Multiple Postsynaptic Protein Levels Are Maintained in a Delayed Form of Compensatory, Experience-Dependent Plasticity in Adult Superior Colliculus

Abbreviated title: Postsynaptic proteins unaffected by visual deprivation

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Abstract

Refinement of developing visual projections is reported to depend on visual experience in rats, cats, ferrets, and monkeys. Visual deprivation in these species delays visual pathway development, reportedly maintaining a juvenile condition and prolonging the critical period for plasticity. Deprivation has been associated with retention of immature NMDA and $GABA_A$ receptors and a juvenile state of chloride transporters. In contrast, receptive fields (RFs) in superior colliculus (SC) and visual cortex (V1) of Syrian hamsters refine normally with spontaneous activity alone, requiring visual experience only to maintain refined RFs in adulthood. Continued visual deprivation into adulthood leads to RF enlargement. This failure to maintain refined RFs and thus preserve high acuity vision in visually deprived adult SC is associated with a loss of GABA and GAD, caused at least in part by a reduction in BDNF-TrkB signaling. In order to explore the postsynaptic mechanism, we examined $GABA_A$ receptor expression levels, location, and subunit composition using Western blotting. In addition, we assayed the $GABA_A$ receptor anchoring protein gephyrin and expression levels of the chloride transporters KCC2 and NKCC2. To test the alternate hypothesis that RFs enlarge in adulthood as a result of immature postsynaptic NMDA receptors, we examined NR2A/2B ratios and levels of the anchoring protein PSD-95. We found no evidence supporting visual deprivation-related postsynaptic alterations in receptors, chloride transporters, or anchoring proteins in adult hamsters. Thus, we argue that visual deprivation continued past puberty reveals a new form of maladaptive, inhibitory plasticity in which, rather than retaining juvenile features and extending the critical period, continued lack of inhibition in adulthood reopens the critical period, reversing the refinement of receptive fields to the detriment of visual acuity. These results suggest that attempts to increase plasticity in adulthood for purposes of rehabilitation or recovery from injury should consider the possibility of unintended negative consequences.

Introduction

Receptive field development in hamster visual pathway is independent of external visual activity

During brain development, synaptic connections are elaborated and then refined to a mature state under the influence of neural activity. Activity due to sensory experience during an early "critical period" is important for shaping some aspects of neural circuit development. In the visual pathway, although spontaneous retinal activity is important for initial pruning, it is historically thought that early visual experience is essential for attainment of refined connectivity patterns (Firth et al., 2005; Katz and Shatz, 1996; Maffei and Galli-Resta, 1990; Meister et al., 1991; Ruthazer and Cline, 2004; Wong et al., 1993). Once these critical periods have closed, plasticity is often limited or even prevented, thus protecting refined circuits from destabilization (Hensch and Quinlan, 2018; Hubel and Wiesel, 1970; Hübener and Bonhoeffer, 2014; Pallas, 2017; Reh et al., 2020; Ribic, 2020; Takesian and Hensch, 2013). In some mammals, visual deprivation is reported to delay or prevent refinement, prolonging critical period plasticity (Cynader and Mitchell, 1980; Fagiolini et al., 1994; Lee and Nedivi, 2002; Mower, 1991; Mower et al., 1985; Nakadate et al., 2012). In apparent contradiction to this common view, spontaneous activity is sufficient for refinement of receptive fields in both visual cortex (V1) and superior colliculus (SC) in dark reared (DR) Syrian hamsters, and early light exposure is necessary only for maintenance of refinement in adulthood (Balmer and Pallas, 2015a; Carrasco and Pallas, 2006; Carrasco et al., 2005; Mudd et al., 2019). Pharmacological activation of TrkB receptors can mimic the effects of early light exposure in DR hamsters, resulting in long-term maintenance of refined receptive fields and visual acuity (Mudd *et al.*, 2019). Without early visual experience, lateral inhibition in SC declines and RFs expand in hamsters, leading to a decrease in visual acuity beginning at about two months of age (Balmer and Pallas, 2015a; Carrasco *et al.*, 2005; Mudd *et al.*, 2019).

Potential mechanisms for how early light maintains adult RF size.

We previously demonstrated that the expansion of RFs in SC of adult, DR subjects was associated with a loss of GABA immunoreactivity. Iontophoretic application of $GABA_A$ agonists *in vivo* restored RFs to an adult size (Carrasco et al., 2011). In addition to a loss of GABA-immunoreactivity, $GABA_A$ receptors in SC and V1 neurons were less effective than in normally reared hamsters (Balmer and Pallas, 2015b; Carrasco *et al.*, 2011).

Incomplete development of RF properties in V1 of visually deprived cats has been associated with a failure in developmental maturation of NMDA and $GABA_A$ receptors (Carmignoto and Vicini, 1992; Chen et al., 2000; Chen et al., 2001; Erisir and Harris, 2003). Although a failure to maintain refined RFs is a different phenomenon than a failure to refine them during a critical period, the mechanism(s) could be similar or convergent. We thus tested the hypothesis that maintenance of refined RFs in adulthood depends on maintenance of mature receptors and other postsynaptic signaling components. This hypothesis predicts that the detrimental, post-critical period receptive field plasticity observed in adulthood results from a deprivation-induced failure to maintain these postsynaptic proteins in their mature state. Contrary to this prediction, we find using Western blot assays that the subunit composition and localization of $GABA_A$ receptors in SC of adult dark reared hamsters with re-expanded RFs are like those seen in normally reared subjects. Furthermore, levels of the synaptic scaffolding proteins gephyrin and PSD-95 are normal, as are the adult expression levels of Cl^- pumps (KCC2/NKCC) and NMDA receptor subunits (NR2A/NR2B). These findings suggest that, although we saw a change in effectiveness of $GABA_A$ receptors previously using pharmacological agents (Balmer and Pallas, 2015b; Carrasco *et al.*, 2011), the loss of RF refinement in adulthood is primarily mediated by reductions in GABA expression in the presynaptic terminals rather than by substantial postsynaptic alterations. This result is at odds with the view that presynaptic changes in the ligand must occur together with corresponding postsynaptic changes in receptor levels. Taken together, our results rule out several hypotheses about the mechanistic basis of this newly identified, maladaptive form of adult neural plasticity, and provide insights into regulation of critical period plasticity that could lead to novel treatment targets for memory disorders and traumatic brain injury.

Materials and Methods

Subjects

A total of 42 adult Syrian hamsters (Cricetidae: *Mesocricetus auratus*) of both sexes were bred within our animal facility and used as subjects in this study. Syrian hamsters are an altricial rodent species that is ideal for studying the developing visual system due to their robust and well-characterized visual responses, short gestation time, and large litters (Carrasco *et al.*, 2005; Chalupa, 1981; Huck et al., 1988; Pratt and Lisk, 1989; Razak et al., 2003). Sexual maturity in this species occurs between P30 and P45 (Schulz and Sisk, 2006). Breeding females were singly housed. Males were introduced and supervised until intromission was observed, after which they were removed. Weanling and older hamsters were housed in single sex social groups of up to 5 adults per cage in standard rodent cages with a variety of enrichment items. Running wheels were not provided because they have been shown to alter the timing of visual cortical plasticity (Baroncelli et al., 2012; Kalogeraki et al., 2014; Tognini et al., 2012). All subjects were provided with *ad libitum* access to rodent chow and water.

Treatment groups

All animals in this study were bred in-house in order to control sensory experiences throughout development. Dams of dark reared (DR) subjects were transferred into total darkness 1-3 days before parturition. An antechamber and light-impenetrable black curtain separated the dark housing room from the hallway, ensuring any accidental openings of the hallway doors did not affect the housing room. Within the dark room, animals were housed inside light-tight stackable cages with a standard HVAC filtration system consistent with the other animal rooms in the animal facility. During general animal husbandry purposes the hamsters were exposed to dim red light at a wavelength not visible to Syrian hamsters (Huhman and Albers, 1994). Normally reared (NR) hamsters were maintained in a standard 12/12 light cycle room from before birth, through the duration of the critical period for RF refinement

maintenance in SC and V1 (P33-P40) (Balmer and Pallas, 2015a; Carrasco *et al.*, 2005; Mudd *et al.*, 2019), and into adulthood.

Western blotting

Animals were euthanized with a sodium pentobarbital-and phenytoin sodium-containing mixture (Euthasol >150 mg/kg IP). Brains were immediately extracted and frozen in 2-methylbutane on dry ice, then stored at -80°C or immediately dissected for preparation of lysates. Individual left and right superficial SC were excised and lysed in RIPA buffer (150mM NaCl, 150mM Tris, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) containing 2% Halt protease inhibitor (ThermoFisher Scientific). Proteins were quantified using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific), mixed with 2X sample buffer, and boiled for 15 minutes at 60 °C. Twenty µg of protein was loaded per well in pre-cast Bio-Rad gels and electrophoresis was carried out at 110 V for 90 minutes in a Bio-Rad electrophoresis tank. Proteins were then transferred onto nitrocellulose membranes at 70 V for 75 minutes, blocked in BSA for 1 h, and probed with primary antibodies overnight. Primary antibodies used in this study included: rabbit anti-$GABA_A$Rα1 (1:1000, Cat#: AGA-001, Alomone Labs); rabbit anti-$GABA_A$Rα2 (1:1000, Cat#: ab72445, Abcam); rabbit anti- $GABA_A$Rα5 (1:1000, Cat#: ab10098, Abcam); rabbit anti-Gephyrin (1:1000, Cat#: ab32206, Abcam); mouse anti-PSD-95 (1:1000, Cat#: ab2723, Abcam); mouse anti-KCC2 (1:1000, Cat#: 75-013, NeuroMab); rabbit anti-NKCC1 (1:1000, Cat#: ab59791, Abcam); rabbit anti-NMDAR2A (1:1000, Cat#: ab133265, Abcam); mouse anti-NMDAR2B (1:1000, Cat#: ab93610, Abcam); and mouse anti-GAPDH (1:1000, Cat#: 600-GAPDH, PhosphoSolutions). Protein bands were labeled using either appropriate IR dye fluorescent secondaries (Li-Cor) or appropriate HRP-conjugated secondary antibodies and imaged on an Odyssey CLx fluorescent imaging system (Li-Cor) or developed with enhanced chemiluminescent (ECL) substrate in a Bio-Rad ChemiDoc Imager. Protein expression levels were quantified as the optical density of the labeled protein normalized against the optical density of the housekeeping protein GAPDH using ImageJ.

Statistical Analysis

A Student's *t*-test was used to compare parametric data with equal variance between treatment groups and a normally distributed control data set. In the case of non-parametric data that were not normally distributed and/or exhibited unequal variance, a Mann-Whitney Rank Sum (U) test was employed. Descriptive statistics for these analyses are provided as mean ± standard error of the mean (SEM) in the text.

Results

Normal brain function relies on the precise regulation of excitatory and inhibitory neuronal activity. Although neuronal signaling is initiated by excitatory, glutamatergic transmission, inhibitory, GABAergic transmission is arguably more important for shaping activity-dependent events later in development, primarily because it prevents circuits from becoming overexcited (Bannai et al., 2009; Hensch et al., 1998; Huang, 2009). The circuits underlying RFs in hamster SC and V1 require early visual experience working through activation of TrkB receptors to maintain their refinement in adulthood (Balmer and Pallas, 2015a; Carrasco *et al.*, 2005; Mudd *et al.*, 2019; Pallas, 2017). The failure to maintain RF refinement in DR animals involves deficits in overall GABA expression and $GABA_A$ receptor function (Carrasco *et al.*, 2011), leading to a loss of lateral inhibition and thus expansion of RFs. In this study we explored several possible ways that visual deprivation during a critical period for RF refinement could affect $GABA_A$ receptor efficacy and other aspects of postsynaptic inhibitory synaptic function in adult SC.

Dark rearing does not affect the subunit composition of $GABA_A$ receptors in adult SC

Previously we showed in DR hamsters that although GAD and GABA-ir declines and $GABA_A$ receptors are less efficient when tested pharmacologically (Carrasco *et al.*, 2011), the level of $GABA_A$ receptor protein as assessed with Western blots for the alpha1 subunit does not change (Mudd *et al.*,

2019). These results raised the question of whether subunit composition of $GABA_A$Rs might be altered by visual deprivation in a way that reduced their effectiveness. Deprivation-induced alterations in both $GABA_A$ and NMDA receptor levels in cat visual cortex have been reported previously and were proposed to be involved in functional deficits (Carmignoto and Vicini, 1992; Chen *et al.*, 2000; Chen *et al.*, 2001; Mower et al., 1986).

$GABA_A$ receptors function as a primary mediator of phasic inhibition in the adult brain. They are pentameric receptors consisting of five subunits grouped around a central chloride ion pore, and the functional characteristics of the receptor largely depend upon the subunit composition (Sigel et al., 1990) and organization (Minier and Sigel, 2004). Of the many subunit arrangements, alpha1 and alpha2 subunits have been linked to synaptic localization of $GABA_A$ receptors, however these two subunit types have different kinetics and are expressed at different points in development. In mice, rats, and cats, receptors containing the alpha2 subunit are widely expressed at birth, whereas alpha1 has low expression and is restricted to a few brain areas (Chen *et al.*, 2001; Dunning et al., 1999; Fritschy et al., 1994; Laurie et al., 1992). During the first several postnatal weeks, alpha1 expression sharply increases, coinciding with a reduction in alpha2 expression. This alpha2-alpha1 switch in dominant subunit expression underlies a developmental decrease in inhibitory postsynaptic current (ipsc) decay time and an increase in ipsc amplitude (Fritschy *et al.*, 1994; Okada et al., 2000; Yu et al., 2006). We reasoned that the alpha2-alpha1 switch, if reversed in adulthood, could underlie the reduction in $GABA_A$ receptor function that we have previously observed in our studies of RF refinement loss in SC {Carrasco, 2011 #9724}. We explored this possibility by examining the expression of each subunit in synaptosome preps of SC in normally reared and visually deprived adults. Hamsters in the visual deprivation group were dark reared from birth. We used Western blotting to measure the amount of synaptic membrane-bound alpha1 and alpha2 $GABA_A$ receptor expression in adult SC. We found that there were no significant differences in either the overall levels of alpha2 protein (NR: 1.21 ± 0.052, n=8; DR: 1.33 ± 0.185, n=8; U=26, n=8, p=0.574; Mann-Whitney Rank Sum Test) **(Figure 1A)**, or the ratio of alpha1/alpha2 expression in the SC between adult DR (1.651 ± 0.277, n=5) and NR hamsters (1.19± 0.084, n=4) (T(7)= -1.436, n=4, p=0.194

Student's t-test) **(Figure 1B)**. These findings argue against a reversion of the normal developmental transition from alpha2 to alpha1 dominant expression as a cause of the deprivation-induced RF enlargement in adult SC and argue that visual experience is not needed to maintain mature $GABA_A$ receptors.

$GABA_A$ receptors can also be expressed extrasynaptically, where they can be activated by ambient GABA derived from synaptic spillover or non-neuronal sources. This low concentration GABA source generates "tonic" inhibition (Farrant and Nusser, 2005), and constitutes a significant (75%) percentage of the total inhibitory forces acting on neurons in hippocampus (Magnin et al., 2019; Mody and Pearce, 2004). Alpha5 subunit-containing receptors are primarily expressed extrasynaptically and have been implicated in regulating the induction of synaptic plasticity for LTP in hippocampus (Saab et al., 2010; Zurek et al., 2012; 2014). To investigate the possible role of alpha5 levels in adult RF maintenance we performed Western blotting and compared between DR (0.527 ± 0.0671, n=11) and NR hamsters (0.554 ± 0.0549, n=11) ($t(20) = 0.308$, $p=0.761$, Student's t-test) **(Figure 2A)**. We found no significant differences in alpha5 protein levels between groups. We also compared the ratio of alpha1/alpha5 between NR (1.027 ± 0.0815, n=10) and DR (0.995 ± 0.0926, n =9) hamsters and found no differences between these groups ($U(18) =35$, $p=0.438$, Mann-Whitney Rank Sum test) **(Figure 2B)**. These results suggest that the localization of alpha1 and alpha5 subunit-containing $GABA_A$ receptors in SC is not being modulated by early visual experience and argue against a deprivation-induced shift in tonic vs synaptic inhibition.

The regulation of $GABA_A$ receptors at the synapse is pivotal for maintaining correct levels of inhibitory synaptic transmission and overall physiological function (Jacob et al., 2008). Impaired trafficking of $GABA_A$ receptors into and out of the membrane could affect their synaptic localization in SC and thus their overall response to synaptically released GABA. $GABA_A$ receptor trafficking is partially regulated by endocytosis: the controlled removal of receptors from the membrane. Receptors are subsequently reinserted into the membrane or undergo lysosomal degradation after longer periods (Kittler et al., 2004). We reasoned that if endocytosis were dysregulated, either by decreased receptor reinsertion

or increased receptor degradation, it could negatively impact the efficacy of $GABA_A$ receptors at the synapse. We examined this possibility by comparing the ratio of membrane-bound to cytosolic alpha1 subunit containing receptors between our treatment groups. We observed no differences in the membrane/cytosolic ratio of alpha1 expressing receptors between DR (0.768 ± 0.044, n=7) and NR (0.778 ± 0.1, n=6) adult hamsters (U(11)= 20.00, p=0.945, Mann-Whitney Rank Sum test) **(Figure 3A)**.

We also examined the possibility that extrasynaptic alpha5 receptor endocytosis-mediated trafficking may be dysregulated and responsible for changes in tonic $GABA_A$ inhibition (Davenport et al., 2021). There were no significant differences in cytosolic or membrane localization of alpha5 subunits between DR (1.320 ± 0.198, n=8) and NR groups (1.753 ± 0.449, n=6) (U(12)=19.00, p=0.573, Mann-Whitney Rank Sum test) **(Figure 3B)**. These results indicate that the overall exo- and endocytosis-mediated trafficking of alpha1 (synaptic) and alpha5 (extrasynaptic) subunit expressing GABA receptors is not responsible for the decreased efficacy of $GABA_A$ receptors observed in RFs that fail to maintain refinement following dark rearing.

Inhibitory and excitatory scaffolding proteins in SC are not affected by dark rearing

One factor influencing the accumulation and confinement of $GABA_A$ receptors at postsynaptic sites is the membrane scaffolding protein gephyrin (Jacob et al., 2005b; Kneussel et al., 1999; Sun et al., 2004; Tretter et al., 2008). Decreased expression of gephyrin results in less clustering (Essrich et al., 1998) and more overall mobility of $GABA_A$ receptors at the synapse (Jacob et al., 2005a). We surmised that decreased gephyrin expression could be responsible for the weaker $GABA_A$ receptor signaling we have observed in neurons with RFs that fail to maintain refinement. Using Western blotting we compared membrane-bound gephyrin expression between DR and NR adults. DR adults (0.786 ± 0.124, n=17) were similar to NR adults (0.736 ± 0.158, n=16) (U(111)=-0.247, p=0.377, Mann-Whitney Rank Sum test) **(Figure 4A)**, a surprising result considering our finding that dark rearing reduces overall GABAergic inhibition. This result indicates that adult gephyrin expression is not affected by dark rearing during the

critical period for maintenance of RF refinement and suggests that if $GABA_A$ receptor accumulation and trafficking is being affected, then it is in a gephyrin-independent manner.

PSD-95 is the primary excitatory (AMPA and NMDA) receptor scaffolding protein in neurons (Chen et al., 2015), and functions in a similar way as gephyrin does for GABA receptors. Although it does not have a direct impact on $GABA_A$ receptor function, PSD-95 has been shown to have an influence on visual plasticity. For example, mice lacking PSD-95 have lifelong ocular dominance plasticity in V1 that results from an increase in the overall expression of silent (AMPA-free) synapses, but normal inhibitory tone (Huang et al., 2015). We examined the possibility that the plasticity involved with the dark rearing induced re-enlargement of RFs could also be mediated by a reduction in adult PSD-95 expression. We found that PSD-95 was not significantly different in DR (0.613± 0.96, n=10) compared to NR adult hamsters (0.486 ± 0.868, n=9) (U(31)=-0.978, p=0.270, Mann-Whitney Rank Sum test) **(Figure 4B)**. These results suggest that a PSD-95 mediated return to silent synapses does not underlie the re-enlargement of RFs in SC following early dark rearing.

NMDA receptors in adult SC have mature subunit compositions despite dark rearing

Excitatory plasticity could also contribute to enlargement of RFs during DR (Huang and Pallas, 2001). In V1 and SC, dark rearing disrupts the substitution of the NMDA receptor NR2A subunit for the NR2B subunit that normally occurs postnatally, limiting NMDAR-dependent excitatory plasticity (due to the shorter open time of NR2A) (Carmignoto and Vicini, 1992; Philpot et al., 2001; Townsend et al., 2004; Zhao and Constantine-Paton, 2007). Thus, lower NR2A expression relative to NR2B could cause RFs to expand through potentiation of excitatory synapses. Our results indicate that the total expression of NR2A in NR (0.680 ± 0.073, n=8) and DR (0.798 ± 0.109, n=6) adult hamsters are similar, as are the expression levels of NR2B (NR: (0.431 ± 0.0445, n=8) DR: (0.484 ± (0.0406)) (T(12)=-0.842, p=0.416, t-test) **(Figure 5A, B)**. The ratio of NR2A/NR2B expression between DR (1.829 ± 0.186, n=5) and NR hamsters (1.440 ± 0.112, n=6) was also similar in adult SC (T(9)=-1.862, p=0.096, t-test) **(Figure 5C)**.

Chloride pumps undergo their normal developmental switch in adult dark reared subjects

Inhibitory GABAergic signaling in neurons is dependent upon the intracellular chloride (Cl^-) concentration. The K^+ Cl^- co transporter (KCC2) is responsible for regulating intracellular Cl^- in mature adult neurons by an outward K^+ current (Rivera et al., 1999), and also regulates the formation, function, and plasticity of glutamatergic synapses (Chevy et al., 2015; Gauvain et al., 2011; Li et al., 2007). Early in development $GABA_A$ receptors are excitatory because the Na^+-K^+-$2Cl^-$ co-transporter 1 (NKCC1) (which mediates Cl^- uptake) is dominant. During the first several weeks after birth in rats NKCC1 is replaced by KCC2 as the dominant Cl^- pump in the brain, shifting $GABA_A$ receptors to become inhibitory (Rivera *et al.*, 1999). Recent findings reveal that in V1, the developmental switch from dominant NKCC1 to dominant KCC2 occurs at the same time as a period of BDNF/TrkB mediated synaptic imbalance – a crucial critical period for the transition of immature neurons to a more mature state (Zhang et al., 2018). We surmised that a shift in the ratio of KCC2:NKCC1 could underlie the reopening of plasticity for RF size refinement in adulthood, leading to re-enlargement of RFs in SC. We examined the expression of KCC2 and NKCC1 in adult SC neurons and observed no significant differences between our treatment groups. KCC2 levels were not significantly different between NR (0.979 ± 0.115, n=8) and DR groups of hamsters (0.963 ± 0.154, n=8) ($T(14)=0.082$, $p=0.936$, t-test) **(Figure 6A)**. The same was true of NKCC1 levels (NR 1.050 ± 0.0419, n=8; DR 1.081 ± 0.0814, n=8) ($T(14)=-0.339$, $p=0.740$, t-test) **(Figure 6B)**, or in the ratio of the two chloride pumps within groups ($T(8)=1.096$, $p=0.305$, t-test) **(Figure 6C)**.

Discussion

Maturation of GABAergic signaling in visual cortex, particularly of the fast-spiking, parvalbumin-containing basket cells, is thought to open and then close the critical period for plasticity (Capogna et al., 2021; Fagiolini et al., 2004; Sale et al., 2010; Toyoizumi et al., 2013). The retinorecipient layers of the superior colliculus have no basket cells and contain very few GABAergic

parvalbumin neurons, and the plasticity described here occurs after the critical period should have closed, suggesting that SC may accomplish plasticity through a different mechanism than visual cortex. On the other hand, our previous studies found that, as in visual cortex (Galuske et al., 2000; Hanover et al., 1999; Huang et al., 1999; Viegi et al., 2002), receptive field plasticity in adult SC is mediated by the BDNF receptor TrkB (Mudd et al., 2019) and a loss of GABA (Balmer and Pallas, 2015b; Carrasco et al., 2011), arguing for mechanistic elements in common. In our previous study of changes in inhibitory signaling associated with deprivation induced RF re-expansion we also described reduced $GABA_A$ receptor efficacy in response to iontophoretically-applied $GABA_AR$ agonists and antagonists in visually deprived compared to normally reared adults (Carrasco et al., 2011; Balmer and Pallas, 2015b). A combined pre- and postsynaptic alteration of synaptic strength has been seen in other sensory deprivation paradigms, including in dark reared and monocularly deprived visual cortex, although with an earlier time course (Carmignoto and Vicini, 1992; Chen et al., 2000; Chen et al., 2001; Villa et al., 2016). The goal of this study was to examine potential postsynaptic mechanisms through which light exposure during an early critical period ensures the long-term stability of visual receptive fields in the superior colliculus. Although our previous results established a correlation between maintenance of RF refinement and GABA expression levels in SC (Carrasco et al., 2011; Mudd et al., 2019) and V1 (Balmer and Pallas, 2015b), potential postsynaptic changes in protein levels had not been examined. We have reported here that at the resolution level of Western blots, visual deprivation-induced failure to maintain refined RFs in SC does not appear to involve changes in $GABA_AR$ or NMDAR subunit composition, inhibitory or excitatory scaffolding protein expression, or chloride pump ratios. These results exclude several possible mechanisms that could explain the reduced $GABA_AR$ signaling reported in DR adult SC (Carrasco et al., 2011), and emphasize that activity-dependent regulation of GABA expression is the primary mechanism underlying TrkB mediated RF refinement maintenance (Mudd et al., 2019). The finding that a change in GABA levels could affect neural plasticity in adulthood has important implications for treatment of memory impairments or brain injury in adults.

A GABA-BDNF feedback loop maintains inhibitory networks, thereby maintaining RF refinement in adulthood

GABA-$GABA_A$R interaction is known to regulate various downstream signaling pathways, and a major regulator of GABA itself is BDNF-TrkB signaling. Our data are consistent with previous studies suggesting a positive feedback loop between the BDNF-TrkB pathway and GABA expression during early development, in which GABA facilitates BDNF expression, and BDNF facilitates the synaptic release of GABA (Gianfranceschi et al., 2003; Hanno-Iijima et al., 2015; Huang et al., 1999; Jovanovic et al., 2004; Kuczewski et al., 2008; Morales et al., 2002; Porcher et al., 2011), in this case maintaining RF size and visual acuity (Mudd et al., 2019). Signaling via the MAPK cascade and the transcription factor cAMP-responsive element-binding protein (CREB) appears to play a substantial role in this process (Obrietan et al., 2002). In early development, GABA and TrkB pathways are co-active and affect multiple cellular actions, including proliferation, migration, and synaptic plasticity (Porcher et al., 2011). BDNF-TrkB interaction leads to dimerization and auto-phosphorylation of TrkB, thereby triggering MAPK, PLC gamma, and PI3K pathways (Yoshii and Constantine-Paton, 2007)**.** These pathways in turn lead to the activation of downstream effectors and mediators to initiate a CREB-dependent transcription process that can lead to an increase in $GABA_A$R levels as well as more BDNF production (Esvald et al., 2020; Huang and Reichardt, 2003; Porcher et al., 2011; Yoshii and Constantine-Paton, 2010). In addition, an increase in the transmembrane localization of $GABA_A$R is mediated by BDNF-dependent inhibition of receptor endocytosis in addition to ongoing reinsertion of the receptor into the membrane (Porcher et al., 2011)**.** This positive feedback regulation is critical in developing neurons and hence constituted a major motivation for the work reported here. In this study, neither the $GABA_A$R subunit composition at the synapse nor subunit composition in the extrasynaptic regions was affected by dark rearing, however. NMDA receptors and chloride transport proteins (KCC2, NKCC2) also remained unchanged from normal in level and composition. This leads to the possibility that GABA expression levels alone are the key factor in RF re-enlargement, as our previous results have supported (Balmer and Pallas, 2015b; Carrasco et al., 2011; Mudd et al., 2019), and suggests that this example of delayed

plasticity after early lack of sensory experience occurs through a different mechanism than described in other types of plasticity resulting from sensory deprivation.

Early visual experience is not necessary for maturation or maintenance of $GABA_A$ receptor subunit composition at the synapse

Synaptic inhibition throughout the central visual pathway is mediated primarily via the neurotransmitter GABA. $GABA_A$ receptors contain fast acting chloride (Cl^-) channels (Pfeiffer et al., 1982; Sigel et al., 1982), are part of the cys-loop ligand-gated ion-channel family and are comprised of a pentameric structure in which five subunits are arranged around a central pore. The subunit composition of $GABA_A$ receptors changes during development from an alpha 2 to alpha 1 dominant condition (Chen et al., 2001; Fritschy et al., 1994; Laurie et al., 1992) and also changes in some disease states (Deidda et al., 2015; Kimoto et al., 2015; Levitt, 2005; Schmidt and Mirnics, 2015; Tang et al., 2021; Tyson and Anderson, 2014) in a way that affects their functional properties (Farrant and Nusser, 2005) and localization (Jacob et al., 2005a). We studied synaptic levels of the $GABA_A$ receptor alpha1 and alpha2 subunits to quantify their expression levels under normal conditions. The overall levels of $GABA_AR$ alpha1 and $GABA_AR$ alpha2 were not altered in DR hamsters when compared to those of NR hamsters, arguing that the altered inhibitory synaptic efficacy that we previously observed was not caused by an immature $GABA_AR$ subunit composition at the synapse.

An increase or decrease in level of any receptor subunit is best understood in context, because different conclusions would be drawn if subunits change independently or in concert. Thus, we also analyzed the alpha1/alpha2 ratios in individual animals. We did not find any change in alpha1/alpha2 ratios in NR compared to DR adult hamsters. These results suggest that early visual experience is not necessary for maturation or for maintenance of mature synaptic $GABA_AR$ subunit composition in adulthood. Thus, the failure to maintain refined RFs cannot be explained by a return to a juvenile-type of $GABA_AR$ subunit arrangement.

Level and localization of the extrasynaptic GABA$_A$R subunit alpha 5 does not change with visual deprivation

GABA$_A$R subunit alpha 5, which is predominantly an extrasynaptic membrane receptor subunit that regulates tonic inhibition, is important in neuronal circuit development, learning and memory (Brady and Jacob 2015), has been implicated in regulating the induction of synaptic plasticity for memory in hippocampus (Saab et al., 2010; Zurek et al., 2012; 2014), and can relocate to the synaptic region in learning and memory deficits (Brady and Jacob 2015, Davenport, Rajappa et al. 2021). Because the excitation/inhibition (E/I) balance could be affected if alpha5 subunit levels changed or if it diffused into the synapse, we compared its expression between NR and DR cases. We did not see any significant changes in the overall levels of GABA$_A$R alpha5, or the alpha1/alpha5 ratio, suggesting that dark rearing-induced RF enlargement is not caused by E/I imbalance via extrasynaptic receptor subunit changes.

Visual deprivation does not affect the rate of endocytosis of GABA$_A$R subunits at the synapse

Because clathrin-dependent endocytosis is likely important for regulating inhibitory signaling and synaptic plasticity (Kittler et al., 2000), we examined the endocytosis of GABA$_A$ receptor alpha1 and alpha5 subunits in normally reared and visually deprived subjects. We did not observe a significant change in endocytosis of either subunit type as assayed by the ratio of membrane-bound to cytosolic fractions. This implies that visual deprivation does not affect the trafficking of the GABA$_A$R subunits between the membrane and the cytosol or the phosphorylation events that maintain the balance between endocytosis and membrane insertion of the receptor subunits.

Early visual experience is not necessary to maintain scaffolding protein levels at the synapse

A key finding of this work is that the expression levels of the postsynaptic scaffold proteins PSD-95 and gephyrin were not altered in adulthood following early visual deprivation, suggesting that any changes in inhibitory function are probably not caused by a gross change in scaffolding protein expression.

Alternatively, the clustering of $GABA_A$Rs at inhibitory synapses in SC may happen in a gephyrin-independent manner (Zita et al., 2007), or total gephyrin expression may not be as important as the formation of gephyrin nanodomains within inhibitory synapses (Pennacchietti et al., 2017). Future studies with different techniques would be required to determine if changes in receptor clustering may be occurring and to what extent gephyrin or PSD-95 may have in mediating any such effects.

Early light exposure is not necessary for maturation of chloride transporters

Given the role that chloride transporters play in maintaining chloride balance inside the neurons and thus in maintaining the E/I balance, we decided to investigate the status of the chloride transporters, KCC2 and NKCC1. It is possible that the chloride pumps revert to their early developmental state, leading to disturbed E/I balance and more excitation in dark reared animals, possibly explaining the RF expansion we observed. We did not observe any changes in the chloride transporters in dark-reared animals compared to normally reared animals, suggesting that the changes in E/I balance due to lack of early experience were not caused by alterations in chloride transporters.

Early light exposure is not necessary for maturation of NMDAR subunit composition

Given that the levels of the important components of synaptic GABAergic inhibition did not differ significantly in normally reared and dark reared conditions, we wanted to investigate if increased excitation is responsible for RF expansion in dark reared animals. We observed that early light experience does not affect overall levels of the NMDAR subunits NR2A and NR2B, and that the NR2A/NR2B ratio in dark reared animals resembles those of the normally reared animals, suggesting that early visual experience does not affect NMDAR subunit composition at the synapse (Monyer et al., 1994).

A few alternative explanations need to be investigated to understand this form of adult neural plasticity. It is possible that the changes leading to the RF expansion and thus the visual acuity deficits take place earlier than the time point that we studied. We should also consider the possibility that

although we studied the most prominent postsynaptic factors, it is possible that other scaffolding proteins and their partners need to be examined, for example gephyrin binding partners including $GABA_AR$ beta2 and beta3 subunits (Kowalczyk et al., 2013), the scaffold protein radixin that binds $GABA_AR$ alpha5 subunit to the actin cytoskeleton (Loebrich et al., 2006) and regulates synaptic $GABA_AR$ density (Hausrat et al., 2015), and gephyrin post-translational modification events that influence inhibitory synaptic plasticity by affecting postsynaptic scaffolding (Zacchi et al.).

In summary, our results argue that visual experience is not necessary to maintain mature levels and composition of these several postsynaptic proteins that are essential for retino-SC synaptic communication, although presynaptic GABA release accounts for a loss of inhibition. Unlike many previous sensory deprivation studies of visual cortex in which both GABA and $GABA_A$ receptors are downregulated (Carmignoto and Vicini, 1992; Chen et al., 2000; Chen et al., 2001; Kilman et al., 2002; Nahmani and Turrigiano, 2014), we report here that in SC $GABA_AR$ levels, subunit composition, and localization in adulthood are unaffected by dark rearing. We also find that the scaffold proteins gephyrin and PSD-95, chloride transporters KCC2 and NKCC2, and NMDA receptor subunits 2A and 2B are not affected by a lack of visual experience. Taken together our current work suggests that this novel, experience-dependent form of adulthood plasticity we observed in the superior colliculus of dark reared hamsters could involve an as yet unidentified, postsynaptic mechanism that explains the reduced $GABA_A$ receptor efficacy seen previously, or it could be caused solely by reduced presynaptic GABA release (Carrasco et al., 2011; Carrasco and Pallas, 2006; Carrasco et al., 2005), thereby challenging the accepted notion that presynaptic changes in ligand availability are associated with matching postsynaptic changes in their receptors. Either possibility is encouraging with respect to understanding this adulthood plasticity we observed and could lead to promising outcomes in adults with memory impairments and traumatic brain injury.

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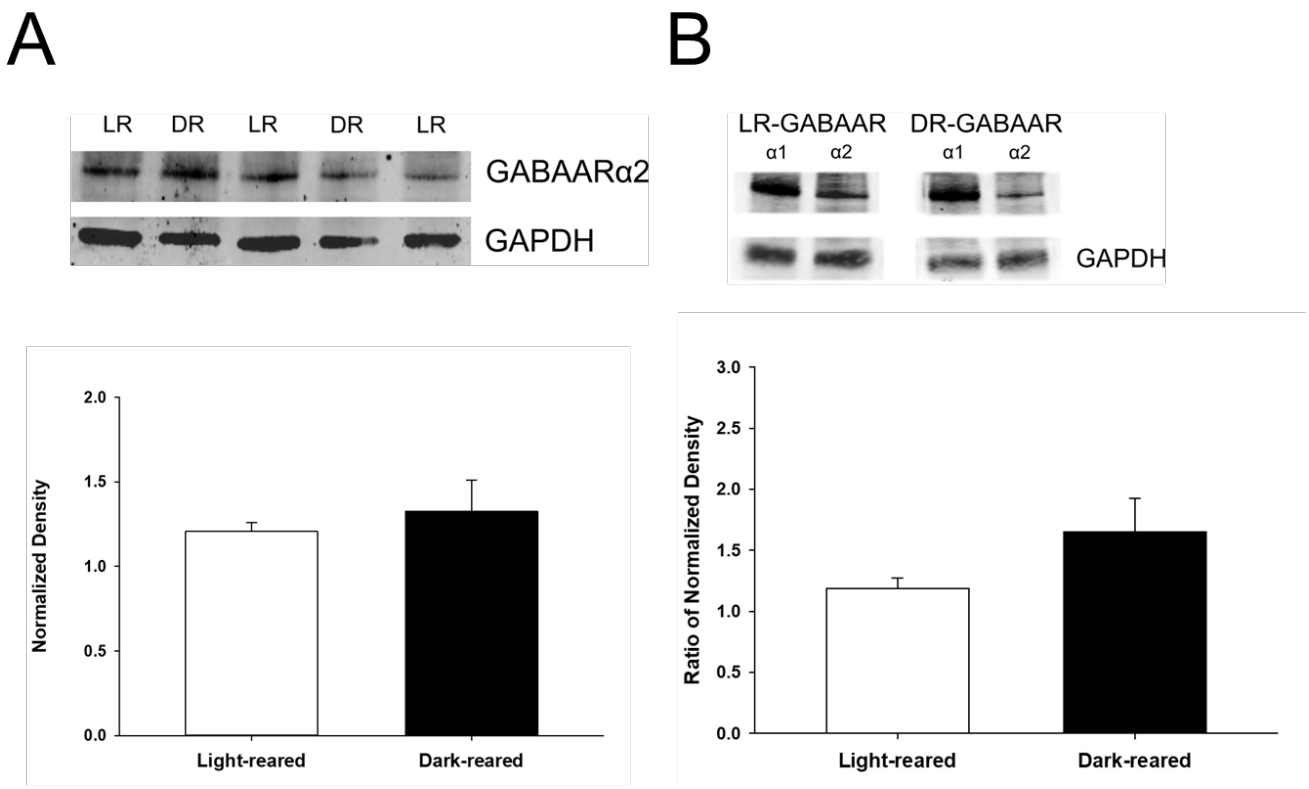

Figure 1. Adult GABA$_A$α2 receptor subunit composition is not affected by early dark rearing. (A) Upper panel: Individual Western blots of normally reared (NR) and dark-reared (DR) treatment groups generated using 20 µg of SC protein per lane. Lower panel: Densitometric analyses of GABA$_A$Rα2 in normally vs. dark reared hamster pups. (B) Comparison of GABA$_A$Rα2 expression with GABAA$_A$Rα1 expression, shown as individual Western blots in the upper panel and as a ratio in the lower panel. Data presented as mean ± SEM. In this and other figures, lanes presented together are from the same gel(s), and each measured protein was normalized against GAPDH as a loading control. These results suggest that the number and ratio of synaptic GABA$_A$α2 receptors are similar in normal and dark reared adult SC.

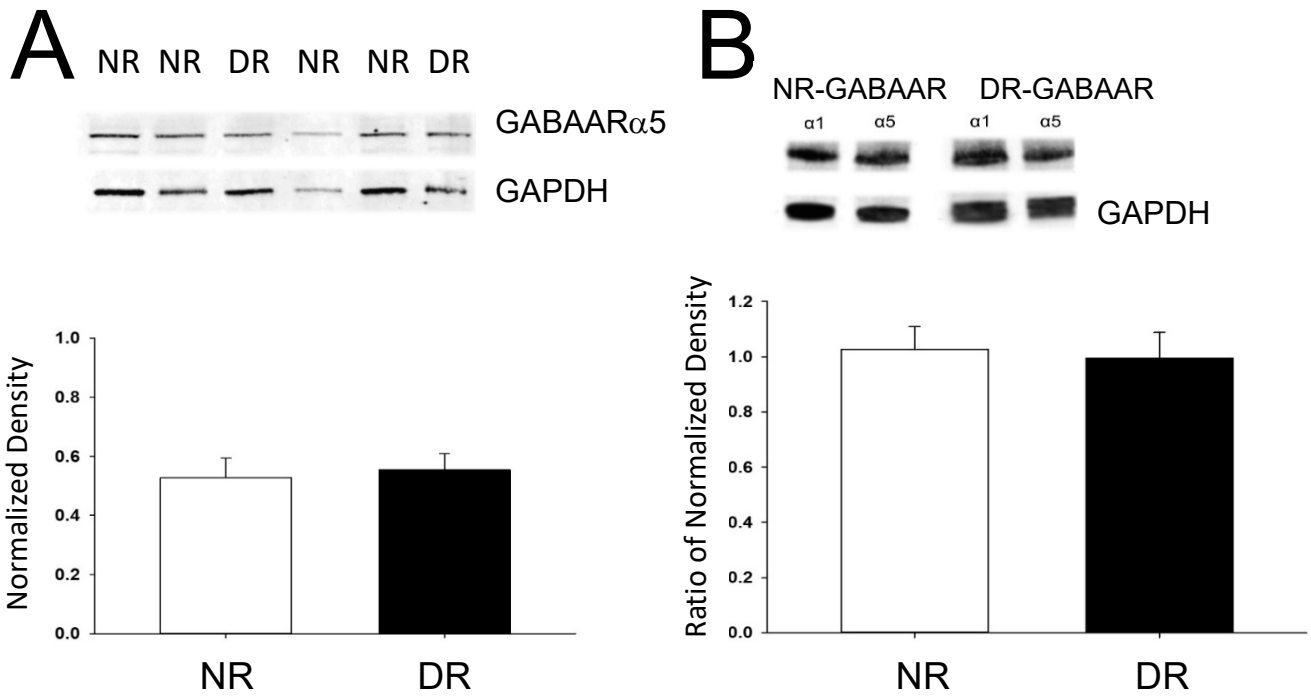

Figure 2. Adult $GABA_A\alpha5$ receptor subunit composition is not affected by early dark rearing. Example blots of NR and DR treatment groups as in Figure 1. All presented lanes are from the same gel(s), and each measured protein was normalized against GAPDH as a loading control. **(A)** GABAARα5 comparison. **(B)** $GABA_AR\alpha1$: $GABA_AR\alpha5$ ratio. All presented lanes are from the same gel(s), and each measured protein was normalized against GAPDH as a loading control. Data presented as mean ± SEM. These results suggest that the number and ratio of synaptic $GABA_A\alpha5$ receptors are similar in normal and dark reared adult SC.

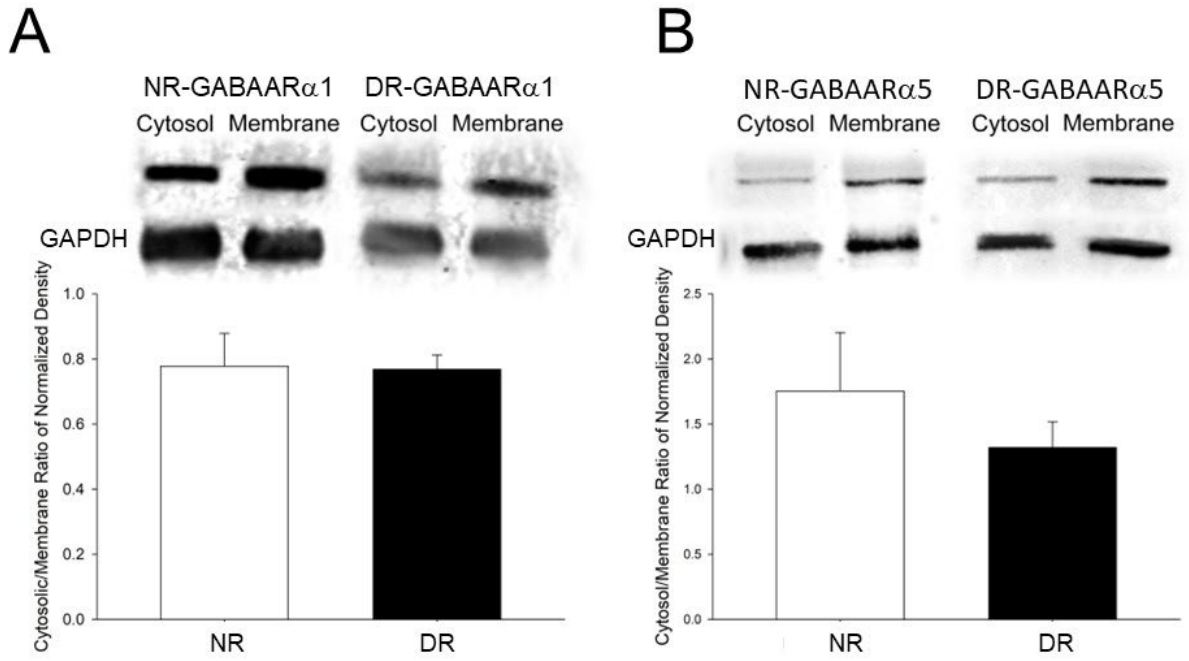

Figure 3. Endocytosis of $GABA_A$ receptors is not affected by early dark rearing. (A) Adult levels of the cytosolic vs. membrane-attached ratio of the extrasynaptically-targeted $GABA_ARα5$ and **(B)** the synaptically-targeted $GABA_ARα1$ subunits were not affected by early light deprivation. Example blots (upper panels) represent bands of labeled intracellular and membrane bound receptor subunit proteins (upper bands) from the same animal, measured as a ratio and compared between NR and DR groups. GAPDH was used as a loading control. Graph data presented as mean relative optical density ± SEM.

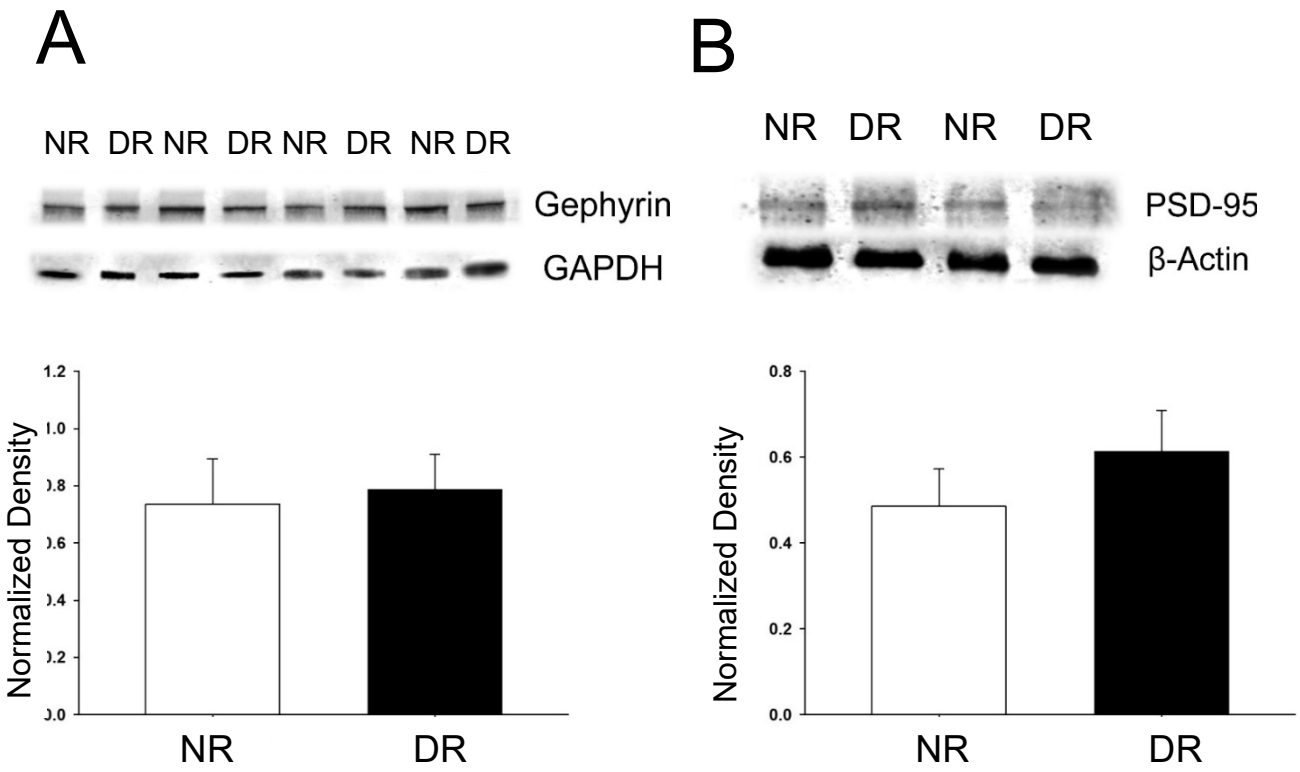

Figure 4. Adult gephyrin and PSD-95 expression are not affected by dark rearing. (A) Gephyrin and (B) PSD-95 expression was consistent between adult (>P90) NR and DR groups. GAPDH and β-actin were used as loading controls. Graph data presented as mean relative optical density ± SEM.

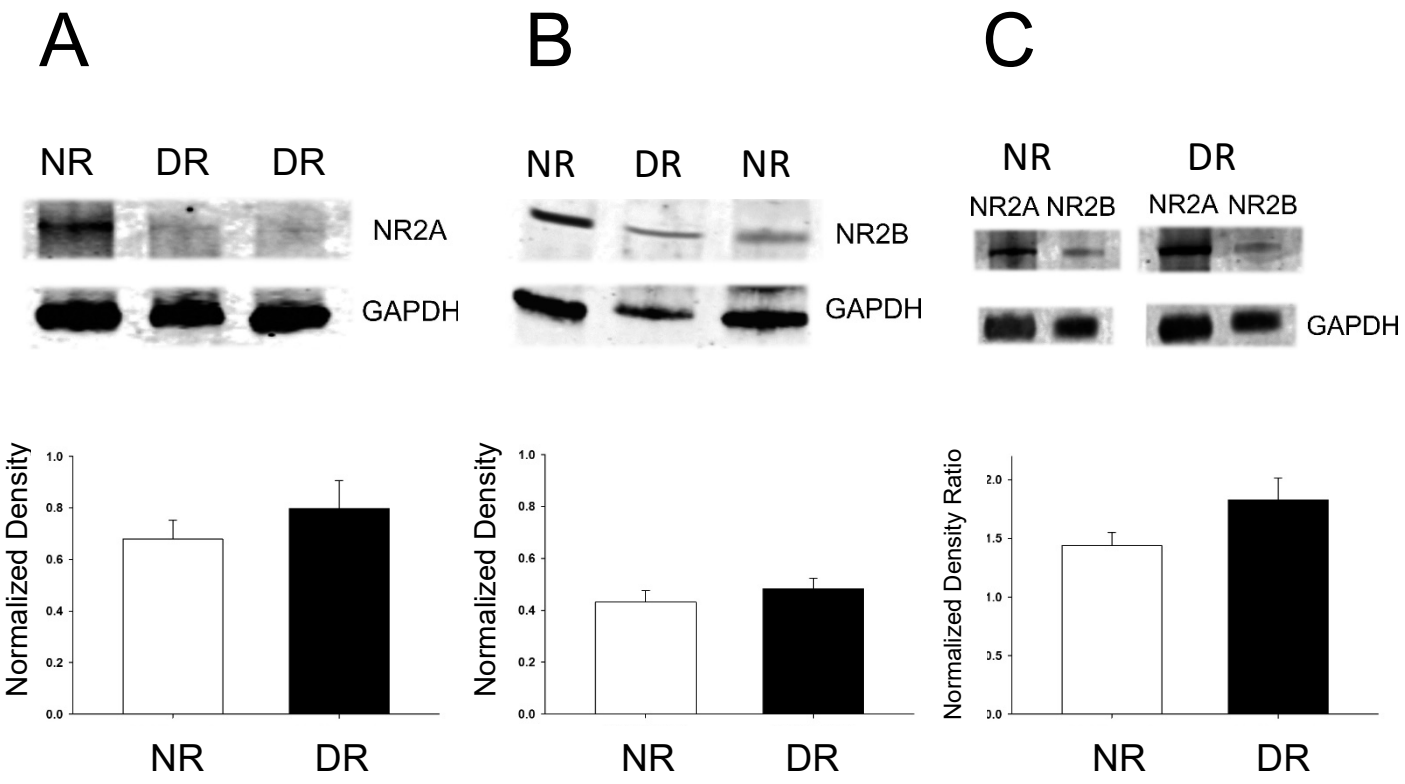

Figure 5. Adult NMDAR subunit compositions are not altered by early dark rearing. Western blot examples and densitometric analyses of **(A)** NR2A, **(B)** NR2B, and **(C)** the ratio of NR2A/NR2B expression in adult (>P90) NR and DR hamsters. Graph data presented as mean relative optical density ± SEM.

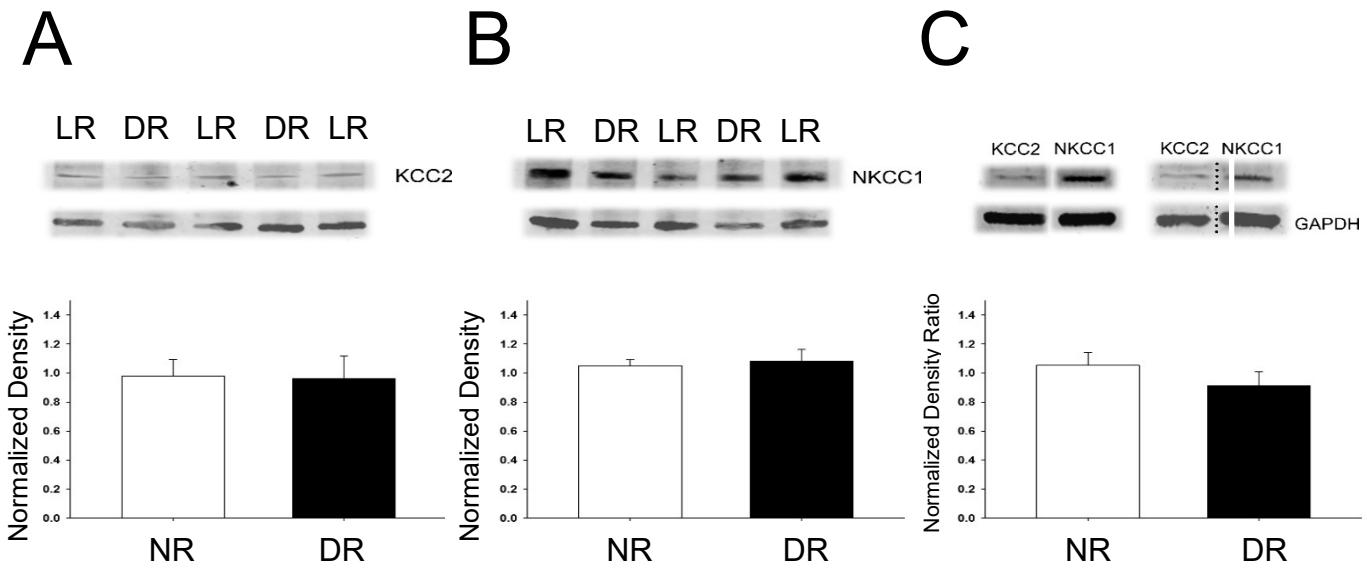

Figure 6. Adult chloride pump expression is not affected by early dark rearing. Western blot examples of NR and DR samples labeled against chloride pumps **(A)** KCC2 and **(B)** NKCC1, **(C)** and a comparison of the within subject ratio of KCC2:NKCC1 in NR and DR adult (>P90) hamsters. Graph data presented as mean relative optical density ± SEM.