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Region-specific expression of NMDA receptor GluN2C subunit in parvalbumin-positive neurons and astrocytes: Analysis of GluN2C expression using a novel reporter model

Aparna Ravikrishnan¹, Pauravi J. Gandhi^{#1}, Gajanan P. Shelkar^{#1}, Jinxu Liu^{#1}, Ratnamala Pavuluri^{#1}, and Shashank M. Dravid^{1,*}

¹Department of Pharmacology, Creighton University, Omaha, NE 68178 USA,

[#] These authors contributed equally to this work.

Abstract

Hypofunction of NMDA receptors in parvalbumin (PV)-positive interneurons has been proposed as a potential mechanism for cortical abnormalities and symptoms in schizophrenia. GluN2C-containing receptors have been linked to this hypothesis due to the higher affinity of psychotomimetic doses of ketamine for GluN1/2C receptors. However, the precise cell-type expression of GluN2C subunit remains unknown. We describe the expression of the GluN2C subunit using a novel EGFP reporter model. We observed EGFP(GluN2C) localization in PV-positive neurons in the nucleus reticularis of the thalamus, globus pallidus externa and interna, ventral pallidum and substantia nigra. In contrast, EGFP(GluN2C) expressing cells did not co-localize with PV-positive neurons in the cortex, striatum, hippocampus or amygdala. Instead, EGFP(GluN2C) expression in these regions co-localized with an astrocytic marker. We confirmed functional expression of GluN2C-containing receptors in the PV-neurons in substantia nigra and cortical astrocytes using electrophysiology. GluN2C was found to be enriched in several first order and higher order thalamic nuclei. Interestingly, we found that a previous GluN2C β -gal reporter model excluded expression from PV-neurons and certain thalamic nuclei but exhibited expression in the retrosplenial cortex. GluN2C's unique distribution in neuronal and non-neuronal cells in a brain region-specific manner raises interesting questions regarding the role of GluN2C-containing receptors in the central nervous system.

Keywords

NMDA receptor; GluN2C; NR2C; parvalbumin; astrocytes; GRIN2C

*Corresponding Author: Dr. Shashank M. Dravid, Department of Pharmacology, Creighton University, School of Medicine, 2500 California Plaza, Omaha, NE 68178, Phone: 402-280-1885, Fax: 402-280-2142, ShashankDravid@creighton.edu.

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Introduction

NMDA receptors are a class of ionotropic glutamate receptors critical for synaptic plasticity associated with learning and memory. NMDA receptors are typically composed of two glycine-binding GluN1 subunits and two glutamate-binding GluN2 subunits, of which there are four types (GluN2A through GluN2D). The identity of the GluN2 subunit designates unique biophysical, pharmacological and signaling properties for the receptor (Traynelis et al., 2010). NMDA receptor hypofunction in parvalbumin (PV)-interneurons in the cortico-limbic region has been suggested to lead to schizophrenia (Belforte et al., 2010; Nakazawa et al., 2012; Gonzalez-Burgos and Lewis, 2012). Supporting this hypothesis, NMDA channel blockers that produce psychosis have a stronger inhibitory effect on the excitability of interneurons compared to pyramidal neurons (Hodayoun and Moghaddam, 2007). Furthermore, NMDA channel blockers inhibit GluN2C and GluN2D subunits with greater affinity than other subunits, suggesting that inhibition of GluN2C and/or GluN2D-containing receptors may underlie psychotic symptoms (Khlestova et al., 2016). In accordance with this hypothesis, we have recently demonstrated that GluN2C knockout mice have impaired excitatory-inhibitory balance, fewer PV-labeled neurons in the prefrontal cortex, and other phenotypes relevant to schizophrenia (Gupta et al., 2016; Hillman et al., 2011). Additionally, lower expression of GluN2C transcripts has been consistently observed in the cortex and thalamus of schizophrenia patients (Catts et al., 2016; Akbarian et al., 1996; Ibrahim et al., 2000; Meador-Woodruff et al., 2003; Beneyto and Meador-Woodruff, 2008; Weickert et al., 2012). Thus, converging findings suggest a critical role for GluN2C and/or GluN2D-expressing PV-interneurons in schizophrenia. However, evidence for the expression of GluN2C and GluN2D in PV-neurons has been lacking. Several recent neuroanatomical and electrophysiological studies have demonstrated expression of GluN2D subunits in interneurons in cortico-limbic regions, including PV-neurons (Perszyk et al., 2016; von Engelhardt et al., 2015; Yamasaki et al., 2014). In contrast, the precise distribution of GluN2C, especially its expression in PV-interneurons, remains unknown.

Early in-situ hybridization and histoblotting studies identified GluN2C expression in cortico-limbic regions, but cell-specific distribution was not addressed in these studies (Monyer et al., 1994; Wenzel et al., 1997). Karavanova et al. used a GluN2C knock-out/ β -galactosidase (β -gal) knock-in mouse line, which was generated by inserting the β -gal cassette at the *GRIN2C* translation initiation site to address cell-specific expression of GluN2C (Karavanova et al., 2007). Immunostaining for β -gal in these mice brains revealed previously unknown expression of GluN2C in several regions, such as the retrosplenial cortex and non-neuronal cells of the striatum and subiculum. Interestingly, this mouse line shows a lack of immunoreactivity for β -gal in the nucleus reticularis (nRT), a region enriched in PV-positive neurons (Hillman et al., 2011). This finding does not align with electrophysiological and histochemical analyses demonstrating enriched GluN2C expression in nRT neurons (Wenzel et al., 1997; Lin et al., 1996; Zhang et al., 2012). We hypothesize that insertion of the reporter cassette and deletion of much of the gene's exons and introns in the β -gal reporter model alters endogenous transcription patterns, and thus fails to identify PV-neuron expression of GluN2C.

Our use of immunohistochemistry to address this question was not successful due to unsatisfactory labeling by commercially available GluN2C antibodies. Similarly, other antibodies lack sensitivity to label regions other than the cerebellum and thalamus (Yamada et al., 2001). Therefore, we used a novel *Grin2C^{tm1}* (EGFP/cre/ERT2)^{Wtsi} mouse line, in which the EGFP reporter is inserted between exons 6 and 7 of the *GRIN2C* allele without deletion of any endogenous elements. Moreover, the reporter model is advantageous in addressing cell-type specific expression, since the reporter (in this case EGFP) is typically expressed as a separate protein and fills the cell body of the expressing cell. Besides confirming GluN2C expression in the nRT, our results also revealed expression of GluN2C in PV-positive neurons in the globus pallidus, ventral pallidum, and substantia nigra. Strikingly, there was no expression of GluN2C in hippocampal, cortical or striatal PV-positive neurons; instead, in these regions GluN2C expression was localized to astrocytes. Together, these findings demonstrate a unique pattern of expression for the GluN2C subunit among NMDA receptor subtypes and raise questions regarding its function in these regions.

Materials and Methods

Animal husbandry

We used the *Grin2C^{tm1}* (EGFP/cre/ERT2)^{Wtsi} mouse line generated on a pure C57BL/6N background obtained from Wellcome Trust Sanger Institute. The *GRIN2C* allele in these mice have loxP sites flanking exons 7 through 9, and inserted into the intron between exons 6 and 7 is one FRT site, an engrailed 2-splice acceptor sequence, an EGFP and cre/ERT2 expression cassette, and a Rox-flanked puromycin resistance cassette. Details of the constructs and design have been previously described (Bradley et al., 2012; Pettitt et al., 2009; Skarnes et al., 2011; White et al., 2013). We also used the NR2C knockout/ β -galactosidase knock-in mice as previously described (Hillman et al., 2011; Karavanova et al., 2007). In this reporter model, the first 11 exons were removed and replaced by an *E.coli nlacZ* gene cassette encoding nuclear-targeted β -gal. The reporter cassette was inserted at the initiator methionine codon. Thus, this reporter construct differs significantly from the EGFP reporter model we used, in which none of the original gene elements are deleted. This feature of the EGFP reporter model increases the likelihood that transcriptional regulatory elements, as well as the putative promoter region, are intact and may thereby facilitate normal gene regulation. All procedures were approved by the Creighton University Institutional Animal Care and Use Committee and conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Gupta et al., 2015; Gupta et al., 2016). Briefly, mice were transcardially perfused with 4% PFA in 0.1 M phosphate buffer (PB) pH 7.4, and brains collected and stored overnight in the same fixative at 4°C. Brains were then transferred successively into solutions of 10%, 20% and 30% sucrose in 0.1 M PB and thereafter frozen at -30°C to -40°C using isopentane. For immunohistochemistry, 20 μ m thick coronal or sagittal sections were cut using a cryostat (Leica CM 1900). After washing, sections were incubated in blocking solution containing 10% normal goat serum in 0.25% Triton-X in 0.01 M PB (PBT) for 1 hour at room

temperature. Following blocking, sections were incubated overnight at 4°C in primary antibodies at appropriate concentrations in PBT (chicken anti-GFP, 1:1000, Invitrogen A10262; rabbit anti-parvalbumin, 1:5000, Swant PV27; mouse anti-NeuN, 1:200, Millipore MAB377; rabbit anti-GABA, 1:1000, Sigma A2052; rabbit anti-Iba1, 1:1000, Wako Chemicals 019–19741; rabbit anti-GFAP, 1:1000, Sigma G9269; mouse anti- β -galactosidase, 1:200, Promega Z378B). The following day, sections were washed and thereafter incubated with the appropriate secondary antibodies conjugated to DyLight 488 (goat antichickens IgG-conjugated, KPL, 1:500 in PBT), or AlexaFluor 594 (goat anti-rabbit or -mouse IgG-conjugated, Invitrogen, 1:500 in PBT) for 2 hours at room temperature. Sections were then washed and mounted with Fluoromount-G (SouthernBiotech, AL). Images were acquired with an Infinity camera (Lumenera Co.) coupled to a widefield epifluorescence microscope (Nikon Eclipse Ci) using the Lumenera Infinity Analyze software (Lumenera Co.). For confocal images equivalent regions, 1024 $\times$ 1024 pixels, were captured using a Leica TCS SP8 MP confocal microscope using a 20 $\times$ or 40 $\times$ objective at 2 $\times$ zoom. An optical section (2- μ m thickness) was taken from each tissue section, and at least three sections per region of interest were analyzed for each animal. A stack of ten images beginning at the surface of the neuron was captured by the z-axis scanning at 0.2 μ m intervals for each neuron. The colocalization of EGFP (GluN2C) with PV was counted manually using NIH ImageJ software. Immunohistochemistry results were confirmed in at least three mice.

Slice electrophysiology

Whole-cell voltage-clamp recordings were obtained from astrocytes in the prefrontal cortex region. After isoflurane anesthesia, mice were decapitated and brains were removed rapidly and placed in ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂, 3 MgCl₂ and 10 glucose saturated with 95% O₂/5% CO₂. Then 300–350 μ m-thick coronal sections were collected using a vibrating microtome (Leica VT1200, Buffalo Grove, IL, USA). After 30–60 minutes of stabilization in slice recording buffer (with 1.5 mM CaCl₂ and 1.5 mM MgCl₂) slices were dye loaded with sulforhodamine 101 (2–4 μ M) for 20 minutes at 37°C. After a brief washing with recording buffer, slices were transferred to the recording chamber. The whole-cell currents were recorded at –60 mV holding potential in the presence of 1 μ M tetrodotoxin, 100 μ M picrotoxin and 20 μ M NBQX. Glass pipettes with a resistance of 5–8 m Ω were filled with an internal solution consisting of (in mM) 110 cesium gluconate, 30 CsCl, 5 HEPES, 4 NaCl, 0.5 CaCl₂, 2 MgCl₂, 5 BAPTA, 2 Na₂ATP, and 0.3 Na₂GTP (pH 7.35). The signal was filtered at 2 kHz and digitized at 10 kHz using an Axon Digidata 1440A analog-to-digital board (Molecular Devices, CA).

For whole-cell recordings from PV and non-PV neurons in the substantia nigra, PV-cre mice (Jackson Labs 008069) were intracranially injected with adeno-associated virus containing a cre-dependent fluorescent protein (AAV2/9-SYN-DIO-hM4D-mCherry, 0.2 μ l of $\sim 3 \times 10^{13}$ units/ml). Two weeks after AAV injection, mice were sacrificed and brain slices were prepared for slice electrophysiology as described above. Voltage-clamp recordings were obtained from both labeled and non-labeled neurons at +40 mV. The patch pipettes (3–5 m Ω) were filled with an internal solution consisting of (in mM) 126 Cs methanesulfonate, 10

HEPES, 8 NaCl, 8 Na₂-phosphocreatine, 2.9 QX314, 0.1 CaCl₂, 4 MgATP, 1 EGTA, and 0.3 Na₂GTP (pH 7.3). Responses to 200 μ M glutamate applied by Picospritzer II with a pulse duration of 20–50 ms were measured. The bath solution contained 0.5 μ M tetrodotoxin, 100 μ M picrotoxin and 10 μ M CNQX to select for NMDA receptor currents. The effect of bath application of 300 μ M D-cycloserine, a superagonist at GluN1/2C receptors, was evaluated. The peak and steady state currents were analyzed using Clampfit by measuring baseline and change in peak or steady state currents after drug application. Data were analyzed by t-test using Graphpad Prism.

Results

Novel transgenic mouse line reveals EGFP(GluN2C) expression in mouse central nervous system

We used a knock-in mouse line (*Grin2C*^{tm1} (EGFP/cre/ERT2)^{Wtsi}) where a cassette with EGFP and tamoxifen-dependent Cre was inserted in the intronic region between exons 6 and 7 of the *GRIN2C* gene. When brain sections from this mouse model were observed under fluorescent microscope, no EGFP signal was detected, suggesting low levels of transcription of the *GRIN2C* gene insufficient to generate a visible EGFP signal. Immunostaining for GFP revealed a distinct expression pattern of the EGFP(GluN2C) in cell bodies of many regions, including the cortex, hippocampus, striatum, thalamic nuclei and cerebellum (Fig. 1). The signal localization to the cell body is consistent with the reporter construct, which generates distinct cytoplasmic EGFP protein driven by endogenous gene transcriptional elements. In addition, certain areas such as the subthalamic nucleus (STN) and somatosensory cortex (SSC), showed intense fluorescence without cell body labeling. Since cytoplasmic EGFP will also be enriched in axons, the non-somatic labeling may represent projections from EGFP(GluN2C) expressing neurons. Consistent with previous reports, the most highly enriched regions of EGFP(GluN2C) expression were the cerebellum and thalamus (Monyer et al., 1994; Wenzel et al., 1997; Watanabe et al., 1992; Buller et al., 1994). In the thalamic nuclei, enriched expression was observed in the nRT, as well as many other nuclei, as detailed later. Expression was also observed in the globus pallidus, ventral pallidum and substantia nigra. In general, the size of cell bodies marked with EGFP appeared smaller in the cortex, hippocampus and striatum compared to those observed in the thalamus and globus pallidus, suggesting potentially different cell types (Fig. 1). These expression patterns are similar to *GRIN2C* mRNA analyses in the Allen mouse brain atlas (Lein et al., 2007) (Table 1).

We further investigated the expression pattern of GluN2C subunits throughout postnatal development by analyzing brains at day 8, 10, 14, 21 and 30 (Fig. 2). EGFP(GluN2C) expression was already observed at postnatal day 8 (P8), with the strongest signals observed in the thalamic nuclei, cerebellum and vestibular nuclei. The ventral thalamus was more strongly labeled than the dorsal part. Strong signals were also detected in layers of the SSC which were generally non-cell-body signals. Faint signals were also observed in the STN. At P10, expression was generally unchanged compared to P8, except for an increase in signal in the granule cell layer of the cerebellum and thalamus and appearance of signal in the STN. Between days 14 and 30, expression in the thalamic nuclei and cerebellum stabilized and

remained unchanged. At P21, expression also appeared in the substantia nigra, and became more distinct and clearly demarcated at P30. No further changes were observed at adulthood. No specific staining was observed in wildtype tissue, confirming the specificity of EGFP labeling.

Heterogeneity of parvalbumin-positive neurons expressing GluN2C subunit

PV-expressing neurons are one of the major classes of GABAergic neurons widely expressed in the brain. Since neurons of the nRT express PV, we tested the expression of EGFP(GluN2C) in PV cells. While EGFP was found to be expressed in certain PV-rich neuronal populations, we unexpectedly found heterogeneity in the PV-neurons expressing EGFP. We found strong EGFP (GluN2C) expression in PV-neurons of the nRT and globus pallidus (Fig. 3a). In contrast, PV-neurons in the cortex, striatum, and hippocampus lacked EGFP expression (Fig. 3b). Thus, there appears to be regional-selectivity in EGFP(GluN2C) expression in PV-positive neurons. The globus pallidus is a component of basal ganglia circuitry important for motor function and other behaviors related to reward and emotion. Given the expression of EGFP(GluN2C) in PV-neurons in the external segment of the globus pallidus (GPe) (Fig. 3a), and the internal segment of the globus pallidus (GPi), we further probed the expression pattern of EGFP(GluN2C) in other nuclei of the basal ganglia.

We observed expression of EGFP(GluN2C) in PV-neurons in the ventral pallidum (Fig. 4a). Further, we examined the STN, the primary target of the GPe, and found that although the EGFP signal was strong, this was not cell body staining (Fig. 4a). No STN labeling was observed in wildtype mice, demonstrating specificity for staining (Fig 2). Co-labeling with PV in the STN further revealed that a majority of the EGFP signal superimposed with PV. Our interpretation of these results is that GluN2C is expressed in the PV-neurons in GPe which send strong projections to STN. Alternatively, the STN labeling may also be a result of projections from other GluN2C-expressing regions. To confirm that the co-localization of PV and EGFP in these studies was not an artifact of cross-reactivity between the two antibodies, we quantified the intensities of signals from PV and EGFP in the cerebellum. Images of the cerebellum showed a distinct non-overlapping expression of PV and EGFP (Fig. 3b). While EGFP expression was restricted to the granule cell layer of the cerebellum, PV expression was seen in the Purkinje cell layer and underlying molecular layer, where there was a complete lack of EGFP signal. These results suggest that the co-localization observed in the basal ganglia nuclei was not due to crossreactivity.

We further found EGFP(GluN2C) expression in the substantia nigra which is organized into two components; substantia nigra compacta (SNc) and substantia nigra reticulata (SNr). The SNc was found to have a larger number of densely packed PV cells, while the SNr had a relatively sparse collection of PV cells (Figs. 1 and 4a). Similar to the GPe, we found strong colocalization of EGFP and PV in the SNc and SNr (Fig. 4a). We further used electrophysiology to test expression of functional GluN2C-containing receptors in PV-neurons of the SNr. We used a PV-cre line and injected an AAV with cre-dependent mCherry reporter in the substantia nigra region. As explained in the methods section, NMDA receptor responses were obtained upon glutamate puff application (in the presence TTX, picrotoxin and CNQX) from both labeled and unlabeled neurons. We tested the effect of bath

application of D-cycloserine which is a superagonist at GluN1/2C receptors but a partial agonist at GluN1/2B receptors (Dravid et al., 2010; Sheinin et al., 2001). We found that NMDA receptor responses in PV-neurons were increased, whereas responses in non-PV-neurons were reduced upon D-cycloserine application (Fig. 4b). These results demonstrate differential NMDA receptor subunit expression in the two cell-types, with PV-neurons possibly enriched in GluN2C subunit and non-PV-neurons enriched in GluN2B subunit.

Cortical, striatal and hippocampal expression of GluN2C subunit in astrocytes but not neurons and microglia

We also observed EGFP(GluN2C) expression in cell bodies in the cortex, striatum and hippocampus. Similar to the β -gal reporter model, we found that the expression in the cortex is not in neurons as indicated by lack of co-localization with the neuronal marker NeuN (Fig. 5a) (Karavanova et al., 2007). In addition, EGFP signal did not co-localize with GABA, suggesting that these are not other classes of interneurons (Fig. 5b). We also did not find co-localization of EGFP with microglia marker Iba1 in the cortex (Fig. 5c), striatum or other regions throughout the brain (data not shown), suggesting that these cells are not microglia. We tested whether GluN2C expression is localized to astrocytes. Interestingly, cortical EGFP(GluN2C) was found to co-localize with GFAP, suggesting that the expression was in astrocytes (Fig. 5d). Indeed, EGFP(GluN2C) expression in cortical astrocytes is consistent with previous electrophysiological analysis of NMDA receptor currents in astrocytes (Lalo et al., 2006; Palygin et al., 2011). EGFP also co-localized with GFAP in the prefrontal cortex, dorsal striatum and nucleus accumbens (Fig. 6a; Table 1). EGFP expression was also observed in hippocampal astrocytes, consistent with previous observations in the β -gal reporter model (Karavanova et al., 2007). We further tested whether the GluN2C subunit contributes to astrocytic NMDA receptor currents by recording from astrocytes in prefrontal cortex of wildtype and GluN2C KO mice. Astrocytes were identified by labeling with SR101 and their typical morphology, although some reports suggest that this dye may also label oligodendrocytes (Hill and Grutzendler, 2014). Whole-cell voltage-clamp recordings were obtained at -60 mV. It should be noted that previous studies have found that astrocytic NMDA receptor currents are weakly Mg^{2+} -sensitive and not significantly blocked by extracellular Mg^{2+} at -60 mV (Lalo et al., 2006; Palygin et al., 2011). Bath application of NMDA and glycine generated inward currents in wildtype but not in GluN2C KO cortical astrocytes (Fig. 6b), demonstrating the presence of GluN2C subunit-containing NMDA receptor in cortical astrocytes.

Thalamic distribution of GluN2C subunit and comparison of the EGFP and β -gal reporter models

Consistent with previous studies, we observed enriched expression of EGFP in several thalamic nuclei (Fig. 7 and 8). These include neurons in nRT, ventral anterior lateral (VAL), ventral posteromedial and posterolateral (VPM and VPL), mediodorsal thalamus (MD), posterior complex (PO), ventral medial thalamus (VM), lateral geniculate dorsal part (LGd), medial geniculate ventral part (MGv), anteroventral (AV) and anterodorsal (AD) thalamus (Table 1). The thalamic nuclei are subdivided into first-order nuclei, which relay sensory information to the cortex, and higher-order nuclei, which relay information from one cortical area to another. GluN2C therefore appears to be expressed in several first-order nuclei such

as VPM, VPL, LGd, MGv and AV, as well as in some higher-order nuclei such as MD, PO and AD. We also observed non-soma EGFP labeling in the barrel cortex, likely representing inputs from the ventrobasal thalamic nuclei (Figs. 1 and 2). We also compared the distribution of GluN2C using the EGFP reporter and β -gal reporter models to address similarities and potential differences in these models. As discussed earlier, one of the main differences in the two models is the expression of GluN2C in the PV-neurons in nRT, globus pallidus and other nuclei in basal ganglia circuitry ((Hillman et al., 2011); Fig. 3; Fig. 8). We also noted that some thalamic nuclei that showed robust EGFP cell body labeling were devoid of β -gal labeling, suggesting that there are additional differences between the two reporter models. For example, the anterior division of the thalamus and LGd are labeled in the EGFP model but not in the β -gal model, and in general, the degree of labeling was lower in the β -gal model for other thalamic nuclei (Figs. 7 and 8; Table 1). We also found that the labeling of neurons in retrosplenial cortex as has been previously described in the β -gal model was absent in the EGFP model (Fig. 7b). In contrast, we found a good degree of co-localization of β -gal with EGFP in hippocampal structures, striatum, SSC and dentate gyrus (Fig. 9). There were also EGFP-alone and β -gal-alone cells in these regions potentially reflecting differences in the transcription of the two alleles. A greater number of EGFP-alone labeled cells were found in some regions such as SSC and the cerebellar granule layer, suggesting potential selectivity in the expression of this allele. Thus, there are crucial differences in the distribution of GluN2C in the two reporter models (Table 1).

Discussion

Expression of GluN2C in PV-neurons in a subset of basal ganglia nuclei

Despite the proposed relationship of GluN2C-expressing PV-interneurons to the NMDA receptor hypofunction hypothesis in schizophrenia, the precise expression pattern of GluN2C subunit remains unknown. Using a reporter model, we found that GluN2C is expressed in PV-neurons of globus pallidus, substantia nigra, ventral pallidum and nRT whereas in the cortico-limbic region and striatum, GluN2C was localized to astrocytes (Schematic; Fig. 10). In this context, it has been found that the interneuron populations in cortico-limbic regions versus the globus pallidus may arise from distinct progenitors and/or mechanisms. Specifically, the ventral medial ganglionic eminence and dorsal preoptic area generate interneurons in the globus pallidus, and ablation of *Nkx2-1* from *Shh*-expressing domain eliminates most of the globus pallidus interneurons but does not affect cortical or striatal interneurons (Flandin et al., 2010; Nobrega-Pereira et al., 2010). Thus, a differential transcriptional programming in PV-neurons of the globus pallidus compared with the cortex and striatum may explain the heterogeneity of GluN2C expression in PV-neurons. We confirmed the functional expression of GluN2C in PV-neurons in the substantia nigra using electrophysiology and D-cycloserine, a superagonist at GluN1/2C receptors. Our ongoing studies also support GluN2C expression in PV-neurons in other basal ganglia nuclei (unpublished observation). Because the basal ganglia circuitry is critical for motor function and emotional behaviors, the regulation of PV-neurons in this circuitry by GluN2C may produce unique behavioral consequences.

Differences in the expression patterns in the EGFP and β -gal reporter models

We found a lack of labeling of PV-neurons in the β -gal reporter model. For example, the nRT neurons which are PV-positive and known to express GluN2C subunit (Wenzel et al., 1997; Lin et al., 1996; Zhang et al., 2012) were devoid of labeling in β -gal reporter model. The EGFP knock-in model was able to accurately demonstrate GluN2C expression in nRT PV-neurons as well as PV-neurons in several basal ganglia nuclei. These findings are supported by the *GRIN2C* mRNA analyses of mouse brain from the Allen Institute for Brain Institute, which shows expression patterns similar to our observations in the EGFP reporter model (Figure 1; Table 1). Importantly, we also confirmed expression of GluN2C in PV-neurons of SNr using electrophysiology and pharmacology. Another major difference in the two reporter models is the expression in neurons in the retrosplenial cortex. The β -gal reporter model, but not the EGFP reporter model, showed expression of GluN2C in the adult retrosplenial cortex ((Karavanova et al., 2007); present study). Interestingly, the Allen developing mouse brain atlas shows expression of *GRIN2C* mRNA in the retrosplenial cortex that appears to be neuronal. However, this expression is seen at an early developmental age (postnatal day 4) and is absent in adulthood in agreement with results from the EGFP reporter model. Thus, both lack of expression and ectopic expression occur in the β -gal reporter model. Overall, our analysis of GluN2C expression using the EGFP reporter model is consistent with the mRNA analysis from the Allen Institute for Brain Science (Table 1) and therefore the expression pattern revealed by the EGFP knock-in mice may represent a more accurate distribution pattern compared to the β -gal reporter model.

The expression patterns in the EGFP and β -gal reporter models most likely differ due to the difference in gene targeting strategies for reporter cassette insertion. In the EGFP-knock-in model the, *GRIN2C* gene is intact with none of the original elements deleted. On the other hand, 11 exons and their adjoining introns are deleted in the β -gal reporter and replaced with the reporter construct. It is known that gene transcription is not only regulated by the putative promoter region but also other elements that may reside elsewhere, including intronic regions. Thus, deletion of large gene elements in the β -gal reporter may alter normal transcription. Because such transcriptional programs may have cell-type specificity, the β -gal reporter may retain expression patterns in astrocytes and excitatory neurons in the thalamus and cerebellum, but may exclude expression from PV-neurons. The cell-type and region-specific expression of the GluN2C subunit, together with the differences between the two reporter models may provide interesting clues regarding regulators of *GRIN2C* gene transcription that may be evaluated in future studies.

Astrocytic expression of the GluN2C subunit

The present study revealed the expression of GluN2C in cortical, striatal and hippocampal astrocytes. Previous studies using the β -gal GluN2C reporter model also demonstrated the expression of GluN2C in non-neuronal cells in the cortex, striatum and amygdala and astrocytes in the hippocampus (Hillman et al., 2011; Karavanova et al., 2007), findings that are further supported by *in situ* studies (Schipke et al., 2001; Dzamba et al., 2013; Conti et al., 1996; Farb et al., 1995) as well as RNA seq studies in humans and mouse cortex and striatum (Cahoy et al., 2008; Zhang et al., 2014; Zhang et al., 2016; Gokce et al., 2016). Importantly, electrophysiological studies support expression of functional NMDA receptors

in cortical astrocytes containing GluN2C subunit (Lalo et al., 2006; Palygin et al., 2011). We found that loss of GluN2C led to abrogation of NMDA-induced currents in cortical astrocytes, further supporting this expression pattern. We have recently demonstrated that ablation of GluN2C leads to cortical abnormalities, including reduction in excitatory neurotransmission, increase in inhibitory synapses, and lower PV labeling (Gupta et al., 2016). A specific reduction in the number of vGluT1 puncta (expressed at cortico-cortical synapses) was observed. In addition, GluN2C KO mice have altered cortical oscillations and working memory, both in basal condition and in response to NMDA receptor antagonist (Hillman et al., 2011; Gupta et al., 2016). Together, our previous and present results raise the possibility of a unique role of astrocytic NMDA receptors in the development and regulation of cortical function. It will be of interest to test this hypothesis in the future using conditional models.

Comparative roles of GluN2C and GluN2D subunits and relevance to subunit composition

Finally, the observation that GluN2C is not expressed in cortical or hippocampal PV interneurons suggests distinct roles for GluN2C and GluN2D in different brain regions. While GluN2C and GluN2D are concurrently expressed in multiple regions, such as the GPe and SNc (Standaert et al., 1994; Wenzel et al., 1995; Wenzel et al., 1996; Wenzel et al., 1997; Kosinski et al., 1998), it remains to be seen whether these patterns of expression are in non-overlapping cell-types. Expression patterns of GluN2D suggest a potentially distinct pattern from GluN2C. For example, GluN2D is expressed in dopaminergic neurons in the SNc (Jones and Gibb, 2005; Brothwell et al., 2008) and excitatory neurons in the STN (Swanger et al., 2015; Standaert et al., 1994). Similarly, in the thalamus, several nuclei such as PO, VPM and VPL appear to be enriched in the GluN2C subunit, while GluN2D appears to be enriched in the midline thalamus, rhomboid nucleus, nucleus reunions, parafascicular nucleus and zona incerta. Some thalamic nuclei, such as nRT, MD and LGd, however, appear to express both GluN2C and GluN2D (Monyer et al., 1994; Wenzel et al., 1997; Watanabe et al., 1992; Buller et al., 1994; Yamasaki et al., 2014). Considering that both subunits have several similar electrophysiological properties but divergent deactivation rates (Traynelis et al., 2010; Vicini et al., 1998), it would be interesting to resolve the roles of individual subunits in the functioning of neurons in these regions. Relevant to this it will also be important to resolve the subunit composition of NMDA receptors in GluN2C enriched cell-types. This is critical since a large proportion of native NMDA receptors are predicted to be triheteromeric receptors and such subunit composition may affect both functional and pharmacological properties (Hansen et al., 2014). Electrophysiology studies have demonstrated that besides GluN2C subunit, cortical astrocytes contain GluN3 subunit (Lalo et al., 2006; Palygin et al., 2011). In contrast, the subunit composition of NMDA receptors in GluN2C-expressing PV-neurons of basal ganglia is unknown. There is also emerging evidence for synapse-specific subunit composition of NMDA receptors within the same cell, as in the case of nucleus reticularis neurons (Fernandez et al., 2017; Deleuze and Huguenard, 2016).

In conclusion, we have identified novel expression patterns for the GluN2C subunit in the basal ganglia circuitry, which may have important implications for motor and behavioral phenotypes. It will also be interesting to test the role of astrocytic NMDA receptors in

cortical function and the effect of selective modulation of GluN2C on circuit function and behavior.

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REFERENCES

- Akbadian S , Sucher NJ , Bradley D , Tafazzoli A , Trinh D , Hetrick WP , Potkin SG , Sandman CA , Bunney WE , Jones EG (1996) Selective alterations in gene expression for NMDA receptor subunits in prefrontal cortex of schizophrenics. *J Neurosci* 16:19–30.8613785
- Belforte JE , Zsiros V , Sklar ER , Jiang Z , Yu G , Li Y , Quinlan EM , Nakazawa K (2010) Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat Neurosci* 13:76–83.19915563
- Beneyto M , Meador-Woodruff JH (2008) Lamina-specific abnormalities of NMDA receptor-associated postsynaptic protein transcripts in the prefrontal cortex in schizophrenia and bipolar disorder. *Neuropsychopharmacology* 33:2175–2186.18033238
- Bradley A , Anastassiadis K , Ayadi A , Battey JF , Bell C , Birling MC , Bottomley J , Brown SD , Burger A , Bult CJ , Bushell W , Collins FS , Desaintes C , Doe B , Economides A , Eppig JT , Finnell RH , Fletcher C , Fray M , Friendewey D , Friedel RH , Grosveld FG , Hansen J , Herault Y , Hicks G , Horlein A , Houghton R , Hrabe de Angelis M , Huylebroeck D , Iyer V , de Jong PJ , Kadin JA , Kaloff C , Kennedy K , Koutsourakis M , Lloyd KC , Marschall S , Mason J , McKerlie C , McLeod MP , von Melchner H , Moore M , Mujica AO , Nagy A , Nefedov M , Nutter LM , Pavlovic G , Peterson JL , Pollock J , Ramirez-Solis R , Rancourt DE , Raspa M , Remacle JE , Ringwald M , Rosen B , Rosenthal N , Rossant J , Ruiz Noppinger P , Ryder E , Schick JZ , Schnutgen F , Schofield P , Seisenberger C , Selloum M , Simpson EM , Skarnes WC , Smedley D , Stanford WL , Stewart AF , Stone K , Swan K , Tadepally H , Teboul L , Tocchini-Valentini GP , Valenzuela D , West AP , Yamamura K , Yoshinaga Y , Wurst W (2012) The mammalian gene function resource: the International Knockout Mouse Consortium. *Mamm Genome* 23:580–586.22968824
- Brothwell SL , Barber JL , Monaghan DT , Jane DE , Gibb AJ , Jones S (2008) NR2B- and NR2D-containing synaptic NMDA receptors in developing rat substantia nigra pars compacta dopaminergic neurones. *J Physiol* 586:739–750.18033813
- Buller AL , Larson HC , Schneider BE , Beaton JA , Morrisett RA , Monaghan DT (1994) The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. *J Neurosci* 14:5471–5484.7916045
- Cahoy JD , Emery B , Kaushal A , Foo LC , Zamanian JL , Christopherson KS , Xing Y , Lubischer JL , Krieg PA , Krupenko SA , Thompson WJ , Barres BA (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. *J Neurosci* 28:264–278.18171944
- Catts VS , Lai YL , Weickert CS , Weickert TW , Catts SV (2016) A quantitative review of the postmortem evidence for decreased cortical N-methyl-D-aspartate receptor expression levels in schizophrenia: How can we link molecular abnormalities to mismatch negativity deficits? *Biol Psychol* 116:57–67.26549579
- Conti F , DeBiasi S , Minelli A , Melone M (1996) Expression of NR1 and NR2A/B subunits of the NMDA receptor in cortical astrocytes. *Glia* 17:254–258.8840166
- Deleuze C , Huguenard JR (2016) Two classes of excitatory synaptic responses in rat thalamic reticular neurons. *J Neurophysiol* 116:995–1011.27281752

- Dravid SM , Burger PB , Prakash A , Geballe MT , Yadav R , Le P , Vellano K , Snyder JP , Traynelis SF (2010) Structural determinants of D-cycloserine efficacy at the NR1/NR2C NMDA receptors. *J Neurosci* 30:2741–2754.20164358
- Dzamba D , Honsa P , Anderova M (2013) NMDA Receptors in Glial Cells: Pending Questions. *Curr Neuropharmacol* 11:250–262.24179462
- Farb CR , Aoki C , Ledoux JE (1995) Differential localization of NMDA and AMPA receptor subunits in the lateral and basal nuclei of the amygdala: a light and electron microscopic study. *J Comp Neurol* 362:86–108.8576430
- Fernandez LMJ , Pellegrini C , Vantomme G , Beard E , Luthi A , Astori S (2017) Cortical afferents onto the nucleus Reticularis thalami promote plasticity of low-threshold excitability through GluN2C-NMDARs. *Sci Rep* 7:12271–017–12552–8.28947779
- Flandin P , Kimura S , Rubenstein JL (2010) The progenitor zone of the ventral medial ganglionic eminence requires Nkx2–1 to generate most of the globus pallidus but few neocortical interneurons. *J Neurosci* 30:2812–2823.20181579
- Gokce O , Stanley GM , Treutlein B , Neff NF , Camp JG , Malenka RC , Rothwell PE , Fuccillo MV , Sudhof TC , Quake SR (2016) Cellular Taxonomy of the Mouse Striatum as Revealed by Single-Cell RNA-Seq. *Cell Rep* 16:1126–1137.27425622
- Gonzalez-Burgos G , Lewis DA (2012) NMDA receptor hypofunction, parvalbumin-positive neurons, and cortical gamma oscillations in schizophrenia. *Schizophr Bull* 38:950–957.22355184
- Gupta SC , Ravikrishnan A , Liu J , Mao Z , Pavuluri R , Hillman BG , Gandhi PJ , Stairs DJ , Li M , Ugale RR , Monaghan DT , Dravid SM (2016) The NMDA receptor GluN2C subunit controls cortical excitatory-inhibitory balance, neuronal oscillations and cognitive function. *Sci Rep* 6:38321.27922130
- Gupta SC , Yadav R , Pavuluri R , Morley BJ , Stairs DJ , Dravid SM (2015) Essential role of GluD1 in dendritic spine development and GluN2B to GluN2A NMDAR subunit switch in the cortex and hippocampus reveals ability of GluN2B inhibition in correcting hyperconnectivity. *Neuropharmacology* 93:274–284.25721396
- Hansen KB , Ogden KK , Yuan H , Traynelis SF (2014) Distinct functional and pharmacological properties of Triheteromeric GluN1/GluN2A/GluN2B NMDA receptors. *Neuron* 81:1084–1096.24607230
- Hill RA , Grutzendler J (2014) In vivo imaging of oligodendrocytes with sulforhodamine 101. *Nat Methods* 11:1081–1082.25357236
- Hillman BG , Gupta SC , Stairs DJ , Buonanno A , Dravid SM (2011) Behavioral analysis of NR2C knockout mouse reveals deficit in acquisition of conditioned fear and working memory. *Neurobiol Learn Mem* 95:404–414.21295149
- Homayoun H , Moghaddam B (2007) NMDA receptor hypofunction produces opposite effects on prefrontal cortex interneurons and pyramidal neurons. *J Neurosci* 27:11496–11500.17959792
- Ibrahim HM , Hogg AJ , Healy DJ , Haroutunian V , Davis KL , Meador-Woodruff JH (2000) Ionotropic glutamate receptor binding and subunit mRNA expression in thalamic nuclei in schizophrenia. *Am J Psychiatry* 157:1811–1823.11058479
- Jones S , Gibb AJ (2005) Functional NR2B- and NR2D-containing NMDA receptor channels in rat substantia nigra dopaminergic neurones. *J Physiol* 569:209–221.16141268
- Karavanova I , Vasudevan K , Cheng J , Buonanno A (2007) Novel regional and developmental NMDA receptor expression patterns uncovered in NR2C subunit-beta-galactosidase knock-in mice. *Mol Cell Neurosci* 34:468–480.17276696
- Khlestova E , Johnson JW , Krystal JH , Lisman J (2016) The Role of GluN2C-Containing NMDA Receptors in Ketamine's Psychotogenic Action and in Schizophrenia Models. *J Neurosci* 36:11151–11157.27807157
- Kosinski CM , Standaert DG , Counihan TJ , Scherzer CR , Kerner JA , Daggett LP , Velicelebi G , Penney JB , Young AB , Landwehrmeyer GB (1998) Expression of N-methyl-D-aspartate receptor subunit mRNAs in the human brain: striatum and globus pallidus. *J Comp Neurol* 390:63–74.9456176
- Lalo U , Pankratov Y , Kirchhoff F , North RA , Verkhratsky A (2006) NMDA receptors mediate neuron-to-glia signaling in mouse cortical astrocytes. *J Neurosci* 26:2673–2683.16525046

- Lein ES , Hawrylycz MJ , Ao N , Ayres M , Bensinger A , Bernard A , Boe AF , Boguski MS , Brockway KS , Byrnes EJ , Chen L , Chen L , Chen TM , Chin MC , Chong J , Crook BE , Czaplinska A , Dang CN , Datta S , Dee NR , Desaki AL , Desta T , Diep E , Dolbeare TA , Donelan MJ , Dong HW , Dougherty JG , Duncan BJ , Ebbert AJ , Eichele G , Estin LK , Faber C , Facer BA , Fields R , Fischer SR , Fliss TP , Frensley C , Gates SN , Glattfelder KJ , Halverson KR , Hart MR , Hohmann JG , Howell MP , Jeung DP , Johnson RA , Karr PT , Kawal R , Kidney JM , Knapik RH , Kuan CL , Lake JH , Laramee AR , Larsen KD , Lau C , Lemon TA , Liang AJ , Liu Y , Luong LT , Michaels J , Morgan JJ , Morgan RJ , Mortrud MT , Mosqueda NF , Ng LL , Ng R , Orta GJ , Overly CC , Pak TH , Parry SE , Pathak SD , Pearson OC , Puchalski RB , Riley ZL , Rockett HR , Rowland SA , Royall JJ , Ruiz MJ , Sarno NR , Schaffnit K , Shapovalova NV , Sivisay T , Slaughterbeck CR , Smith SC , Smith KA , Smith BI , Sodt AJ , Stewart NN , Stumpf KR , Sunkin SM , Sutram M , Tam A , Teemer CD , Thaller C , Thompson CL , Varnam LR , Visel A , Whitlock RM , Wohnoutka PE , Wolkey CK , Wong VY , Wood M , Yaylaoglu MB , Young RC , Youngstrom BL , Yuan XF , Zhang B , Zwingman TA , Jones AR (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445:168–176.17151600
- Lin YJ , Bovetto S , Carver JM , Giordano T (1996) Cloning of the cDNA for the human NMDA receptor NR2C subunit and its expression in the central nervous system and periphery. *Brain Res Mol Brain Res* 43:57–64.9037519
- Meador-Woodruff JH , Clinton SM , Beneyto M , McCullumsmith RE (2003) Molecular abnormalities of the glutamate synapse in the thalamus in schizophrenia. *Ann N Y Acad Sci* 1003:75–93.14684436
- Monyer H , Burnashev N , Laurie DJ , Sakmann B , Seeburg PH (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529–540.7512349
- Nakazawa K , Zsiros V , Jiang Z , Nakao K , Kolata S , Zhang S , Belforte JE (2012) GABAergic interneuron origin of schizophrenia pathophysiology. *Neuropharmacology* 62:1574–1583.21277876
- Nobrega-Pereira S , Gelman D , Bartolini G , Pla R , Pierani A , Marin O (2010) Origin and molecular specification of globus pallidus neurons. *J Neurosci* 30:2824–2834.20181580
- Palygin O , Lalo U , Pankratov Y (2011) Distinct pharmacological and functional properties of NMDA receptors in mouse cortical astrocytes. *Br J Pharmacol* 163:1755–1766.21449975
- Perszyk RE , DiRaddo JO , Strong KL , Low CM , Ogden KK , Khatri A , Vargish GA , Pelkey KA , Tricoire L , Liotta DC , Smith Y , McBain CJ , Traynelis SF (2016) GluN2D-Containing N-methyl-D-Aspartate Receptors Mediate Synaptic Transmission in Hippocampal Interneurons and Regulate Interneuron Activity. *Mol Pharmacol* 90:689–702.27625038
- Pettitt SJ , Liang Q , Rairdan XY , Moran JL , Prosser HM , Beier DR , Lloyd KC , Bradley A , Skarnes WC (2009) Agouti C57BL/6N embryonic stem cells for mouse genetic resources. *Nat Methods* 6:493–495.19525957
- Schipke CG , Ohlemeyer C , Matyash M , Nolte C , Kettenmann H , Kirchhoff F (2001) Astrocytes of the mouse neocortex express functional N-methyl-D-aspartate receptors. *FASEB J* 15:1270–1272.11344110
- Sheinin A , Shavit S , Benveniste M (2001) Subunit specificity and mechanism of action of NMDA partial agonist D-cycloserine. *Neuropharmacology* 41:151–158.11489451
- Skarnes WC , Rosen B , West AP , Koutsourakis M , Bushell W , Iyer V , Mujica AO , Thomas M , Harrow J , Cox T , Jackson D , Severin J , Biggs P , Fu J , Nefedov M , de Jong PJ , Stewart AF , Bradley A (2011) A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 474:337–342.21677750
- Standaert DG , Testa CM , Young AB , Penney JB (1994) Organization of N-methyl-D-aspartate glutamate receptor gene expression in the basal ganglia of the rat. *J Comp Neurol* 343:1–16.8027428
- Swanger SA , Vance KM , Pare JF , Sotty F , Fog K , Smith Y , Traynelis SF (2015) NMDA Receptors Containing the GluN2D Subunit Control Neuronal Function in the Subthalamic Nucleus. *J Neurosci* 35:15971–15983.26631477

- Traynelis SF , Wollmuth LP , McBain CJ , Menniti FS , Vance KM , Ogden KK , Hansen KB , Yuan H , Myers SJ , Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. *Pharmacol Rev* 62:405–496.20716669
- Vicini S , Wang JF , Li JH , Zhu WJ , Wang YH , Luo JH , Wolfe BB , Grayson DR (1998) Functional and pharmacological differences between recombinant N-methyl-D-aspartate receptors. *J Neurophysiol* 79:555–566.9463421
- von Engelhardt J , Bocklisch C , Tonges L , Herb A , Mishina M , Monyer H (2015) GluN2D-containing NMDA receptors mediate synaptic currents in hippocampal interneurons and pyramidal cells in juvenile mice. *Front Cell Neurosci* 9:95.25859181
- Watanabe M , Inoue Y , Sakimura K , Mishina M (1992) Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport* 3:1138–1140.1493227
- Weickert CS , Fung SJ , Catts VS , Schofield PR , Allen KM , Moore LT , Newell KA , Pellen D , Huang XF , Catts SV , Weickert TW (2012) Molecular evidence of N-methyl-D-aspartate receptor hypofunction in schizophrenia. *Mol Psychiatry*
- Wenzel A , Fritschy JM , Mohler H , Benke D (1997) NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. *J Neurochem* 68:469–478.9003031
- Wenzel A , Scheurer L , Kunzi R , Fritschy JM , Mohler H , Benke D (1995) Distribution of NMDA receptor subunit proteins NR2A, 2B, 2C and 2D in rat brain. *Neuroreport* 7:45–48.8742413
- Wenzel A , Villa M , Mohler H , Benke D (1996) Developmental and regional expression of NMDA receptor subtypes containing the NR2D subunit in rat brain. *J Neurochem* 66:1240–1248.8769890
- White JK , Gerdin AK , Karp NA , Ryder E , Buljan M , Bussell JN , Salisbury J , Clare S , Ingham NJ , Podrini C , Houghton R , Estabel J , Bottomley JR , Melvin DG , Sunter D , Adams NC , Sanger Institute Mouse Genetics Project, Tannahill D , Logan DW , Macarthur DG , Flint J , Mahajan VB , Tsang SH , Smyth I , Watt FM , Skarnes WC , Dougan G , Adams DJ , Ramirez-Solis R , Bradley A , Steel KP (2013) Genome-wide generation and systematic phenotyping of knockout mice reveals new roles for many genes. *Cell* 154:452–464.23870131
- Yamada K , Fukaya M , Shimizu H , Sakimura K , Watanabe M (2001) NMDA receptor subunits GluRepsilon1, GluRepsilon3 and GluRzeta1 are enriched at the mossy fibre-granule cell synapse in the adult mouse cerebellum. *Eur J Neurosci* 13:2025–2036.11422443
- Yamasaki M , Okada R , Takasaki C , Toki S , Fukaya M , Natsume R , Sakimura K , Mishina M , Shirakawa T , Watanabe M (2014) Opposing role of NMDA receptor GluN2B and GluN2D in somatosensory development and maturation. *J Neurosci* 34:11534–11548.25164652
- Zhang Y , Buonanno A , Vertes RP , Hoover WB , Lisman JE (2012) NR2C in the thalamic reticular nucleus; effects of the NR2C knockout. *PLoS One* 7:e41908.22848654
- Zhang Y , Chen K , Sloan SA , Bennett ML , Scholze AR , O'Keefe S , Phatnani HP , Guarnieri P , Caneda C , Ruderisch N , Deng S , Liddelow SA , Zhang C , Daneman R , Maniatis T , Barres BA , Wu JQ (2014) An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci* 34:11929–11947.25186741
- Zhang Y , Sloan SA , Clarke LE , Caneda C , Plaza CA , Blumenthal PD , Vogel H , Steinberg GK , Edwards MS , Li G , Duncan JA , Cheshier SH , Shuer LM , Chang EF , Grant GA , Gephart MG , Barres BA (2016) Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. *Neuron* 89:37–53.26687838

- GluN2C is expressed in parvalbumin-positive neurons in a subset of basal ganglia nuclei.
- GluN2C is localized to astrocytes in majority of telencephalon including cortex, hippocampus, striatum and amygdala.
- NMDA receptor currents in parvalbumin-positive neurons of substantia nigra and cortical astrocytes are GluN2C-dependent

Fig. 1. General expression pattern of EGFP(GluN2C).

Immunohistochemical labeling with EGFP antibody was performed. High expression of EGFP(GluN2C) was observed in cerebellar granule neurons and the thalamus (**a, b**).

Expression was also observed in the striatum, cortex and hippocampus (**c-e**) where the labeled cell bodies were smaller in size than those observed in the thalamus. Distinct cellular expression was also observed in the globus pallidus externa (GPe) (**f**), nucleus reticularis (nRT) (**g**) and substantia nigra reticulata and compacta (SNr and SNc) (**h**). The detected signal is EGFP driven by the endogenous *GRIN2C* promoter. St, Striatum; CTX, cortex; CA1, Cornu Ammonis 1; CA2, Cornu Ammonis 2; DG, dentate gyrus; Mol, molecular layer; VPL, ventral posteromedial thalamus; VPM, ventral posterolateral thalamus; PO, posterior complex

Fig. 2. Developmental expression pattern of EGFP(GluN2C).

The expression of EGFP (GluN2C) was tested across different ages. Expression was observed at all ages tested. At postnatal day 8, high expression was observed in the ventral thalamus. In general, the expression patterns looked very similar at ages 10–30 days, suggesting that the EGFP(GluN2C) expression reaches an adult-like pattern very early in development. No specific signal was observed in wildtype mice with no reporter cassette.

Fig. 3. Heterogeneity of EGFP(GluN2C) expression in parvalbumin-containing neurons. Co-labeling for PV and EGFP was performed. **a.** EGFP cells strongly co-localized with PV-neurons in the globus pallidus externa (GPe) and nucleus reticularis (nRT). **b.** No co-localization of PV with EGFP was observed in the hippocampal CA1, striatum, somatosensory cortex (SSC) or cerebellum.

Fig. 4. Expression of EGFP(GluN2C) in basal ganglia.

a. Expression of EGFP (GluN2C) and its co-localization with PV was tested in the ventral pallidum (VP), subthalamic nuclei (STN) and substantia nigra pars reticulata (SNr). Cell body staining of EGFP was observed in VP and SNr which co-localized with PV. In contrast, non-cell body labeling was observed in the STN, similar to PV expression in this region. **b.** PV-neurons in the substantia nigra were labeled by injecting AAV with a cre-dependent fluorescent marker. Whole-cell recordings were obtained at +40 mV and NMDA receptor responses were obtained by applying a puff of 200 µM glutamate in the presence of picrotoxin, TTX and CNQX. Effect of bath application of 300 µM D-cycloserine was evaluated. A significant increase in the peak amplitude of currents from PV-positive neurons and a reduction in currents from PV-negative neurons was observed upon D-cycloserine application (* $P < 0.05$, paired t-test, $n = 3$).

Fig. 5. Cortical EGFP(GluN2C) expression in astrocytes but not in neurons and microglia. Immunohistochemistry was performed for EGFP and markers for **a.** neurons (NeuN), **b.** inhibitory neurons (GABA), **c.** microglia (Iba1) and **d.** astrocytes (GFAP) in cortical regions. No co-localization of EGFP was found with NeuN, GABA and Iba1. EGFP co-localized with the astrocytic marker GFAP.

Fig. 6. Expression of EGFP(GluN2C) in astrocytes in cortex, striatum and hippocampus.

a. EGFP co-localization was observed with GFAP in the prefrontal cortex (PFC), dorsal striatum (DS) and hippocampus (HPC). **b.** Electrophysiology recordings from prefrontal cortex astrocytes in brain slices. The astrocytes were labeled by SR101 dye, and whole-cell voltage-clamp (holding potential of -60 mV) recordings were obtained from wildtype and GluN2C KO mice in the presence of $1\mu\text{M}$ tetrodotoxin, $100\mu\text{M}$ picrotoxin and $20\mu\text{M}$ NBQX. Application of NMDA ($50\mu\text{M}$) and glycine ($20\mu\text{M}$) to the bath led to inward currents in wildtype but not in GluN2C KO astrocytes ($n=5$).

Fig. 7. Thalamic nuclei-specific distribution of EGFP(GluN2C) and comparison between the EGFP reporter model and β-gal reporter model.

a. High expression of EGFP(GluN2C) was observed in the nucleus reticularis (nRT), posterior complex (PO), ventral posteromedial and posterolateral (VPM and VPL), mediodorsal thalamus (MD), ventrolateral (VL), ventromedial (VM), anterodorsal (AD), anteroventral (AV) and anteromedial (AM) nuclei. GluN2C expression was low in the zona incerta (ZI), and parafascicular thalamus (PF). The β-gal reporter model excluded EGFP(GluN2C) expression in several thalamic nuclei including nRT and the anterior thalamic nuclei. **b.** The β-gal reporter model exhibited expression in the retrosplenial cortex, which was absent in EGFP reporter model.

Fig. 8. Comparison of expression patterns in the EGFP and β-gal reporter models in the thalamus and globus pallidus.
a. Sections with expression patterns of reporters in the thalamus. Details are included in Table 1. **b.** The β-gal reporter model also excluded expression of GluN2C in other PV-expressing cells, such as the globus pallidus externa (GPe).

Fig. 9. Comparison between the EGFP reporter model and β -gal reporter model in cerebellum and cortico-limbic regions.

Co-localization studies were conducted in mice heterozygous for EGFP and the β -gal reporter. Several EGFP and β -gal co-localized cell bodies were observed in the cerebellar granule layer, striatum, somatosensory cortex (SSC) and dentate gyrus. In addition, both EGFP-alone and β -gal-alone cell bodies were observed suggesting differential expression of the two alleles in different cells. However, EGFP-alone cells were greater in number in the cerebellar granule cell layer and SSC.

Fig. 10. Schematic representation of GluN2C expression pattern in mouse brain.

Schematic depicting the cell-type specific expression of EGFP(GluN2C) in the mouse brain. EGFP(GluN2C) co-localization is observed with PV-neurons in the nucleus reticularis (nRT), globus pallidus externa (GPe), substantia nigra (SN) and ventral pallidum (VP). In the cortex, striatum and hippocampus, EGFP(GluN2C) expression was found in astrocytes

Table 1:
Distribution of EGFP(GluN2C) in novel reporter model.

The distribution of EGFP(GluN2C) signal in various brain regions is indicated. + expression, – lack of expression and ± sparse expression.

Region	EGFP signal in cell body	Cell type, localization of staining	Expression based on β-gal model [#]	Correlation to available mRNA expression data [^]
Telencephalon				
<i>Cortex</i>				
Prefrontal cortex and other cortical regions	+	Cell bodies of astrocytes [*]	+ (astrocytic [*])	+
Somatosensory cortex (SSC)	+	Cell bodies of astrocytes [*] ; also non-cell body labeling in barrels	+ (astrocytic)	+
Retrosplenial cortex	+	Cell bodies of astrocytes [*]	+ (primarily neuronal [*])	+ (appears astrocytic in adult but neuronal at postnatal day 4)
<i>Basal ganglia nuclei</i>				
Nucleus accumbens (NAc)	+	Cell bodies of astrocytes [*]	+	+
Dorsal striatum (DS)	+	Cell bodies of astrocytes [*]	+ (astrocytic)	+
Globus pallidus extemus (GPe)	+	Cell bodies of PV-neurons [*]	–	+
Globus pallidus intemus (GPi)	+	Cell bodies of PV-neurons [*]	–	+
Ventral pallidum (VP)	+	Cell bodies of PV-neurons [*]	–	+
Substantia nigra pars compacta (SNc)	+	Cell bodies of PV-neurons [*]	–	+
Substantia nigra pars reticulata (SNr)	+	Cell bodies of PV-neurons [*]	–	+
Other midbrain nuclei	–		–	–
<i>Limbic system nuclei</i>				
Hippocampus (CA1/CA2/CA3/DG/subiculum)	+	Cell bodies of astrocytes [*]	+ (astrocytic [*])	+
Basolateral amygdala	+	Cell bodies of astrocytes [*]	+ (astrocytic [*])	+
Diencephalon				
<i>Thalamus</i>				
Anteroventral (AV)	+	Cell bodies	+	+
Anterodorsal (AD)	+	Cell bodies	–	+
Anteromedial (AM)	+	Cell bodies	+	+
Thalamic reticular nucleus (nRT)	+	Cell bodies of PV-neurons [*]	–	+

Region	EGFP signal in cell body	Cell type, localization of staining	Expression based on β -gal model [#]	Correlation to available mRNA [^] expression data [^]
Ventral Posterolateral nucleus (VPL)	+	Cell bodies	+	+
Ventral Posteromedial nucleus (VPM)	+	Cell bodies	$\pm$	+
Ventral antero-lateral nucleus (VAL)	+	Cell bodies	+	+
Ventral medial nucleus (VM)	+	Cell bodies	$\pm$	+
Posterior thalamic nucleus (Po)	+	Cell bodies	+	+
Lateral dorsal nucleus (LD)	–	Cell bodies	–	–
Lateral posterior nucleus (LP)	$\pm$	Cell bodies	–	$\pm$
Lateral geniculate dorsal part (LGd)	+	Cell bodies	–	+
Lateral geniculate ventral part (LGv)	–	Cell bodies	–	–
Medial geniculate nucleus (MG)	+	Cell bodies	+	+
Subparafascicular nucleus	+	Cell bodies	+	+
Mediodorsal nucleus (MD)	+	Cell bodies	+	+
Zona Incerta (ZI)	–	Non-cell body strong fluorescent signal	–	–
Paraventricular thalamus (PVT)	–		–	–
Paracentral nucleus (PC)	–		–	$\pm$
Intermediodorsal thalamus (IMD)	–		–	–
Central medial thalamus (CM)	–		–	–
Central Lateral nucleus (CL)	–		–	$\pm$
Nucleus of reunions (RE)	–			
Parafascicular nucleus (Pf)	–		–	–
Subthalamic nucleus (STN)	–	Non-cell body strong fluorescent signal	–	–
Cerebellum				
Granular layer	+	Cell bodies	+	+
Molecular layer	–		–	–

* cell type confirmed by co-localization experiments with cell-type specific antibodies

[#] confirmed either in the present study, and/or by previous studies that used the β -gal reporter model (Karavanova et al., 2007; Hillman et al., 2011)

[^] Based on available ISH data bank generated by the Allen Institute for Brain Science (Lein et al., 2007)