Representative traces of hypothalamic neurons coexpressing NL-2 with GlyR α 1 (upper left) or GlyR α 1 and GlyR β (upper right) in the presence of BIC (40 μ M) and CNQX (20 μ M). Bottom traces show recordings in the presence PTX (100 μ M, in conjunction with BIC and CNQX). **(D)** PTX blocked $83.9 \pm 9\%$ of α 1-sIPSC_{Gly} ($n = 11$), and $43 \pm 10\%$ of α 1 β -sIPSC_{Gly} ($n = 12$).

In neurons expressing NL-2 and GlyR α 1 β , PTX application in the presence of BIC and CNQX blocked 43 ± 10 % of the events ($n = 12$) (Fig. 6.C, right), indicating that exogenous GlyR α 1 and β subunits formed GlyR heteromers at the synaptic sites, which are resistant to PTX inhibition.

To ensure that the exogenous receptors were expressed at the cell membranes, we also tested the effect of PTX on whole-cell current amplitudes in transfected neurons. In agreement with the data from synaptic currents, PTX blocked a significantly larger portion of the current amplitude in neurons transfected with GlyR α 1 and NL2 (58.6 ± 5.7 %, $n = 11$), comparing to the neurons expressing GlyR α 1 β and NL2 (13.1 ± 2.7 %, $n = 13$, $p < 0.001$). Together, our results suggest that the postsynaptic organization of receptors is crucial in remodeling the presynaptic composition of neurons, which in turn will help to determine the neurotransmission phenotype.

DISCUSSION

In the present work, we have identified a novel inducible plasticity in cultured hypothalamic neurons. We demonstrate a phenotypic switch from purely GABAergic transmission to one mediated by both GABA and glycine. Previous studies have demonstrated high levels of GlyR expression in forebrain regions such as hypothalamus, hippocampus and neocortex, by using electrophysiological and immunohistochemical methods. Here, we have also shown that in cultured hypothalamic neurons the functional GlyRs are expressed at high levels similar to that of GABA_A-Rs. In agreement with the previous *in situ* hybridization and RT-PCR analyses (Fujita et al., 1991; Malosio et al., 1991)), the presence of both GlyR α -homomers and $\alpha\beta$ -heteromers is demonstrated in our experiments (Figure 1). However, high levels of GlyRs do not appear to contribute to the inhibitory neurotransmission in cultured hypothalamic neurons, since the sIPSCs are completely blocked by the selective GABA_A-R antagonist BIC. The lack of glycinergic synaptic currents might be attributed to the non-synaptic localization of glycine receptors, as shown by previous immunohistochemical and electron microscopic studies in the hippocampus and hypothalamus (Brackmann et al., 2004; Danglot et al., 2004; Deleuze et al., 2005). Non-synaptic localization of GlyRs might be a common phenomenon in the forebrain regions where high levels of receptor expression are not coupled to a function in neurotransmission. Instead, the non-synaptic GlyRs are involved in cortical development (Flint et al., 1998), suppressing hyperexcitability in hippocampus (Chattipakorn and McMahon, 2003; Zhang et al., 2008), and

osmoregulation in hypothalamus (Hussy et al., 2001). Taurine, released by neurons in cortex (Flint et al., 1998) and glial cells in hypothalamus (Hussy et al., 1997) and hippocampus (Mori et al., 2002) is a proposed endogenous ligand for the extrasynaptic GlyRs. Taurine activation of GlyRs occurs via mechanisms that are distinct from conventional action potential and calcium dependent modes of neurotransmission. Nonetheless, given that a substantial number of glycine IR afferents have been demonstrated in these regions (Rampon et al., 1996), one should not rule out an *in vivo* activation of glycinergic synaptic currents.

Our results suggest that the inducible changes in the neurotransmission phenotype depend upon NL-2 along with the receptor expression on the postsynaptic membranes. Previous studies have shown that NL-2 can selectively induce formation and/or stabilization of GABAergic synapses (Chih et al., 2005; Varoqueaux et al., 2006; Chubykin et al., 2007; Huang and Scheiffele, 2008). NL-2 exerts its synaptogenic effects by stabilizing both the postsynaptic machinery and inhibitory presynaptic innervation through trans-synaptic interactions with neurexins. Our recent study has also shown that NL-2 can co-aggregate with GABA_AR γ 2 subunit on HEK 293T cells and induce strong presynaptic GABAergic innervation from cocultured neurons onto HEK cells (Dong et al., 2007).

Here, we show that NL-2 can also induce formation of glycinergic synapses between neurons and HEK 293T cells (Figure 2). All of the three subtypes of GlyRs, α 1, and α 2 homomeric GlyRs and α 1 β heteromeric

GlyRs, are capable of forming functional apparatus together with NL-2. The recombinant receptors assembled at the artificial synapses resemble the endogenous receptors located at the glycinergic synapses in spinal cord and brain stem (Takahashi et al., 1992; Singer et al., 1998; Singer and Berger, 1999; Legendre, 2001), represented by the kinetic characterization of the synaptic currents mediated by individual GlyR subtypes (Figure 2). Previous studies have suggested that the synaptic receptors in mature neurons are predominantly $\alpha 1\beta$ heterooligomeric, whereas the neonatal forms of synaptic GlyRs are mostly the $\alpha 2$ homooligomeric subtype. We have observed that $\alpha 2$ homomeric GlyRs are formed less effectively than the $\alpha 1$ homomeric or $\alpha 1\beta$ heteromeric receptors, as judged by the significant reduction in both the amplitude and the frequency of the synaptic events mediated by the $\alpha 2$ GlyRs. It has been previously shown that GlyR α subunits can form functional chloride channels when expressed in HEK 293T cells (Kirsch and Betz, 1995); however, surface labeling reveals a diffuse expression in the plasma membrane (Meier et al., 2000). It is therefore of interest to determine whether GlyR α homomers are clustered on the HEK 293T cells when specifically co-expressed with NL-2.

Data presented here also shows that both GABA_A-R and GlyRs can cluster at the synaptic sites to form 'mixed' synapses, with distinct kinetics (Figure 4). Therefore, it is tempting to propose that NL-2 might function as a postsynaptic organizing molecule to recruit inhibitory postsynaptic receptors opposing to the presynaptic terminals. Our study implies that NL-2 is

associated with GlyR α subunits; however, biochemical assays are required to address whether NL-2 directly interacts with GlyRs. Other interesting questions include whether NL-2 discriminates between GlyRs and GABA_ARs or if there is a competitive mechanism between the two receptors for association with NL-2.

The effect of picrotoxin applications on transfected HEK 293T cells clearly shows that not only $\alpha 1$ homomeric but also $\alpha 1\beta$ heteromeric GlyRs are formed at the induced glycinergic synapses (Figure 6). Given that HEK 293T cell expression system is a less complex model when compared to neurons, i.e. devoid of extensively regulated intracellular trafficking mechanisms (Luscher and Keller, 2004), it is expected that both receptor subtypes are assembled at the synapses. Previous reports have suggested the presence of $\alpha 1$ homomers in functional glycinergic synapses (Legendre, 1997; Racca et al., 1997). Accordingly, we also demonstrate that functional GlyR $\alpha 1$ homomers can accumulate at the induced synapses formed on neurons. However, GlyR β subunit is thought to be crucial in clustering and stabilization of GlyRs at synaptic sites, which directly interacts with the scaffolding protein gephyrin (Kirsch et al., 1993; Kirsch et al., 1995; Meyer et al., 1995; Feng et al., 1998). Therefore, endogenous gephyrin's interaction with the exogenous GlyR β subunit might account for the postsynaptic localization of the heteromeric GlyRs. In contrast, our results support a clustering mechanism for GlyR $\alpha 1$ homomers independent of the anchoring function of gephyrin. This finding does not contradict with previous reports (Meier et al., 2000). Although our functional data

do not discriminate between two different clustering mechanisms for homomeric or heteromeric GlyRs, NL-2 appears a likely mediator for clustering both GlyR subtypes at postsynaptic sites.

GABA and glycine corelease is considered as a common phenomenon of the inhibitory neurotransmission rather than an exception. Evidence in support of this view includes the widespread colocalization of the synthetic enzyme for GABA, glutamic acid carboxylase (GAD), with neuronal transmembrane glycine transporter, GlyT2 in midbrain, cerebellum and brain stem (Tanaka and Ezure, 2004). Additionally, a common vesicular transporter, VIAAT, mediates loading and release of both GABA and glycine from the same synaptic vesicles (Wojcik et al., 2006). Moreover, functional corelease of GABA and glycine from the same presynaptic vesicle has been demonstrated in the spinal cord (Jonas et al., 1998) and brain stem (O'Brien and Berger, 1999; Nabekura et al., 2004; Awatramani et al., 2005), resulting in co-activation of GABA_A and glycine receptors on the postsynaptic sites. Here we also demonstrate that the mIPSCs recorded from neurons co-expressing glycine and GABA_A receptors together with NL-2 display dual-component events comprising GABA_A-R and GlyR mediated components (Figure 5). Our results implicate that in phenotypically altered hypothalamic neurons, GABA and glycine can be released from a single presynaptic vesicle and co-activate the corresponding receptors at the same postsynaptic density. GABA and glycine cotransmission have crucial implications in timing and strength of neuronal inhibition to regulate excitation. For instance in spinal cord, it is suggested that fast glycinergic components effectively

hyperpolarize the cell, whereas the slower GABAergic components control shunting and time course of inhibition. Differential modulation of GABA_A and glycine receptors may also allow rapid and precise adaptation of inhibition in the neuronal network (Dugue et al., 2005).

Our experiments so far did not reveal the origin of the phenotypic switch. However, the neurotransmitter plasticity induced by glycine receptor and NL-2 overexpression could be explained by at least two different hypotheses. First, the cultured hypothalamic neurons may release both GABA and glycine continuously. However, glycinergic synaptic currents are not detected in cultured neurons because GlyRs are not localized at synapses. Upon coexpression of NL-2 and GlyR subunits, NL-2 can cluster functional receptors at synaptic terminals, which allows detection of STR-sensitive glycinergic events. This explanation does not contradict with previous studies, which have demonstrated the presence of glycine IR terminals (Rampon et al., 1996) and non-synaptic localization of GlyRs in hypothalamus (Deleuze et al., 2005).

Alternatively, the ability of the postsynaptic neuron to influence the properties of presynaptic terminal may result in the inducible plasticity of neurotransmitter phenotype. Postsynaptic expression of functional receptors and cell adhesion molecules could modify the inhibitory presynaptic organization, which results in (co)packaging of glycine into synaptic vesicles. Previous studies have provided evidence in support of this explanation. For instance, postsynaptic overexpression of active calmodulin dependent protein kinase II (CaMKII) is shown to induce the remodeling of presynaptic inputs with an increase in both

quantal amplitude and synaptic contacts (Pratt et al., 2003). Furthermore, postsynaptic PSD-65 and NL-1 complex can also modify the presynaptic release in a retrograde way (Futai et al., 2007). Transsynaptic interactions between NL-1 and presynaptic β -neurexin have been suggested as a mediator for the retrograde modulation, by functionally connecting the presynaptic release machinery to postsynaptic sites. It should be noted that a target dependent plasticity in the neurotransmission phenotype is not compatible with previously described forms of phenotypic switch, where presynaptic neurotransmitter content governs the postsynaptic receptor selection (Nabekura et al., 2004; Borodinsky and Spitzer, 2007). Additional tests are required to distinguish between these two hypotheses.

In conclusion, our results suggest an inducible plasticity of the neurotransmission phenotype upon postsynaptic expression of a synaptic cell adhesion molecule NL-2 and postsynaptic receptors (glycine receptors). Furthermore, we propose that the switch in the inhibitory synaptic phenotype identified in our study is not restricted to hypothalamic neurons, but rather a general mechanism of plasticity in the CNS. This mechanism would be extremely important in the regulation of excitation and synaptic transmission by temporal precision in networks that are involved in a wide range of complex behaviors.

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