Title

Localisation and stress-induced plasticity of $GABA_A$ receptor subunits within the cellular networks of the mouse dorsal raphe nucleus

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Abstract

The dorsal raphe nucleus (DRN) provides the major source of serotonin to the central nervous system (CNS) and modulates diverse neural functions including mood. Furthermore, DRN cellular networks are engaged in the stress-response at the CNS level allowing for adaptive behavioural responses, whilst stress-induced dysregulation of DRN and serotonin release is implicated in psychiatric disorders. Therefore, identifying the molecules regulating DRN activity is fundamental to understanding DRN function in health and disease. $GABA_A$ receptors ($GABA_A$Rs) allow for brain region, cell-type and subcellular domain specific GABA-mediated inhibitory currents and are thus key regulators of neuronal activity. Yet, the $GABA_A$R subtypes expressed within the neurochemically diverse cell-types of the mouse DRN are poorly described. In this study, immunohistochemistry and confocal microscopy revealed that all serotonergic neurons expressed immunoreactivity for the $GABA_A$R alpha2 and 3 subunits, although the respective signals were co-localised to varying degrees with inhibitory synaptic marker proteins. Only a topographically located sub-population of serotonergic neurons exhibited $GABA_A$R alpha1 subunit immunoreactivity. However, all GABAergic as well as non-GABAergic, non-serotonergic neurons within the DRN expressed $GABA_A$R alpha1 subunit immunoreactivity. Intriguingly, immunoreactivity for the $GABA_A$R gamma2 subunit was enriched on GABAergic rather than serotonergic neurons. Finally, repeated restraint stress increased the expression of the $GABA_A$R alpha3 subunit at the mRNA and protein level. The study demonstrates the identity and location of distinct $GABA_A$R subunits within the cellular networks of the mouse DRN and that stress impacts on the expression levels of particular subunits at the gene and protein level.

Introduction

The dorsal raphe nucleus (DRN) provides the major source of serotonin to the central nervous system (Jacobs and Azmitia 1992) and modulates the activity of distinct brain regions, notably those involved in mood-related functions such as the amygdala and prefrontal cortex (Petrov et al. 1994; Vertes 1991) . Allied to its role in mental function is the contribution of the DRN in determining adaptive behavioural responses to psychosocial stressors (Waselus et al. 2011). Engagement of both the serotonergic and non-serotonergic neuronal networks comprising the DRN is integral to processing such various forms of stress-related information (Roche et al. 2003; Shikanai et al. 2012; Challis et al. 2013) resulting in the release of serotonin in a brain region and stimulus specific manner (Kirby et al. 1995; Kirby et al. 1997; Adell et al. 1997). Since altered DRN function is implicated in stress-related mental illnesses (Baumann et al. 2002), identifying the neurotransmitter receptors which underpin cellular communication within the DRN is fundamental to understanding the changes in DRN neuronal activity and serotonin release during different brain-states or disease profiles.

GABAergic inputs onto serotonergic neurons, which arise locally, or from distant sources signalling *via* $GABA_A$Rs, appear to play a central role in shaping DRN serotonin neuronal activity and stress-induced DRN behaviours (Gervasoni et al. 2000; Celada et al. 2001; Challis et al. 2013; Crawford et al. 2013; Soiza-Reilly et al. 2013). Therefore, the precise location of distinct $GABA_A$R subtypes is likely to be central to cellular communication between the functionally distinct cell-types of the DRN and thus coordinated serotonin release. $GABA_A$Rs are composed of five interacting subunit proteins forming an associated anion channel. Nineteen $GABA_A$R subunits have been cloned (Olsen and Sieghart 2009) with the subunit combinations determining the biophysical (Farrant and Nusser 2005; Belelli et al. 2009; Eyre et al. 2012) and pharmacological (Rudolph and Knoflach 2011)

properties of the receptor. As a consequence, the brain-region and cell-type specific expression patterns of various $GABA_AR$ subunits (Hortnagl et al. 2013; Fritschy and Mohler 1995; Wisden et al. 1992) manifest in diverse $GABA_AR$-mediated inhibitory currents throughout the brain, the kinetics of which, within the DRN, are influenced by psychosocial stressors (Kirby et al. 2008; Crawford et al. 2013).

$GABA_AR$ subunit expression patterns have been reported for the rat DRN (Gao et al. 1993; Pirker 2000; Fritschy and Mohler 1995), but not the mouse DRN, despite considerable inter-species differences in their DRN neurochemistry (Fu et al. 2010). Furthermore, although there is a convergence of $GABA_AR$ expression (Vithlani et al. 2013) and exposure to stress (Binder and Nemeroff 2010) with mental illnesses, it is unclear whether stress directly influences $GABA_AR$ subtypes expressed within the DRN. Having recently shown functional contribution of $GABA_AR$-mediated inhibitory postsynaptic currents to DRN serotonergic neuronal excitability (Maguire et al. 2013), this study goes on to demonstrate the identity and the location of the $GABA_AR$ alpha and gamma2 subunits expressed within the cellular networks of the mouse DRN. We also reveal that repeated stress results in the selective up-regulation of the $GABA_AR$ alpha3 subunit expression at both the mRNA and protein level.

Materials and methods

All procedures involving experimental animals were performed in accordance with the Animals (Scientific Procedures) Act, 1986 (UK) and associated procedures. Every effort was made to minimise any pain or discomfort to the animals.

Animals

Adult C57BL/6J male mice and transgenic mice which expressed green fluorescent protein (GFP) as a reporter molecule driven by the promoter of the gene for the GABA synthesising enzyme glutamic acid decarboxylase 67 (GAD67), (GAD67-GFP) (Tamamaki et al. 2003) were used to determine the native expression patterns of particular $GABA_AR$ subunits.

Tissue preparation

Animals were perfusion-fixed as follows: anaesthesia was induced with isofluorane and maintained with pentobarbitone (1.25 mg/kg of bodyweight; i.p.). The animals were perfused transcardially with 0.9 % saline solution for 1 minute, followed by 12 minutes fixation with a fixative consisting of 1% paraformaldehyde, 15% v/v saturated picric acid, in 0.1 M phosphate buffer (PB), pH 7.4. The brains were kept in the same fixative solution overnight at 4°C. Coronal sections of the DRN, 60 μm thick, were prepared on a Vibratome and stored in 0.1 M PB containing 0.05% sodium azide.

Immunohistochemical reactions

Immunohistochemical procedures were according to those used in (Corteen et al. 2011). Four C57BL/6J and two GAD67-GFP transgenic animals were used to confirm the native patterns of the immunoreactivity. A proteolytic antigen retrieval method was used to localise membrane–bound epitopes according to (Watanabe et al. 1998; Lorincz and

Nusser 2008). Briefly, the tissue sections were incubated at 37°C for 10 minutes in 0.1 M PB followed by 15 minutes in 0.2 M HCl containing 1 mg/ml pepsin (Sigma, UK) after which they were washed thoroughly in Tris-buffered saline containing 0.3% triton (TBS-Tx) for 30 minutes. Non-specific binding of secondary antibodies was blocked by incubating sections with 20% normal horse serum for 2 hours at room temperature. The tissue sections were incubated with cocktails of primary antibodies (Table 1), diluted in TBS-Tx, overnight at 4°C. After washing with TBS-Tx, sections were incubated in a mixture of appropriate secondary antibodies conjugated with either Alexa Fluor 405 (Jackson ImmunoReserach) Alexa Fluor 488 (Invitrogen, Eugene, OR), indocarbocyanine (Cy3; Jackson ImmunoResearch), and indodicarbocyanine (Cy5; Jackson ImmunoResearch) for 2 hours at room temperature. Sections were washed in TBS-Tx and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Antibody specificity

Although the specificity of all the antisera against the $GABA_A$R subunits used in this study have been reported upon extensively in other publications (see Table 1), tissue from $GABA_A$R alpha1 subunit-specific gene deleted mice (Sur et al. 2001), $GABA_A$R alpha2 subunit-specific gene deleted mice (Dixon et al. 2008) and $GABA_A$R alpha3 subunit-specific gene deleted mice (Yee et al. 2005) was used in the current study to confirm the specificity of the respective immunoreactivity patterns under current reaction conditions and within specific brain regions investigated. Method specificity was also tested by omitting the primary antibodies in the incubation sequence. To confirm the absence of cross reactivity between IgGs in double and triple immunolabelling experiments, some sections were processed through the same immunohistochemical sequence, except that only an individual primary antibody was applied with the full complement of secondary antibodies.

Image acquisition

Sections were examined with a confocal laser-scanning microscope (LSM710; Zeiss, Oberkochen, Germany) using either a Plan Apochromatic 63x DIC oil objective (NA1.4) (pixel size 0.13 µm) or a Plan Apochromatic 100x DIC oil objective (NA1.46) (pixel size 0.08 µm). Z-stacks were used for routine evaluation of the labelling. All images presented represent a single optical section. These images were acquired using sequential acquisition of the different channels to avoid cross-talk between fluorophores, with the pinholes adjusted to one airy unit. Images were processed with the software Zen2008 Light Edition (Zeiss, Oberkochen, Germany) and exported into Adobe Photoshop. Only brightness and contrast were adjusted for the whole frame, and no part of a frame was enhanced or modified in any way.

Quantification of the density of gephyrin and neuroligin2 as well as their degree of colocalisation on TPH-immunopositive somata and dendrites

Immunoreactivity for molecular markers of inhibitory synapses, namely gephyrin (Essrich et al. 1998) and neuroligin2 (NL2) (Varoqueaux et al. 2004) were used to gain a perspective of the location of putative GABAergic synapses on serotonergic neurons within the DRN. Tryptophan hydroxylase (TPH) immunoreactivity was used to visualise serotonergic neurons. The quantitative method used is according to our previous reports (Corteen et al. 2011; Gunn et al. 2013; Maguire et al. 2013). A total of 9 tissue sections, 3 per animal, 3 animals, were used to quantify: 1) the density of individual gephyrin and NL2 immunoreactive clusters on TPH immunopositive profiles; 2) the density of gephyrin and NL2 immunoreactive clusters which colocalised with one another and; 3) the proportion of individual gephyrin and NL2 immunoreactive clusters which were located on either TPH-immunopositive somata or dendrites. Preliminary investigations revealed no discernible gradients in the intensity of gephyrin and NL2 signals in the rostro-caudal planes. Therefore, tissue sections at the rostral-caudal midline level of the DRN (Bregma ~ -4.60)

from each animal were selected for analyses. Two fields of view (FOV) were randomly selected within the DRN ventromedial sub-region of each tissue section. A Z-stack consisting of three optical sections was acquired for each FOV with a Plan Apochromatic x100 (NA1.4) DIC oil immersion objective. The dimensions of the optical sections were 84.94μm x 84.94μm in the X & Y planes and 1 μm thick in the Z plane. Optical sections were spaced 2 μm apart. In all cases, triple immunofluorescence (TPH-neuroligin2-gephyrin) was acquired using sequential acquisition of the different channels. The number of individual clusters (gephyrin or NL2) within an optical section associated with TPH immunoreactivity was manually counted using ImageJ software and the density determined by dividing the number of clusters by the area of the optical section. Co-localisation of individual clusters (gephyrin and NL2) from the different channels was visually confirmed in the X, Y and orthogonal planes and manually counted using ImageJ and the density then calculated. Finally, the proportion of NL2 and gephyrin immunoreactive clusters located on either somatic or dendritic compartments was determined.

Quantification of the relative proportion of TPH-immunopositive cells expressing $GABA_AR$ alpha1 subunit immunoreactivity

A previous study of the rat reported that the $GABA_AR$ alpha1 subunit is predominantly expressed by GABAergic non-serotonergic neurons and to a lesser extent by serotonergic neurons in the DRN (Gao et al. 1993). Preliminary investigations confirmed this similar restricted expression pattern of this subunit in the DRN of the mouse. However, it was noticeable that the TPH-$GABA_AR$ alpha1 subunit immunopositive neurons were not randomly distributed throughout the mouse DRN, but were concentrated within specific sub-regions of the nucleus. We therefore quantified the ratio of TPH-$GABA_AR$ alpha1 subunit immunopositive cells to total TPH immunopositive cells as well as the location of this population of cells, in three animals, nine tissue sections per animal which were 70

µm-thick. For a particular animal, three sections representing either the rostral (~ -4.24 mm Bregma), midline (~ -4.6 mm Bregma) or caudal (~ -4.96 mm Bregma) (total of nine tissue sections per animal) extents of the DRN were used for quantification. This was repeated in 3 animals. For each tissue section, the proportion of TPH-GABA$_A$R alpha1 subunit immunopositive cells to total TPH-immunopositive cells was quantified within the distinct sub-regions of the DRN. For the rostral sections, the DRN was subdivided into dorsal (dDRN) ventral (vDRN) and medial interfasicular (ifDRN) regions. For the midline sections, the DRN was subdivided into dorsal, ventral, medial interfasicular and lateral (lDRN) regions. For the caudal sections, the DRN was subdivided into dorsal, ventral regions and medial interfasicular regions. To unequivocally confirm that GABA$_A$R alpha1 subunit immunoreactive clusters were located on TPH-immunopositive neurons, Z-stacks were acquired throughout the full extent of the tissue section using a Plan Apochromatic 63X DIC oil objective (NA1.3). The dimensions of the optical sections were 224 µm x 224 µm x 0.9 µm (X, Y, Z). The optical sections within a Z-stack were spaced 7 µm apart in the Z-plane with 0.5 µm overlap between optical sections. ImageJ software (NIH) was used to manually count the number of TPH-GABA$_A$R alpha1 subunit-immunopositive neurons in relation to the total number of TPH-immunopositive cells within the different DRN sub regions. The mean ± SEM number of total TPH and TPH-GABA$_A$R alpha1 subunit-immunopositive cells within the DRN sub-nuclei at rostral, midline and caudal planes of three animals are presented as well as the proportion of TPH-GABA$_A$R alpha1 subunit-immunopositive cells to total TPH immunopositive cells at the rostral, midline and caudal planes.

Quantification of the relative proportion of NL2 immunoreactive clusters which co-localise with $GABA_AR$ alpha2 & 3 subunit immunoreactive clusters on TPH-immunopositive somata and dendrites

The quantitative method used is according to our previous report (Corteen et al. 2011). Tissue from five animals (three tissue sections per animal) was used to quantify the relative proportion of NL2 immunoreactive clusters which co-localised with $GABA_AR$ alpha2 and $GABA_AR$ alpha3 subunit immunoreactive puncta on TPH-immunopositive dendrites and somata with the intention of estimating what proportion of inhibitory synapses on such cell surfaces are likely to contain $GABA_ARs$ composed of these subunits. Preliminary investigations revealed no discernible gradients in the intensity for signal for either the $GABA_AR$ alpha2 or $GABA_AR$ alpha3 subunits in the rostro-caudal planes. Therefore, tissue sections at the rostral-caudal midline level of the DRN from each animal were selected for each of the following immunohistochemical reactions: 1) TPH-$GABA_AR$ alpha2 subunit-NL2 and; 2) TPH-$GABA_AR$ alpha3 subunit-NL2. Two fields of view (FOV) were randomly selected within the DRN ventromedial sub-region of each tissue section. A Z-stack consisting of three optical sections was acquired for each FOV with a Plan Apochromatic x100 (NA1.4) DIC oil immersion objective. The dimensions of the optical sections were 84.94µm x 84.94µm in the X & Y planes and 1 µm thick in the Z plane. Optical sections were spaced 2 µm apart. In all cases, triple immunofluorescence (TPH-NL2-alpha2 or TPH-NL2-alpha3) was acquired using sequential acquisition of the different channels. The number of clusters within an optical section associated with TPH immunoreactivity was manually counted using ImageJ software. Co-localisation of individual clusters from the different channels was visually confirmed in the X, Y and orthogonal planes and manually counted using ImageJ. The proportion of NL2 clusters which co-localised with either $GABA_AR$ alpha2 or alpha3 subunit clusters was computed and expressed as the number of puncta per 1000 um^2 of DRN.

Repeated restraint stress

The DRN-5HT system is central to integrating stress-related information within the CNS and the adoption of adaptive or maladaptive behavioural responses (Waselus et al. 2011) with $GABA_AR$ functional plasticity within the cellular networks of the DRN central to such processes (Crawford et al. 2013; Kirby et al. 2008; Kirby et al. 2000). However, it is currently unclear whether exposure to stress directly impacts on the level of expression of particular $GABA_AR$ subtypes and the particular cell-types involved. With a view to investigating potential $GABA_AR$ plasticity in adaptive, rather than maladaptive responses to stressful experiences, we deliberately used a mild restraint stress protocol which does not induce a chronic stress phenotype (Buynitsky and Mostofsky 2009) nor anxiogenic-like behaviour (Seifi et al. 2014). However, this protocol has been shown to robustly engage the DRN-serotonergic system, amongst other brain regions (Shoji and Mizoguchi 2010; Keshavarzy et al. 2014). Animals were divided into stress and control experimental groups one week prior to the start of the experiment in order to allow adaptation to the new cage environment before commencing the stress, To deliver restraint, mice aged postnatal day (PND) 40 were inserted tail first into a Broome rodent restrainer (Harvard Apparatus # 52-0470) for 30 minutes per day. Mice were restrained within the tube, but not fully immobilised. They thus were able to move slightly backwards and forwards in the device. During restraint stress, mice were kept individually in standard housing cages containing a thin layer of corn cob. After the restraint stress, animals were removed to their home cages. The restraint stress was delivered at set times during the day for eight consecutive days. The time-period of eight days was chosen empirically as the minimum length of time required to reliably induce statistically significant changes in animal behaviour and $GABA_AR$ subunit expression, based on pilot experiments. To prevent habituation to the repeated stressor over the course of the experiment, the duration and the degree of restraint was subtly varied between days, as detailed in Table 2. To apply escapable restraint, the animal was inserted into the tube and restrained for 1 minute, after which the

stopper was removed and the animal was able to emerge from the restrainer and explore the novel environment of the cage, with the restrainer left in the cage. Control mice remained in their original cages and were left undisturbed in this home environment. On day 9 the tissue from control and stress animals was processed for either $GABA_AR$ mRNA or immunohistochemical analyses.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

To assess how stress might impact upon the mRNA encoding $GABA_AR$ subunits within the DRN, one day after the final stress episode (day 9), the mice were euthanized by cervical dislocation and their brains rapidly removed. Fourteen control and fourteen stress animals were used for these analyses. Two millimetre thick tissue sections containing the DRN were obtained using a brain matrix (Harvard apparatus; #726233). From these sections, a tissue punch of 2 mm diameter was used to extract the DRN which was then placed in RNAlater® for post hoc RNA extraction. Each tissue punch was removed from the RNAlater® and placed in RLT lysis buffer (Qiagen) and was disrupted with a rotor homogeniser. Total RNA was extracted from the tissue lysate using RNeasy® RNA Purification kit (Qiagen) according to the manufacturer's instructions. Subsequently, purified RNA was reverse transcribed to cDNA at 37 °C using M-MuLV reverse transcriptase and Oligo(dT)18 primers.

Multiplex qPCR

Multiplex qPCR was used to simultaneously analyse the expression of both the housekeeping gene (*Gapdh*) and a second gene of interest. The genes investigated were: *Gabra1* (the $GABA_AR$ alpha1 subunit); *Gabra2* (the $GABA_AR$ alpha2 subunit); *Gabra3* (the $GABA_AR$ alpha3 subunit), and *Gabrg2* (the $GABA_AR$ gamma2 subunit). qPCR was performed according to Carter et al. (2013); for each sample 2 µl cDNA was mixed with nuclease free water, mastermix (Roche) and the primers and probes of housekeeping

gene and gene of interest, tagged with VIC (primer limited) and FAM respectively, to yield a 10 µl total reaction volume. The primers and probes used in this study are displayed in Table 3. The qPCR was performed under the following cycling conditions: 95°C for 10 minutes and 55 cycles of 95°C for 10 sec and 60°C for 30 sec.

Analysis of RNA expression levels

The relative standard curve method was used for quantitative determination of the amount of the gene of interest in relation to the amount of the housekeeping gene (*Gapdh*). Standard curves were generated for *Gabra1*, *Gabra2*, *Gabra3*, and *Gabrg2* using serial dilutions of cDNA from whole mouse brain. Each measurement was performed in triplicate and each C_t value was then converted into ng RNA using linear regression analysis of the standard curve (Microsoft Excel). Each ng RNA value was normalised against the ng housekeeping gene level within the same sample. Mean ng RNA levels were computed from the normalised measurements and compared across stress and control experimental groups. qRT-PCR data is presented as relative change in gene expression above or below control.

Quantification of the relative proportion of $GABA_AR$ alpha3 subunit and gephyrin immunoreactive clusters on TPH immunopositive somata and dendrites in tissue from repeated restraint stress and control mice

One day after the final stress episode (day 9), tissue was prepared for immunohistochemical analyses as above using three control and three stress animals. The quantitative method used to identify how stress influences $GABA_AR$ subunit expression in the DRN is according to Lorenzo et al. (2007). Tissue from six animals, three stress and three control, were used to quantify the relative proportion and degree of co-localisation of $GABA_AR$ alpha3 subunit and gephyrin immunoreactive puncta on TPH-immunopositive dendrites and somata. Tissue sections at the rostra-caudal midline level of the DRN

(approximately -4.60 Bregma) from each animal were selected for the following immunohistochemical reaction: TPH-GABA$_A$R alpha3 subunit-gephyrin. Two fields of view (FOV) were randomly selected within the ventromedial sub-region of each tissue section. The ventromedial sub-region was selected because of data within this study showing that inhibitory innervation is enriched on the dendritic domains of TPH-immunopositive neurons and TPH dendrites are densest within the ventromedial sub-region. A Z-stack consisting of three optical sections was acquired for each FOV with a Plan Apochromat x100 (NA1.4) DIC oil immersion objective. The dimensions of the optical sections were 84.94 µm x 84.94 µm in the X & Y planes and 1 µm thick in the Z plane. Optical sections were spaced 2 µm apart. Triple immunofluorescence was acquired using sequential acquisition of the different channels. The number of GABA$_A$R alpha3 subunit, gephyrin and co-localised GABA$_A$R alpha3 subunit-gephyrin clusters within an optical section associated with TPH immunoreactivity was manually counted using ImageJ (NIH) software. The numbers of clusters for each optical section within a field of view were combined and the means ± SD for all FOV within and between sections were pooled within either control or stress groups. Quantitative data are reported as density of clusters per 1000 um^2.

Statistical analysis

All quantitative data are presented as the mean ± SEM unless otherwise stated. The data were tested for normality using a Shapiro-Wilk test. An Independent Student's T-test or Mann-Whitney test was used for normally and non-normally distributed data, respectively. In all cases, SPSS was used for statistical analyses. GraphPad was used for graphical presentation of the data.

Results

Inhibitory synaptic marker proteins are preferentially located on dendritic compartments of DRN serotonergic neurons

Molecular markers of inhibitory synapses were used to initially gain a perspective of the location of putative GABAergic synapses on the somatic and dendritic domains of serotonin neurons within the DRN. Immunolabelling for gephyrin, a protein enriched in inhibitory synapses due to its role in clustering $GABA_A$Rs and strychnine-sensitive glycine receptors (Essrich et al. 1998), was widely associated with TPH-immunopositive profiles, although immunoreactive clusters appeared to be preferentially located on dendritic rather than their somatic compartments (Fig. 1a1). Immunoreactivity for NL2 (Fig. 1a2), a protein exclusively expressed at inhibitory synapses (Varoqueaux et al. 2004) (Poulopoulos et al. 2009) closely matched the pattern of gephyrin. Both gephyrin and NL2 immunoreactivity displayed significant co-localisation throughout the rostral-caudal and dorsal-ventral extents of the DRN (Fig. 1a3). Quantification of gephyrin immunoreactivity revealed that (mean ± SEM) 37.7 ± 2.7 clusters per 1000 μm^2 were located on TPH immunopositive profiles, of which, 7% were located on somata and 93% on dendrites. Furthermore, quantification of NL2 immunoreactivity revealed that (mean ± SEM) 55.4 ± 4.9 clusters per 1000 μm^2 were located on TPH immunopositive profiles of which, 7% were located on somata and 93% on dendrites. Finally, the density of colocalised gephyrin-NL2 immunopositive clusters was (mean ± SEM) 29 ± 3 clusters per 1000 μm^2 which computes to 76% of gephyrin immunoreactive clusters being colocalised with those immunoreactive for NL2 whereas 53% of NL2 immunoreactive clusters colocalised with those immunoreactive for gephyrin (Fig. 1b).

Data on only the $GABA_A$R alphas 1, 2, 3 and gamma2 subunits are presented since we found no evidence for the expression of the $GABA_A$R alpha 4, 5 and delta subunits in the

DRN, whilst the expression of all other subunits (beta; gamma1, 3; epsilon; pi) was not investigated. The specificity of the antibodies against either the $GABA_AR$ alpha1, alpha2 or alpha3 subunits was confirmed in tissue from wild-type (WT) mice and tissue from either $GABA_AR$ alpha1 (Fig. 2a, b), alpha2 (Fig. 2c, d) or alpha3 (Fig. 2e, f) subunit-specific gene-deleted mice respectively. There were no discernible differences in the intensity of TPH immunoreactivity in the DRN of WT and $GABA_AR$ alpha1, alpha2 or alpha3 subunit-specific gene-deleted mice suggesting that the absence of these subunits did not impact on the expression of this enzyme within the DRN (Fig. 2).

The $GABA_AR$ alpha1 subunit is expressed by a minority of serotonin neurons and all GABAergic neurons within the DRN

$GABA_AR$ alpha1 subunit immunoreactivity was evident throughout the DRN in both rostral-caudal and dorso-ventral extents although it appeared to be enriched particularly in the lateral wing sub-regions (Fig. 2a2). Numerous $GABA_AR$ alpha1 subunit immunoreactive profiles were interspersed between those immunopositive for TPH, demonstrating the multitude of cells which expressed this subunit within the DRN (Fig. 3a1). The $GABA_AR$ alpha1 subunit signal was equally enriched on somatic as well as dendritic compartments of cells as demonstrated by its high degree of co-localisation with the dendritic marker protein microtubule associated protein (MAP-2) (Fig. 3a2, 3). This location of the $GABA_AR$ alpha1 subunit signal on neuronal somata is in stark contrast to the signal for the $GABA_AR$ alphas 2 and 3 subunits which was preferentially restricted to dendritic domains (see below). A previous study in the DRN of the rat reported that the $GABA_AR$ alpha1 subunit is mainly expressed by non-serotonergic, GABAergic neurons of the DRN (Gao et al. 1993). $GABA_AR$ alpha1 subunit immunoreactive signal was localised to three neurochemically diverse cell types within the DRN (Fig. 3b,c); i) a sub-population of TPH-immunopositive neurons which displayed $GABA_AR$ alpha1 subunit signal on their somata (Fig. 3b1); ii) all

GFP-GAD67 immunopositive neurons (Fig. 3B2) and; iii) a population of DRN neurons which were immunonegative for both TPH and GAD67-GFP (Fig. 3b3).

Double labelling with gephyrin revealed extensive co-localisation between gephyrin and $GABA_AR$ alpha1 subunit immunoreactivity (Fig. 3b3). The gephyrin signal was predominantly enriched on dendritic domains, apart from a subpopulation of GAD67-GFP immunolabelled neurons situated within the ventral DRN, which, qualitatively, exhibited uncharacteristically intense somatic gephyrin signal. Qualitative observations revealed that these ventral GAD67-GFP neurons were also much larger than dorsal GAD67-GFP neurons (Fig. 3c3). This distribution and diverse morphology could suggest the presence of at least two sub-populations of GABAergic neurons within the DRN; i) one population which possesses lower levels of synaptically localised alpha1 subunit containing $GABA_ARs$ (alpha1-$GABA_ARs$) on their somata and; ii) a second sub-population, which possess higher levels of synaptically localised alpha1-$GABA_ARs$ on their somata. The predominance of $GABA_AR$ alpha1 subunit signal on most of the non-TPH immunopositive neurons suggests that $GABA_ARs$ containing this subunit will have a major influence on the excitability and function of these neurons, which are thought to provide local circuit inhibition onto the principal serotonergic neurons of the DRN. Therefore, while alpha1-$GABA_ARs$ are not widely expressed on serotonin neurons, their modulation *in vivo*, for example with subunit-specific ligands such as zolpidem, could have a profound impact upon brain serotonin levels, by altering the levels of GABA released onto serotonergic neurons.

The ratio of TPH-$GABA_AR$ alpha1 subunit immunopositive cells to those TPH immunopositive cells which did not express this subunit was quantified throughout the rostro-caudal axis of the DRN (Fig. 4a-c). TPH-$GABA_AR$ alpha1 subunit immunopositive cells were enriched at the rostral extent of the DRN (Bregma ~ -4.24) (Fig. 4a1), with ~

21% of TPH labelled cells in rostral DRN also displaying the $GABA_AR$ alpha1 subunit signal (mean ± SEM, total number of TPH immunopositive cells, 220 ± 35 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 46 ± 6, N = 3 animals) (Fig. 4a3). Of the cells counted at this rostral location the following numbers (mean ± SEM) were located within these specific DRN sub-nuclei; a) dDRN, total number of TPH immunopositive cells, 66 ± 14 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 18 ± 4; b) vDRN, total number of TPH immunopositive cells, 77 ± 33 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 13 ± 6; c) ifDRN, total number of TPH immunopositive cells, 85 ± 17 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 15 ± 2 (Fig. 4 a2).

The proportion of TPH labelled cells which co-expressed the $GABA_AR$ alpha1 subunit signal decreased considerably from the rostral to caudal planes. Although the midline DRN (Bregma ~ -4.60) (Fig. b1) has the majority of TPH immunopositive cells, only ~ 7% of TPH labelled cells co-labelled for the $GABA_AR$ alpha1 subunit (mean ± SEM, total number of TPH immunopositive cells, 728 ± 54 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 50 ± 4, N = 3 animals) (Fig. 4b3). Of the cells counted at this DRN midline location the following numbers (mean ± SEM) were located within these specific DRN sub-nuclei; a) dDRN, total number of TPH immunopositive cells, 172 ± 32 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 19 ± 6; b) vDRN, total number of TPH immunopositive cells, 246 ± 47 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 13 ± 4; c) ifDRN, total number of TPH immunopositive cells, 109 ± 11 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 3 ± 2; d) lDRN, total number of TPH immunopositive cells, 219 ± 24 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 17 ± 5 (Fig. 4 b2).

TPH and $GABA_AR$ alpha1 subunit immunopositive cells were least abundant in caudal DRN (Bregma ~ -4,96) (Fig. 4c1), with only ~ 5% of TPH labelled cells also displaying

immunoreactivity for the $GABA_AR$ alpha1 subunit (mean ± SEM, total number of TPH immunopositive cells, 244 ± 46 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 11 ± 4, N = 3 animals) (Fig. 4c3). Of the cells counted at this DRN caudal location the following numbers (mean ± SEM) were located within these specific DRN sub-nuclei; a) dDRN, total number of TPH immunopositive cells, 174 ± 35 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 7 ± 4; b) vDRN, total number of TPH immunopositive cells, 122 ± 34 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 10 ± 2; c) ifDRN, total number of TPH immunopositive cells, 90 ± 10 versus TPH-$GABA_AR$ alpha1 subunit immunopositive cells, 4 ± 1 (Fig. 4 c2). Considering the extensive topographical organisation of the DRN serotonergic neurons in terms of their efferent outputs, with neurons located in different sub-divisions providing serotonergic supply to disparate brain regions (Vertes 1991; Lee et al. 2003; Xu and Hokfelt 1997), the distribution pattern of the $GABA_AR$ alpha1 subunit signal on TPH immunopositive neurons could have a highly specific influence over DRN-serotonin mediated regulation of distinct brain regions.

$GABA_AR$ alpha2 subunit immunoreactivity is predominantly located on the dendrites of serotonergic neurons

Whilst the immunoreactivity pattern of the $GABA_AR$ alpha1 subunit appeared continuous, or clustered along somatic and dendritic plasma membranes (Fig. 3), immunoreactivity for the $GABA_AR$ alpha2 subunit was wholly clustered and located predominantly on TPH immunolabelled dendrites, with fewer clusters located on cell bodies (Fig. 5a2). Immunoreactivity of the $GABA_AR$ alpha2 subunit exhibited the highest degree of co-localisation with the inhibitory synaptic marker protein NL2 out of all subunits examined (Fig. 5b). The density (mean ± SEM number of clusters per 1000 μm^2) of NL2 immunoreactive clusters located on TPH immunopositive profiles was 57.5 ± 2.4 of which 26.3 ± 1.6 were co-localised with $GABA_AR$ alpha2 subunit immunoreactive puncta which

suggests that within the DRN, alpha2-subunit containing $GABA_AR$s (alpha2-$GABA_AR$s) are located in ~ 45% of putative inhibitory synapses located on serotonergic neurons.

The $GABA_AR$ alpha3 subunit is expressed on serotonergic and GABAergic neurons of the DRN

An overview of the midbrain region containing the DRN revealed that $GABA_AR$ alpha3 subunit immunoreactivity was closely associated with that of TPH illustrating the significant expression of this subunit on serotonergic neurons (Fig. 2e2). $GABA_AR$ alpha3 subunit signal appeared clustered (Fig. 6a2). However, in contrast to the relatively uniformly sized clusters of the $GABA_AR$ alpha2 subunit, qualitative observations revealed the $GABA_AR$ alpha3 subunit immunopositive clusters to be notably more variable in both size and shape. Compared with the $GABA_AR$ alpha2 signal, fewer NL2 immunoreactive clusters (Fig. 6a1) co-localised with those of the $GABA_AR$ alpha3 subunit (Fig. 6a3): the density (mean ± SEM number of clusters per 1000 μm^2) of NL2 immunoreactive clusters located on TPH immunopositive profiles was 47.7 ± 0.3 of which 13 ± 2 were co-localised with $GABA_AR$ alpha3 subunit immunoreactive puncta which suggests that within the DRN, alpha3-$GABA_AR$s are located in ~ 27% of putative inhibitory synapses located on serotonergic neurons. The antibodies against the $GABA_AR$ alpha2 & 3 subunits were both raised in the same species thus precluding double labelling experiments to ascertain the degree, if any, of co-localisation between clusters immunoreactive for these subunits. Such data would provide a perspective as to whether these subunits are targeted to overlapping or distinct populations of synapses on serotonergic neurons, as is the case for noradrenergic neurons in the locus coeruleus (Corteen et al. 2011).

A considerable proportion of $GABA_AR$ alpha3 subunit immunopositive clusters did not appear to co-localise with clusters immunoreactive for NL2. This finding could denote either the presence of extrasynaptically located alpha3-$GABA_AR$ subtypes on the

serotonergic neurons of the DRN, similar to other brain regions (Marowsky et al. 2012), or the reaction conditions employed did not optimally allow for the detection of the synaptically located $GABA_AR$ alpha3 subunit signal. To examine the latter, we performed parallel reactions using tissue containing the thalamic reticular nucleus (nRT), since this brain region, in adulthood, exclusively expresses the $GABA_AR$ alpha3 subunit within inhibitory synapses (Fritschy 1998; Browne et al. 2001; Studer et al. 2006) (Fig. 6b). Predictably, parvalbumin immunopositive profiles of the nRT (Fig. 6b1) were decorated by $GABA_AR$ alpha3 subunit immunoreactive clusters (Fig. 6b2) which displayed complete co-localisation with NL2 (Fig. 6b3). This disparate immunolocalisation pattern of the $GABA_AR$ alpha3 subunit across different brain regions provides evidence for a brain region and cell-type specific expression profile of the $GABA_AR$ alpha3 subunit.

Within the DRN, we also observed a proportion of $GABA_AR$ alpha3 subunit clusters which was not associated with TPH immunopositive profiles. In order to identify which non-serotonergic cell types express the $GABA_AR$ alpha3 subunit in the DRN, quadruple immunofluorescence for TPH, the $GABA_AR$ alpha1 subunit, the $GABA_AR$ alpha3 subunit and GFP was performed using tissue from the GAD67-GFP mouse model (Fig. 6c). Relatively sparse $GABA_AR$ alpha3 subunit puncta co-localised with $GABA_AR$ alpha1 subunit puncta on non-TPH profiles in the DRN (Fig. 6c1, 2). This $GABA_AR$ alpha3 subunit immunoreactivity was localised to GAD67-GFP immunopositive somata and $GABA_AR$ alpha1 subunit labelled dendrites (Fig 6c3, 4). At least a proportion of $GABA_AR$ alpha3 subunit immunopositive non-serotonergic neurons were GABAergic. However, because GAD67-GFP immunoreactivity did not label distal dendrites of GABAergic neurons of the DRN, we cannot rule out the possibility that a third, non-serotonergic, non-GABAergic, cell type in the DRN also expresses the $GABA_AR$ alpha3 subunit.

Compensatory changes in the levels of co-expressed $GABA_AR$ subunits in various brain regions have been reported following the constitutive deletion of a particular subunit (Peng et al. 2002; Schneider Gasser et al. 2007; Kralic et al. 2006). In order to assess whether such changes occur in the DRN, the level of $GABA_AR$ alpha3 subunit immunoreactivity was compared in tissue from WT and alpha1$^{-/-}$ (Fig.7a) or alpha2$^{-/-}$ (Fig. 7b) mice. There was a striking increase in the intensity of $GABA_AR$ alpha3 subunit immunoreactivity in the DRN, on both TPH immunopositive and immunonegative profiles of alpha1$^{-/-}$ mice (Fig. 7b1-3) compared to WT mice (Fig. 7a1-3). Such changes need to be borne in mind when assessing alterations in DRN function from alpha1$^{-/-}$ mice. In contrast, no difference was detected in the intensity of $GABA_AR$ alpha3 subunit immunoreactivity in the DRN of alpha2$^{-/-}$ mice (data not shown).

The $GABA_AR$ gamma2 subunit is enriched on non-serotonergic neurons

Within the DRN, GAD67-GFP immunopositive neurons exhibited the highest levels of $GABA_AR$ gamma2 subunit immunoreactivity (Fig. 8a2) with the signal being localised to somatic and dendritic compartments on these putative GABAergic neurons (Fig. 8a1). Furthermore, widespread co-localisation between the $GABA_AR$ alpha1 and gamma2 subunit signal on GAD67-GFP immunopositive neurons was evident (Fig. 8a3, 4) as well as on TPH-immunopositive profiles which co-localised with $GABA_AR$ alpha2 and alpha3 subunit signals (Fig. 8b, c).

CRH immunoreactive puncta are associated with $GABA_AR$ molecular machinery on serotonergic and non-serotonergic neurons of the DRN

DRN neuronal activity is influenced by stress and the effect of stress on serotonin release in DRN projection regions is both stressor and brain region specific (Adell et al. 1997). Within the DRN, information related to stressful stimuli is conveyed predominantly by the stress neuropeptide corticotrophin releasing hormone (CRH) which, in the rat, is contained

in axon terminals and cell bodies (Valentino et al. 2001; Waselus et al. 2005). The bath application of CRH to acute brain slices containing the DRN decreases serotonergic neuronal activity by enhancing both the local release of GABA as well as the postsynaptic GABA receptor sensitivity (Kirby et al. 2008). Furthermore, the intracerebroventricular administration of CRH directly influences DRN neuronal excitability and serotonin release in DRN target fields (Price et al. 1998; Price and Lucki 2001; Kirby et al. 2000). We therefore investigated a possible anatomical basis for an interaction between mouse DRN $GABA_AR$ and CRH systems by examining the association of the $GABA_AR$ molecular machinery, with that of CRH on the cell types of the DRN. CRH immunoreactive profiles were closely opposed to a sub-population of gephyrin-immunoreactive puncta located on TPH immunopositive profiles (Fig. 9a1-3), as well as being closely opposed to $GABA_AR$ alpha1 subunit immunoreactivity which outlined the membrane of a non-TPH neuron (Fig. 9b). This suggests that within the DRN, CRH is released into a sub-population of $GABA_AR$-containing synapses on both principal and non-principal neurons.

Repeated stress alters $GABA_AR$ expression at the mRNA level in a subunit specific manner

Since stress has been shown to influence DRN neuronal activity (Lemos et al. 2011; Wood et al. 2013) this raises the question as to whether stress has a direct effect on DRN $GABA_AR$ expression. If so, identifying the $GABA_AR$ subtypes which underlies such stress-induced plasticity of the DRN will be vital in devising therapeutic strategies against stress-related mental illnesses. We therefore investigated whether stress directly influences the expression profile of specific $GABA_AR$ subunits within the DRN. Repeated stress significantly increased the level of expression of the mRNA encoding for the $GABA_AR$ alpha2 subunit by 13% (N=14 animals; $p = 0.007$; Student's T test) and that of the $GABA_AR$ alpha3 subunit by 27% (N=14 animals; $p = 0.011$; Student's T test). However, exposure to stress had no significant effect on the level of the mRNA encoding for the

$GABA_AR$ alpha1 subunit (N=14 animals; p = 0.365; Student's T test) and the $GABA_AR$ gamma2 subunit (N=14 animals; p = 0.386; Student's T test) (Fig. 10a). We then investigated whether this stress-induced increase in the $GABA_AR$ alpha3 subunit encoding mRNA translated to a change in $GABA_AR$ alpha3 subunit expression at the protein level on TPH immunopositive profiles of the DRN. Exposure to stress significantly increased the density of $GABA_AR$ alpha3 subunit immunoreactive clusters located on TPH-immunopositive profiles (mean ± SEM; control, 32 ± 0.5 clusters per 1000um^2 versus 57 ± 0.7 clusters per 1000um^2; p <0.001, Mann Whitney; N = 3 control animals, 3 stress animals) (Fig. 10b, c). Thus, this stress paradigm increased the expression of the $GABA_AR$ alpha3 subunit within the DRN at both the mRNA and protein level.

To speculate on the functional contribution of such a stress-induced increase in $GABA_AR$ alpha3 subunit expression on serotonergic neuronal activity, we quantified the changes in the degree of co-localisation between $GABA_AR$ alpha3 subunit-immunoreactive clusters and gephyrin, a synaptic anchoring protein. Gephyrin directly interacts with the $GABA_AR$ alpha3 subunit (Tretter et al. 2011) and can therefore be used to infer potential changes in synaptic/extrasynaptic density ratios. Stress did not produce a change in the mean ± SEM density (number of cluster *per* 1000 μm^2) of gephyrin immunoreactive clusters localised to TPH immunolabelled profiles (control, 28 ± 0.6 versus stress, 29 ± 0.3; p = 0.480 , Mann Whitney; N = 3 control animals and 3 stress animals). However, stress increased the mean density ± SEM (number of clusters per 1000 μm^2) of $GABA_AR$ alpha3 subunit immunoreactive clusters which were co-localised with those immunoreactive for gephyrin on TPH immunopositive profiles (control, 6 ± 0.4 *versus* stress, 10 ± 0.2; p = 0.019, Mann Whitney; N = 3 control and 3 stress animals). A proportion of $GABA_AR$ alpha3 subunit immunoreactive clusters were not co-localised with those immunoreactive for gephyrin and probably represent an extrasynaptic pool of alpha3 subunit containing $GABA_AR$ subtypes. Repeated stress significantly increased the mean density ± SEM (number of clusters per

1000um^2) of these $GABA_AR$ alpha3 subunit immunoreactive clusters which did not co-localise with gephyrin (control, 27 ± 0.4 *versus* stress, 46 ± 0.6; $p < 0.001$, Mann Whitney; N = 3 control and 3 stress animals) (Fig. 10d). Collectively, these data suggest that stress increases both the putative synaptic and extrasynaptic pools of alpha3-$GABA_AR$ subtypes on the serotonin neurons of the DRN.

Discussion

The study demonstrates the diversity in the regional, cell-type and domain specific expression patterns of four $GABA_AR$ subunits within the mouse DRN and that the repeated stress paradigm used in this study directly influences the level of expression of specific $GABA_AR$ subunits at the mRNA and protein levels, on individual, identified cell-types. Collectively, these data demonstrate the rich molecular repertoire of the $GABA_AR$ apparatus within the neurochemically and functionally diverse cell-types of the DRN and that the expression levels of specific $GABA_AR$ subtypes can be shaped by the environment. We recently reported on the relative contributions of $GABA_AR$-mediated phasic and tonic inhibitory postsynaptic currents to serotonergic neuronal excitability at the single cell level (Maguire et al. 2013). Therefore, this expression analysis provides a platform for investigating and interpreting the eventual influence of these specific $GABA_AR$ subtypes on the DRN-serotonin system in the context of intact DRN cellular networks in the behaving animal.

The potential role $GABA_AR$ alpha1 subunit in the DRN networks

$GABA_AR$ alpha1 subunit immunoreactivity within the DRN was confined to all cells which expressed the molecular signature of GABA, namely GAD67, as well as a population of TPH-immunopositive neurons located preferentially in rostral regions of the DRN. The prediction arising from this expression pattern is that the activation of alpha1-$GABA_AR$s on these different cell-types, the subsequent dampening of excitability and the consequent reduced quantal release of GABA, will have contrasting effects on serotonin release from the DRN due to the divergent projection patterns of these alpha1-$GABA_AR$ immunopositive cell-types. Different populations of GABAergic neurons within the DRN project to either local serotonin-expressing neurons (Challis et al. 2013), or to other brain regions such as

the prefrontal cortex, nucleus accumbens, and lateral hypothalamus (Bang and Commons 2012). Local circuit GABAergic neurons mono-synaptically contact DRN serotonergic neurons and inhibit their activity, impacting on DRN-associated behaviours (Challis et al. 2013; Soiza-Reilly et al. 2013). Thus, the activation of alpha1-$GABA_A$Rs on DRN GABAergic interneurons is likely to decrease excitability of these interneurons and result in a disinhibition of the principal cells with a consequent increase in serotonin release. Conversely, activation of alpha1-$GABA_A$Rs on the select population of rostrally-located serotonergic neurons should decrease their neuronal activity leading to decreased serotonin release. Alpha1 subunit expressing serotonergic neurons were enriched dorsally, in rostral DRN, such anatomically located DRN serotonin neurons project to the caudate putamen, amygdala, thalamus, nucleus accumbens and the neocortex (O'Hearn and Molliver 1984; Waselus et al. 2011). It is tempting to speculate that DRN serotonin release within these brain regions must be under the strict temporal control of somatically located, fast synaptic $GABA_A$R neurotransmission mediated by alpha1 containing $GABA_A$R subtypes, rather than dendritic, slower synaptic $GABA_A$R neurotransmission mediated by alpha2 and alpha3 containing $GABA_A$Rs. The functional significance of alpha1 subunit containing $GABA_A$R-mediated inhibition of specific, topographically organised DRN serotonin neurons remains to be determined. However, it may shape DRN serotonin release in a brain region specific manner and contribute to the involvement of the DRN in a vast array of behaviours

The $GABA_A$R alpha2 and 3 subunits in the DRN

The overall appearance of the $GABA_A$R alpha2/3 subunit immunoreactivity differed greatly to that of the alpha1 subunit. $GABA_A$R alpha2/3 subunit labelling was clustered and enriched on the dendritic domains of DRN serotonergic neurons. The quantitative data revealed that 45% and 36% of NL2 clusters located on TPH-immunopositive profiles co-localised with alpha2 and alpha3 subunit clusters, respectively, indicating that the majority

of GABAergic postsynaptic inhibitory currents on DRN principal neurons are mediated by alpha2/3-GABA$_A$Rs. A further point of note was the proportion of GABA$_A$R alpha3 immunoreactive clusters which did not co-localise with NL2. This pattern of expression, which did not appear to be restricted to particular serotonergic neurons, suggests a component of non-synaptically located alpha3-GABA$_A$R subtypes, which predictably might mediate tonic GABAergic inhibition in the DRN. Indeed, in our recent functional study using somatic whole-cell patch clamp recordings of acute DRN slices (Maguire et al. 2013), GABA$_A$R-mediated tonic inhibitory currents were detectable, but only in ~30% of serotonergic neurons. The apparent discrepancy between the proportion of serotonergic neurons exhibiting a GABA-mediated tonic conductance and the immunohistochemistry may result from the preferential location of GABA$_A$R alpha3 subunit immunoreactive clusters on TPH-immunopositive dendrites (Fig. 8) precluding their detection with somatic recordings. Indeed, cell-types, such as the principal neurons of the basolateral amygdala, in which robust alpha3-GABA$_A$Rs mediated tonic currents are detectable, have GABA$_A$R alpha3 subunit immunoreactivity enriched on their somata; see Fig. 3G of (Marowsky et al. 2012). Alternatively, the levels of ambient GABA experienced by some neurons may not be sufficient (greater than 10 μM- see Maguire et al., 2013) to activate such extrasynaptic alpha3-GABA$_A$Rs.

The importance of such persistent patterns of inhibition in particular neurons is likely to indicate the requirement for sustained modulation of neuronal activity compared to brief periods of phasic inhibition. This pattern of persistent inhibitory regulation of neuronal activity could be particularly important for diffuse modulatory systems such as serotonergic pathways which provide brain-wide release of their neurotransmitters during the processing of on-going sensory stimuli, or a specific behavioural state. In turn, alterations in the levels of expression of such extrasynaptically located GABA$_A$R subtypes might dynamically regulate serotonin release resulting in altered behaviours or even DRN-

serotonin associated disorders. Behavioural inflexibility, the inability to thrive in a constantly changing environment is a feature of mood disorders. Therefore, in terms of regulating mood for example, the balance between slow persistent and fast, brief pulses of inhibition may provide the neuron with the flexibility to seamlessly adapt its firing pattern in response to external inputs such as various stressors. Thus, the changes in synaptic:extrasynaptic expression ratios of particular $GABA_AR$ subunits and the accompanying changes in phasic and tonic currents in the context of animal models of mental illnesses could provide novel insights to the changes in neuronal activity which underlie such behaviour.

The $GABA_AR$ gamma2 subunit in the DRN

$GABA_AR$ gamma2 subunit heterozygous knockout mice display reduced $GABA_AR$ clustering and exhibit an anxiogenic-like behavioural phenotype (Crestani et al. 1999) which infers a role for gamma2-$GABA_ARs$ mediated inhibition in such disorders. Indeed, classical benzodiazepine agents with anxiolytic activity potentiate GABAergic neurotransmission preferentially at gamma2-alpha2/3-beta-$GABA_AR$ subtypes (Rudolph and Knoflach 2011). The evidence for the involvement of alpha2-$GABA_ARs$ in anxiety and anxiolytic drug effects is clear (Low et al. 2000; Smith et al. 2012; Engin et al. 2012; Koester et al. 2013). It is thus surprising that in this study, immunoreactivity for the $GABA_AR$ gamma2 subunit was enriched on DRN non-serotonergic cells which co-expressed the $GABA_AR$ alpha1 and 3 subunits with noticeably lower levels of $GABA_AR$ gamma2 signal associated with $GABA_AR$ alpha2/3 subunit immunoreactivity on serotonergic neurons, suggesting a lesser role for alpha2-$GABA_ARs$ in DRN-benzodiazepine mediated anxiolysis. The use of mutant mouse models in which specific $GABA_AR$ alpha subunits are either rendered insensitive to benzodiazepines (Wieland et al. 1992; Benson et al. 1998; Rudolph and Knoflach 2011) or deleted, in a cell-specific manner (Wisden 2010; Kos 2004) will be invaluable in determining the precise cell-types

which mediate the behavioural effects of ingested $GABA_AR$-specific ligands or possibly underlie associated disorders.

Stress and $GABA_AR$ expression in the DRN

Overwhelming evidence indicates that $GABA_ARs$ are integral to DRN stress circuitry, although their precise roles are difficult to unequivocally define. Acute swim stress in rats results in an increase in the frequency of GABAergic miniature inhibitory postsynaptic currents on serotonergic neurons (Lamy and Beck 2010), while five days of social defeat attenuates inhibitory synaptic input onto serotonergic neurons (Crawford et al. 2013). Furthermore, the application of the stress neuropeptide CRH to acute slices of the DRN has both direct and indirect effects on GABAergic currents onto serotonergic neurons (Kirby et al. 2008). The current data build on these studies by providing a high resolution analysis of the changes in the expression of specific $GABA_AR$ subunits within identified cell-types of the DRN.

Multiple and distinct mechanisms are likely in place to modulate this stress-induced $GABA_AR$ alpha3 subunit expression. Glucocorticoids, as a result of the HPA activation, are central molecular players of the stress response and signal both peripherally and centrally to engage metabolic and neural process required for dealing with adversity. As such, the signal transduction cascades initiated by such steroid hormones and their derivatives are well placed to dynamically regulate gene transcription in response to an external stimulus. Chronic exposure to glucocorticoids has been shown to elevate $GABA_AR$ subunit mRNA expression within the hippocampus (Orchinik et al. 1995). As the DRN expresses high levels of glucocorticoid receptor (Aronsson et al. 1988), glucocorticoid mediated regulation of transcription may mediate the stress induced increases in $GABA_AR$ subunit mRNA in the DRN shown here. Interestingly, $GABA_AR$ subunit mRNA expression within the LC, a region of enriched glucocorticoid receptor expression, was not significantly affected by this

stress protocol. The obvious caveat is that stress might affect $GABA_AR$ subunit expression within the LC at the protein level. Nevertheless, this suggests that multiple mechanisms are in place to regulate $GABA_AR$ mRNA expression in a brain region specific manner. Other potential mechanisms may include epigenetic influences such as dinucleotide methylation of cytosine-phosphodiester bond-guanine (CpG) islands (Weaver et al. 2006) and histone modification (Renthal et al. 2007; Uchida et al. 2011). Both mechanisms have received considerable attention recently in mediating the effects of environmental factors on brain function, behaviours and psychiatric diseases (Tsankova et al. 2007). Bioinformatic analysis of the *Gabra3* gene revealed the absence of a CpG island within or near the *Gabra3* promotor sequence; this suggests that if epigenetic mechanisms do contribute to the regulation of *Gabra3* gene transcription, it is unlikely to be mediated by changes in the methylation status of CpG dinucleotides. Several different types of post translational histone modifications exist. However, acetylation and methylation are the most widely studied. Histone deacetylase inhibitors have been shown to influence $GABA_AR$ subunit mRNA expression in the nucleus accumbens (Kennedy et al. 2013). Therefore, it is possible that post translational histone modifications may also contribute to stress induced alterations in $GABA_AR$ subunit mRNA plasticity within the DRN. Evaluation of epigenetic changes in $GABA_AR$ subunit expression could represent new avenues for investigating how environmental factors shape brain function and behaviour.

In addition to increased $GABA_AR$ alpha3 mRNA levels, we also show that stress increases the expression of the $GABA_AR$ alpha3 subunit at the protein level. Through extensive intracellular signalling cascades, encompassing a vast array of proteins, trafficking pathways dynamically regulate the distribution and number of diverse $GABA_AR$ subtypes within the cell membrane (Luscher et al. 2011). Due to the complexity of $GABA_AR$ trafficking, multiple points along the secretory or endocytotic pathway may be targeted to influence $GABA_AR$ expression and in turn neuronal activity. Clathrin-mediated endocytosis

is an important factor in regulating $GABA_AR$ expression (Kittler et al. 2000). The phosphorylation status of beta subunits of $GABA_ARs$ has a major role in regulating the clathrin mediated endocytosis of such receptors. When specific residues of the $GABA_AR$ beta subunits are phosphorylated, the affinity between the $GABA_AR$ beta subunit and clathrin adaptor protein (AP2) is reduced thereby attenuating receptor endocytosis (Kittler et al. 2005). A number of proteins contribute to the phosphorylation of $GABA_AR$ subunits including protein kinase A, C and B (PKA, PKC, PKB) as well as calmodulin dependent kinase II (CaMKII). Furthermore, neurosteroids have recently been implicated in the phosphorylation and membrane insertion of $GABA_ARs$ (Abramian et al. 2014). A potential trigger of such secondary messenger cascades is likely to the endogenous stress hormone CRH. Receptors for CRH are coupled to diverse intracellular signalling pathways which activate kinase pathways including PKA and PKC (Hauger et al. 2009). Since CRH together with both receptors for CRH, CRH-R1 and R2 are expressed and mediate the effects of stress on DRN neuronal activity (Kirby et al. 2000), CRH is the likely candidate through which stress can dynamically regulate the expression of $GABA_AR$ subtypes via PKA or PKC mediated phosphorylation of $GABA_AR$ beta subunits.

One particularly intriguing finding of this study was that repeated stress impacts upon $GABA_AR$ alpha3 subunit expression at both mRNA and protein levels. This is surprising as the constitutive activity of endoplasmic reticulum (ER) degradation enzyme results in it being more efficient for a cell to recycle endocytosed $GABA_ARs$ rather than transporting newly assembled receptors from the ER to the cell membrane. Thus, the reach of environmental stimuli to both the gene and protein levels suggests that even this mild stress paradigm exerted a significant demand on the alpha3-$GABA_AR$ machinery of the cell necessitating the engagement of a variety of intracellular signalling cascades including those likely to be less energy efficient. These processes are likely to serve the cell well during short-term bouts of stress, as is the case in this paradigm. Indeed, since we

deliberately used a mild stress protocol which numerous studies have shown does not manifest in a chronic stress phenotype, it is speculative that such stress-induced increases in the levels of $GABA_AR$ alpha3 subunit expression parallels or contributes to adaptive, rather than maladaptive responses to stressful life experiences. In turn, during severe chronic periods of stress, especially episodes which trigger mental disorders, such molecular pathways could be exhausted. Thus, the expectation is that longer periods of stress will eventually manifest in lower levels of $GABA_AR$ expression within the DRN. If so, this could lead to, for example, an anxiogenic profile which might be ameliorated by benzodiazepine treatment which augments the activity of the ensuing lower levels of $GABA_ARs$. Such data could provide a wholly novel avenue on the biological mechanisms which underpin adaptive or maladaptive stress pathways and may contribute to the identification of newer and more effective drug targets for the treatment of stress-related psychiatric disorders.

In conclusion, the study demonstrates the molecular identity and the location of the $GABA_AR$ subunits which are likely to determine some of the most salient effects of GABA within the DRN-serotonin system, which are known to influence the excitability of the DRN and consequently behaviour. Furthermore, the evidence that stress directly impacts on the level of expression of the $GABA_AR$ alpha3 subunit provides a distinct molecular target for future studies investigating the mal/adaptive consequences of stress-induced alterations in the function of the DRN-serotonin system.

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Legends

Fig. 1

Immunohistochemical localisation of inhibitory synaptic marker proteins on serotonergic neurons of the DRN

(a1) shows gephyrin immunoreactive clusters enriched on dendritic compartments of putative serotonergic neurons identified with tryptophan hydroxylase (TPH) immunoreactivity, with notably fewer gephyrin clusters present on somatic domains. (a2) shows that neuroligin2 (NL2) immunoreactive clusters displayed a similar distribution pattern to that of gephyrin, with NL2 immunoreactivity being enriched on TPH-immunopositive dendrites. (a3) numerous NL2 clusters co-localise with gephyrin. (b) quantification of the density of individual and colocalised gephyrin and NL2 immunoreactive clusters. Scale bars 5 µm.

Fig. 2

Confirmation of the specificity of the immunoreactivity patterns produced by antibodies against the $GABA_AR$ alpha1, alpha2 or alpha3 subunits in the DRN using tissue from WT and $GABA_AR$ subunit-specific gene-deleted mice processed and imaged under identical conditions

(a1) shows an overview of TPH immunoreactivity in tissue from WT (alpha1$^{+/+}$) mouse. (a2) shows an overview of the pattern of $GABA_AR$ alpha1 subunit immunoreactivity in the DRN of WT tissue with extensive signal evident throughout most of the DRN although the signal is particularly enriched in the lateral wing sub-regions. (b1) shows an overview of TPH immunoreactivity in tissue from the DRN of a $GABA_AR$ alpha1 subunit-specific gene-deleted mouse (alpha1$^{-/-}$). No differences in the levels of TPH signal in the DRN of WT and alpha1$^{-/-}$mice were detectable (b2) shows that no specific $GABA_AR$ alpha1 subunit immunoreactivity was detectable in tissue from an alpha1$^{-/-}$ mouse. (c1) shows the

localisation of NL2 on tryptophan hydroxylase (TPH) immunoreactive profiles of the DRN indicating the distribution of putative inhibitory synapses on serotonergic processes within the DRN of WT mouse. (c2) shows the pattern of $GABA_AR$ alpha2 subunit immunoreactivity in the same field of view. (d1) shows the distribution and intensity of TPH and NL2 immunoreactivity in the DRN of an alpha2$^{-/-}$mouse. (d2) shows that no specific $GABA_AR$ alpha2 subunit immunoreactivity was detectable in tissue from alpha2$^{-/-}$ mice. (e1) shows TPH immunoreactivity in the DRN of WT mouse. (e2) shows the pattern of $GABA_AR$ alpha3 subunit immunoreactivity in the same field of view. Note how $GABA_AR$ alpha3 subunit immunoreactivity closely follows the pattern of TPH immunoreactivity. (f1) an overview of the pattern of TPH and (f2) $GABA_AR$ alpha3 subunit immunoreactivity in the DRN of an alpha3$^{-/-}$ mouse showing the absence of any specific signal for this subunit. Scale bars (a, b, e, f) 50 μm; (c, d) 10 μm.

Fig. 3

Immunolocalisation of the $GABA_AR$ alpha1 subunit in neurochemically diverse cell-types of the DRN

(a1 – a3) the $GABA_AR$ alpha1 subunit immunoreactive profiles are widely distributed throughout the DRN. (A1) an overview, taken from the ventromedial sub-region of the DRN showing an overlay of the immunoreactive signals for the $GABA_AR$ alpha1 subunit, the dendritic marker protein microtubule associated protein 2 (MAP2) and TPH indicating that such $GABA_AR$ alpha1 subunit immunopositive cells comprise a significant proportion of the neurons composing the DRN. (a2) is a magnified view of the inner boxed area in (a1) showing $GABA_AR$ alpha1 subunit immunoreactivity which is closely associated with numerous MAP2 immunolabelled dendrites as well as being on a soma (asterisk) which in (a3) is TPH-immunonegative. (a3) shows TPH immunoreactivity associated with somatic as well as dendritic (arrowheads) profiles. Images from (b) and (c) were acquired from the dorsal and ventral regions of the DRN respectively and demonstrate $GABA_AR$ alpha1

subunit immunoreactivity in neurochemically distinct cell-types and its varying association with gephyrin immunoreactivity across these sub-regions of the DRN. Immunoreactivity for the $GABA_AR$ alpha1 subunit was evident in cells which were (b1) TPH immunopositive (#), (b2, c1, 2) TPH immunonegative, GAD67-GFP immunopositive (*) and (b1 & 2) a discrete population of cells not labelled by either TPH or GAD67-GFP (+). (b3) shows the strong association of gephyrin with $GABA_AR$ alpha1 subunit immunoreactive dendrites apart from (c3) which shows that the GAD67-GFP $GABA_AR$ alpha1 subunit immunolabelled neurons shown in (b,c2) exhibit a relative enrichment of gephyrin immunoreactivity on their somata and proximal dendrites. In panel (b), the inserts shows magnified views of the boxed areas. (b4) and (c4) are overlays of all the individual (b) and (c) panels respectively. Scale bar 10 μm.

Fig. 4

$GABA_AR$ alpha1 subunit-immunopositive TPH labelled cells are differentially distributed throughout the rostra-caudal and ventro-medial extents of the DRN.

(a1, b1, c1) schematics of the anatomical coordinates and the DRN sub-nuclei whereby the number of total TPH and TPH-$GABA_AR$ alpha1 subunit-immunopositive cells at the rostral, midline and caudal planes were quantified respectively according to (Paxinos and Franklin 2004). (a2, b2, c2) graphical representation of the number of total TPH and TPH-$GABA_AR$ alpha1 subunit-immunopositive cells within the different DRN sub-nuclei at the rostral, midline and caudal planes respectively. Bars represent means with lines indicating SEM. N = 3 animals. (a3, b3, c3) shows the proportion of TPH-$GABA_AR$ alpha1 subunit-immunopositive cells relative to the total number of TPH immunopositive cells at the rostral, midline and caudal planes respectively. lDRN, lateral region of the DRN; ifDRN, interfasicular region of the DRN; vDRN, ventral region of the DRN.

Fig. 5

Immunolocalisation of the $GABA_AR$ alpha2 subunit in the DRN

(a1) shows an overview of NL2 immunoreactivity in the DRN indicating the distribution of putative inhibitory synapses. (a2) shows an overview of $GABA_AR$ alpha2 subunit immunoreactivity. Note that the immunoreactive clusters are largely uniformly sized and associated with TPH immunopositive dendrites with only dispersed clusters evident on TPH immunopositive somata (asterisks). (a3) an overlay of (a1) and (a2) indicating the extensive co-localisation of $GABA_AR$ alpha2 subunit clusters with those of NL2. (b) magnified views of the boxed regions in (a). The arrowheads point to (b1) NL2 immunopositive clusters and (b2) $GABA_AR$ alpha2 subunit immunopositive clusters which in (b3) co-localise. Note that some NL2 immunopositive clusters do not co-localise with $GABA_AR$ alpha2-immunoreactive clusters (arrows). Scale bar (a) 10 µm, (b) 5 µm.

Fig. 6

Immunolocalisation of the $GABA_AR$ alpha3 subunit in the DRN

(a1) shows that NL2 immunoreactivity preferentially located on TPH immunopositive dendrites. (a2) $GABA_AR$ alpha3 subunit-immunoreactive clusters were preferentially localised to TPH immunopositive dendrites rather than somata and appeared more variable in both size and shape when compared with those of the $GABA_AR$ alpha2 subunit (Figure 5). (a3) a subpopulation of NL2 immunoreactive clusters present on TPH immunopositive profiles co-localised with the notably larger sized $GABA_AR$ alpha3 subunit clusters (arrowheads). In contrast, the smaller sized $GABA_AR$ alpha3 subunit clusters together with the diffuse signal did not co-localise with NL2-immunoreactive signal (arrows). The inserts in (a1-3) are magnified views of the boxed areas in the respective panels. (b) shows the distribution of $GABA_AR$ alpha3 subunit immunoreactivity in the thalamic reticular nucleus (nRT), a brain region known to express only this $GABA_AR$ alpha

subunit and specifically in inhibitory synapses. (b1) NL2 immunoreactive puncta decorated parvalbumin labelled processes within the nRT. (b2) $GABA_AR$ alpha3 subunit immunoreactivity presented as large distinct clusters associated with parvalbumin immunopositive positive profiles. (b3) shows that the majority $GABA_AR$ alpha3 subunit immunoreactivity colocalise with NL2 immunoreactivity within the nRT indicating that the association of this subunit with inhibitory synaptic markers is cell-type specific. (c) Sparse, discrete $GABA_AR$ alpha3 subunit immunopositive clusters decorated non-serotonergic, $GABA_AR$ alpha1 subunit, GAD67-GFP immunopositive profiles in the DRN. (c1) shows $GABA_AR$ alpha3 subunit immunoreactive clusters on TPH-immunopositive profiles (arrowheads) whilst a proportion of $GABA_AR$ alpha3 immunoreactive clusters were not associated with TPH immunopositive profiles (arrows). (c2) shows $GABA_AR$ alpha3 subunit clusters not associated with TPH-immunopositive profiles co-localised with $GABA_AR$ alpha1 immunopositive signal. (c3) some of these $GABA_AR$ alpha3 subunit-immunopositive, TPH-immunonegative neurons were immunopositive for GFP-GAD67 signal. Scale bar 10µm.

Fig. 7

The constitutive brain-wide deletion of the $GABA_AR$ alpha1 subunit increases $GABA_AR$ alpha3 subunit immunoreactivity the DRN

(a1, b1) no discernible differences were evident in the intensity of gephyrin and TPH immunoreactivity in the DRN of WT (alpha1$^{+/+}$) and $GABA_AR$ alpha1 subunit-specific gene-deleted (alpha1$^{-/-}$) mouse. This also serves to confirm that the comparative images were taken at identical focal planes (a2, b2) the deletion of the alpha1 subunit results in a dramatic increase in the intensity of $GABA_AR$ alpha3 subunit immunoreactivity. Note that WT and alpha1$^{+/+}$ mouse tissue was processed, reacted and imaged under identical conditions. (a3, b3) An overlay shows an apparent increase in the extent of co-localisation between gephyrin and $GABA_AR$ alpha3 subunit immunoreactive clusters similar to the

degree of $GABA_AR$ alpha1 subunit-gephyrin co-localisation in Figure 3b. The insert in (B3) shows dense $GABA_AR$ alpha3 subunit immunoreactivity which is co-localised with that of gephyrin and outlines the somato-dendritic plasma membrane of a non-TPH labelled cell in the DRN, reminiscent of $GABA_AR$ alpha1 subunit-immunopositive neurons shown in Figure 3b. There was no evidence of such $GABA_AR$ alpha3 subunit-enriched cells in DRN tissue from WT mice. Scale bars 10μm.

Fig. 8

The association between $GABA_AR$ gamma2 subunit immunoreactivity with immunoreactivity for the $GABA_AR$ alpha1, 2 and 3 subunits in the DRN

(a) shows that the majority of the $GABA_AR$ gamma2 subunit immunoreactivity in the DRN is contained on $GABA_AR$ alpha1 subunit GAD67-GFP-immunopositive neurons. (a1) shows immunoreactivity for non-TPH expressing cells which are immunopositive for GFP-GAD67 and likely represent local-circuit GABAergic interneurons. (a2) these GAD67-GFP-immunopositive neurons exhibited strong immunoreactivity for the $GABA_AR$ gamma2 subunit which was localised to their somatic and dendritic compartments. (a3) $GABA_AR$ alpha1 subunit immunoreactivity patterns closely followed that of the $GABA_AR$ gamma2 subunit being localised to the somatic and dendritic domains of these GAD67-GFP-immunopositive neurons. (a4) shows widespread co-localisation between $GABA_AR$ gamma2 subunit and $GABA_AR$ alpha1 subunit immunoreactive clusters within the DRN. The insert is a magnified view of a GAD67-GFP immunopositive neuron (*). (b-c) a proportion of $GABA_AR$ gamma2 subunit immunopositive puncta co-localised with $GABA_AR$ alpha2 subunit and $GABA_AR$ alpha3 subunit clusters on TPH immunopositive profiles. Arrows highlight $GABA_AR$ gamma2 immunoreactive puncta not co-localised with alpha2/alpha3 puncta, whereas arrowheads highlight $GABA_AR$ gamma2 puncta co-localised with alpha2/alpha3 puncta. (b1) and (c1) show $GABA_AR$ gamma2 subunit immunoreactive clusters on the somatic and dendritic compartments, but enriched on the

dendritic domains of TPH immunopositive and TPH immunonegative profiles (arrows). (b2, c2) shows $GABA_AR$ alpha2 and alpha3 subunit immunoreactivity decorating the dendritic and somatic domains of TPH immunopositive profiles. (b3, c3) $GABA_AR$ gamma2 subunit immunoreactive clusters displayed minimal co-localisation with $GABA_AR$ alpha2 and alpha3 subunit immunoreactive clusters. A magnified view of the boxed area highlights the sparse co-localisation of $GABA_AR$ gamma2 subunit immunoreactive clusters with $GABA_AR$ alpha2 and with alpha3 immunoreactive clusters (arrowheads). Scale bars 10 μm.

Fig. 9

Corticotrophin releasing hormone (CRH) immunoreactive puncta are associated with gephyrin and $GABA_AR$ alpha1 subunit immunoreactive clusters on TPH and non-TPH profiles within the DRN

(a) shows isolated CRH immunoreactive puncta closely opposed to gephyrin immunoreactive clusters on TPH immunopositive profiles which are likely to represent $GABA_AR$ alpha2-3 subunit immunopositive synapses (arrowheads). (b) shows intense CRH immunoreactive signal closely opposed to $GABA_AR$ alpha1 subunit immunoreactivity which outlines the membrane of a non-TPH cell within the DRN (arrowheads). Scale bar 5 μm

Fig. 10

Repeated restraint stress alters the mRNA and protein levels of specific $GABA_AR$ subunits within the DRN

(a) the relative expression levels of mRNA encoding for $GABA_AR$ subunits above or below control. In the DRN, the influence of stress on levels of mRNA expression of $GABA_AR$ subunits was subunit specific, with the alpha2 and alpha3 subunit encoding mRNA levels significantly increased above control. In contrast, no significant differences in the $GABA_AR$ alpha1 and gamma2 subunit encoding mRNA levels were observed. (N = 14 animals per

group). (b) and (c) are representative images of the levels of $GABA_AR$ alpha3 subunit immunoreactivity on TPH immunopositive profiles within the DRN in tissue from control and stress animals respectively, processed and imaged under identical conditions (d) graphical representation of the quantification of the stress-induced changes in $GABA_AR$ alpha3 subunit and gephyrin immunoreactivity on TPH immunopositive profiles within the DRN. Bars represent means with lines indicating SEM. N = 3 animals per group, *p <0.05, ***p <0.001; Mann Whitney. Scale bar 5 μm.

Table 1
Details and characterisation of antibodies used in this study

Primary antibodies	**Species (raised in)**	**Source/code**	**Dilution**	**Specificity reference**
$GABA_A$R alpha1 subunit	Rabbit	Werner Sieghart, Antigen sequence -α_{1N} amino acids 1-9 Rabbit # 21/7, bleed # 04/10/1999	1:5000	(Pirker et al. 2000; Poltl et al. 2003; Corteen et al. 2011) Knockout mouse, this study
$GABA_A$R alpha2 subunit	Guinea pig	Jean-Marc Fritschy, University of Zurich, Zurich, Switzerland Antigen sequence $\alpha_{2N.}$ amino acids 1-9	1:1000	(Fritschy and Mohler 1995; Corteen et al. 2011) Knockout mouse, this study
$GABA_A$R alpha3 Subunit	Guinea pig	Jean-Marc Fritschy, University of Zurich, Zurich, Switzerland Antigen sequence $\alpha_{3N.}$ amino acids 1-15	1: 5000	(Corteen et al. 2011; Fritschy and Mohler 1995) Knockout mouse, this study
$GABA_A$R gamma2 subunit	Guinea pig	Jean-Marc Fritschy, University of Zurich, Zurich, Switzerland Antigen sequence Antigen sequence $\alpha_{3N.}$ amino acids 1-29	1: 3000	(Fritschy and Mohler 1995)
$GABA_A$R gamma2 subunit	Rabbit	Synaptic systems #224 003	1: 1000	Labelling pattern as published with other antibodies. Antibody extensively used in the literature (Essrich et al. 1998; Eyre et al. 2012; Fan et al. 2012)
Tryptophan hydroxylase	Sheep	Millipore #AB1541	1: 3000	Raised to rabbit recombinant TPH. Labelling pattern as published with other antibodies
Gephyrin	Mouse	Synaptic Systems #147 021	1: 500	(Pfeiffer et al. 1984)
Neuroligin2	Rabbit	Synaptic Systems #129 203	1: 1000	Labelling pattern as published with other antibodies.
GFP	Chicken	Aves Labs Inc. #GFP-1020	1: 5000	
Parvalbumin	Mouse	Swant #253	1: 2000	Labelling pattern as published with other antibodies.
Corticorticotrophin releasing hormone	Guinea Pig	Peninsula Laboratories #T-5007	1: 3000	Labelling as published with other antibodies

Table 2

Schedule of the repeated restraint stress protocol

Day 1	2pm: restraint
Day 2	10am: restraint
Day 3	2pm: animal placed in cage with restrainer but not restrained
Day 4	10am: ‘escapable’ restraint
Day 5	2 pm: restraint
Day 6	10am: ‘escapable’ restraint
Day 7	10am: restraint
Day 8	2pm: restraint

Table 3

Primers and probes used for qRT-PCR

Gene	Primers and Probes (Life Technologies™)
gabra1	Mm00439046_m1
gabra2	Mm00433435_m1
gabra3	Mm01294271_m1
gabrg2	Mm00433489_m1
gapdh	Mm99999915_g1

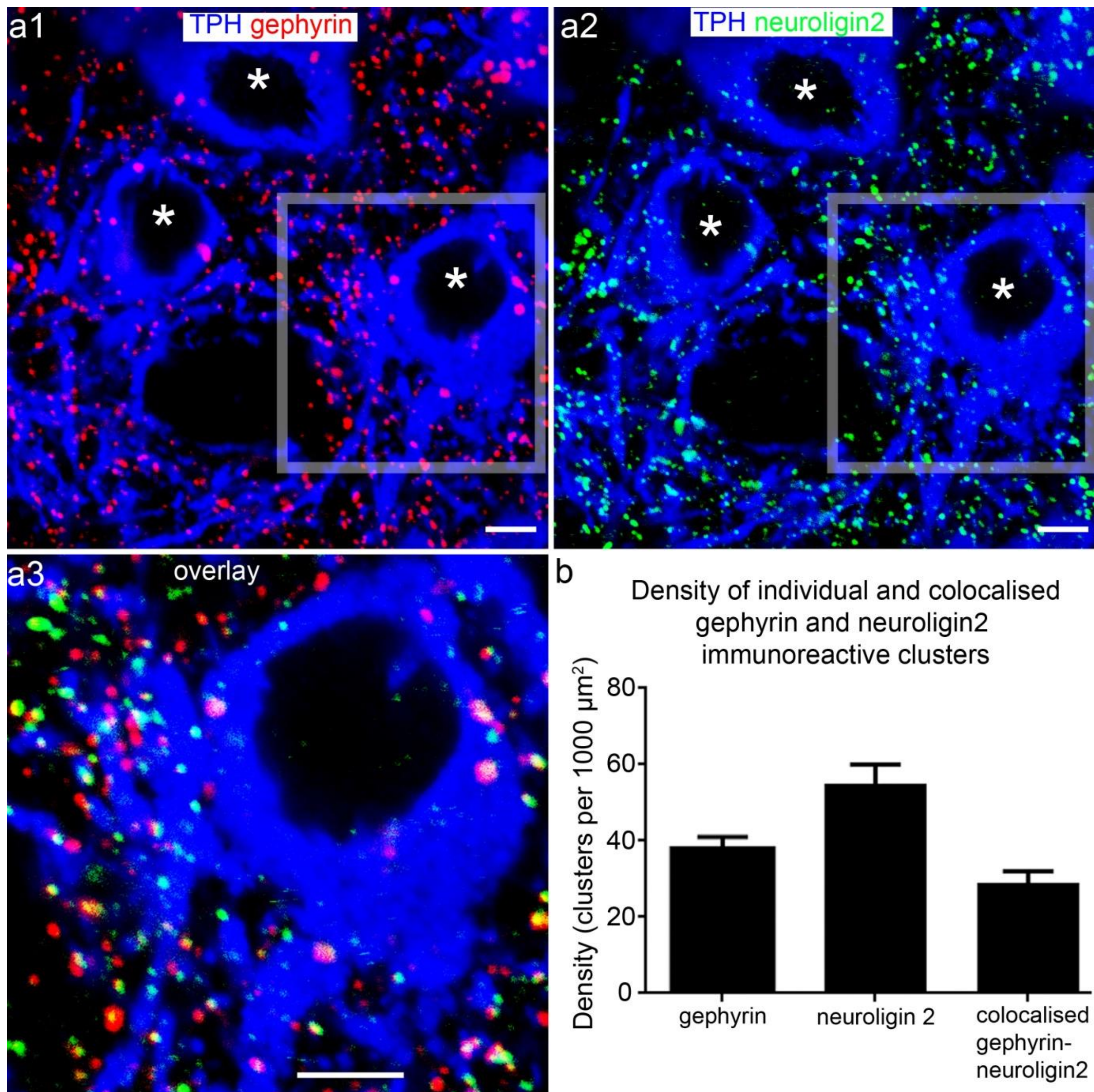
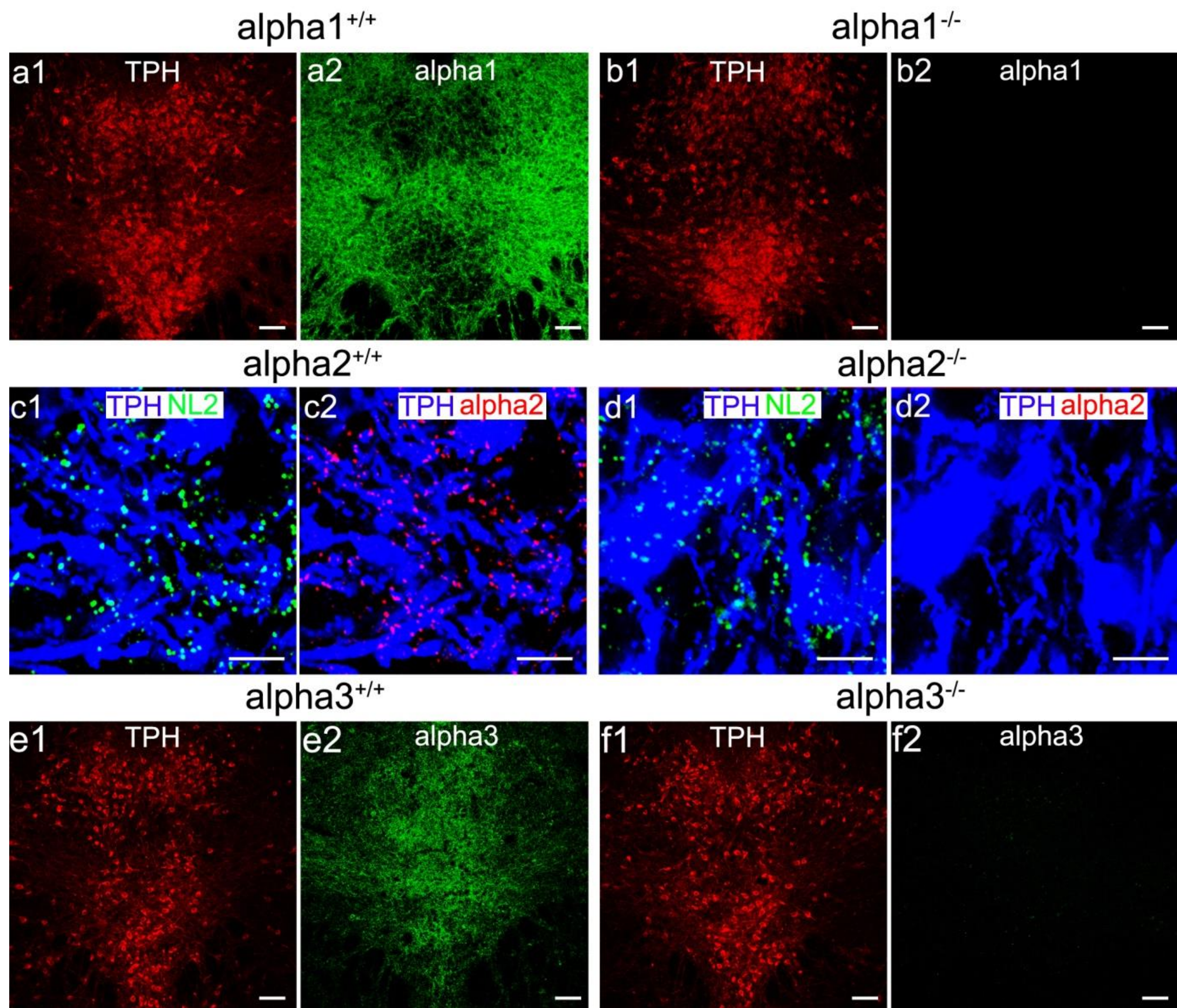


Fig1



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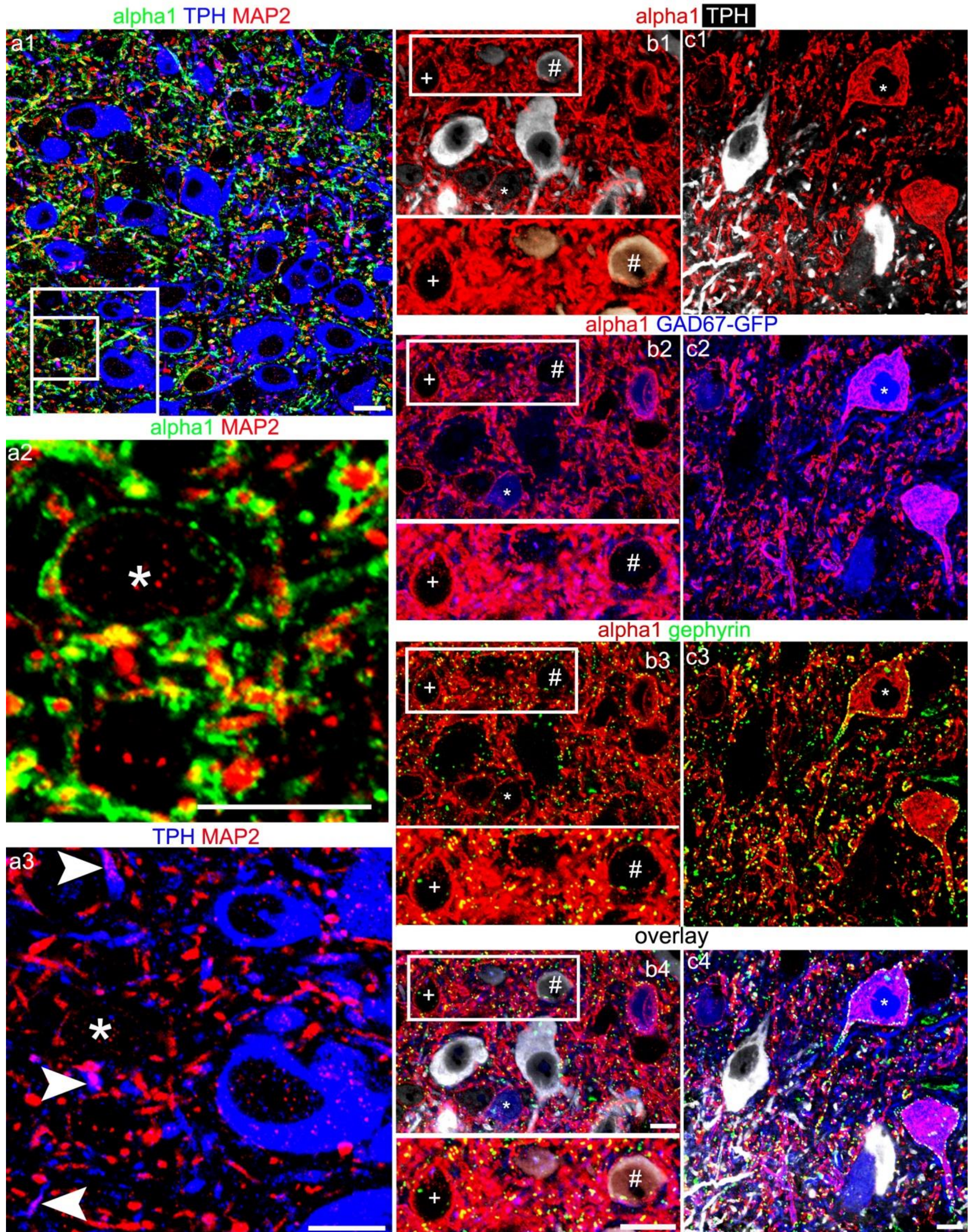

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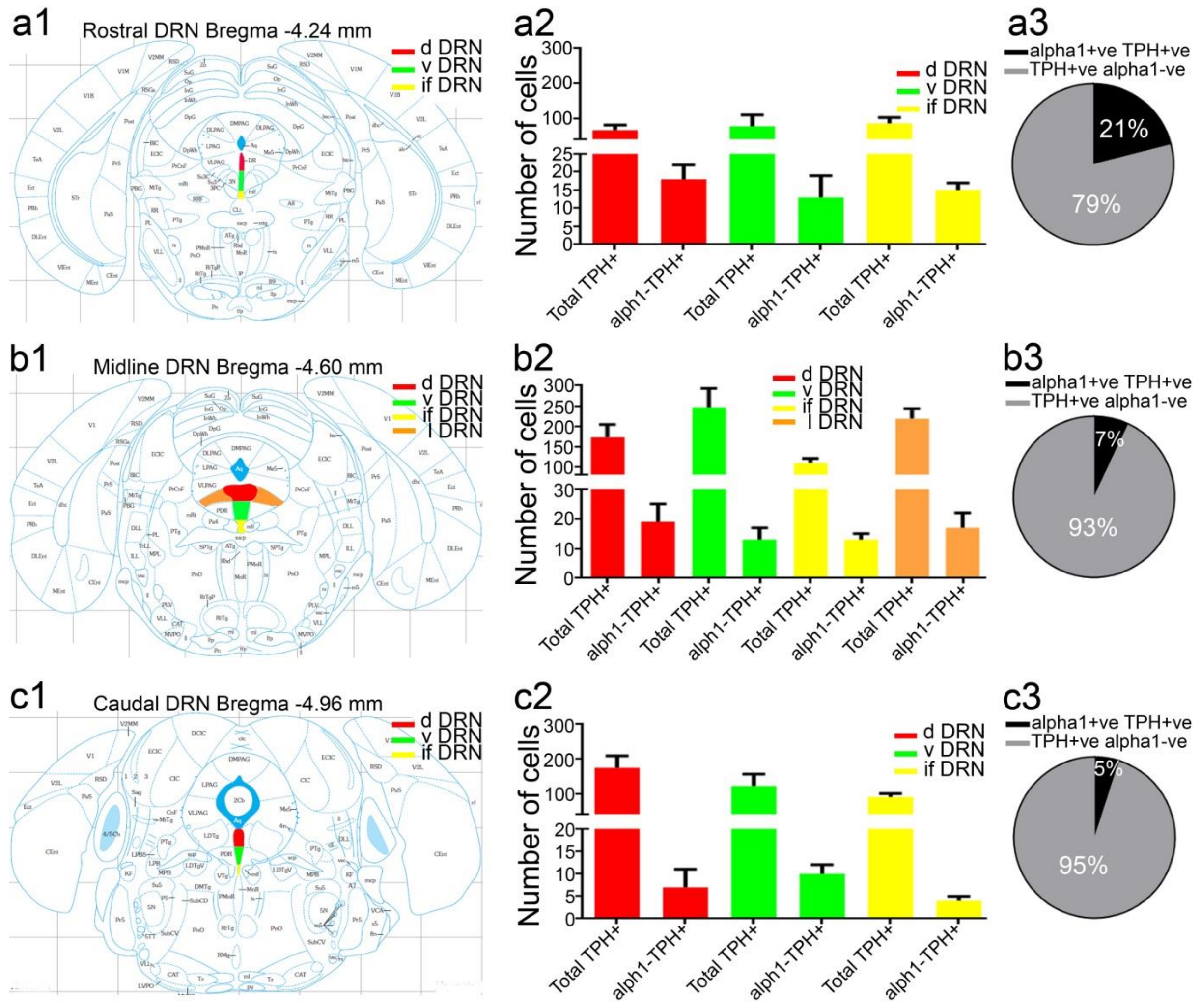

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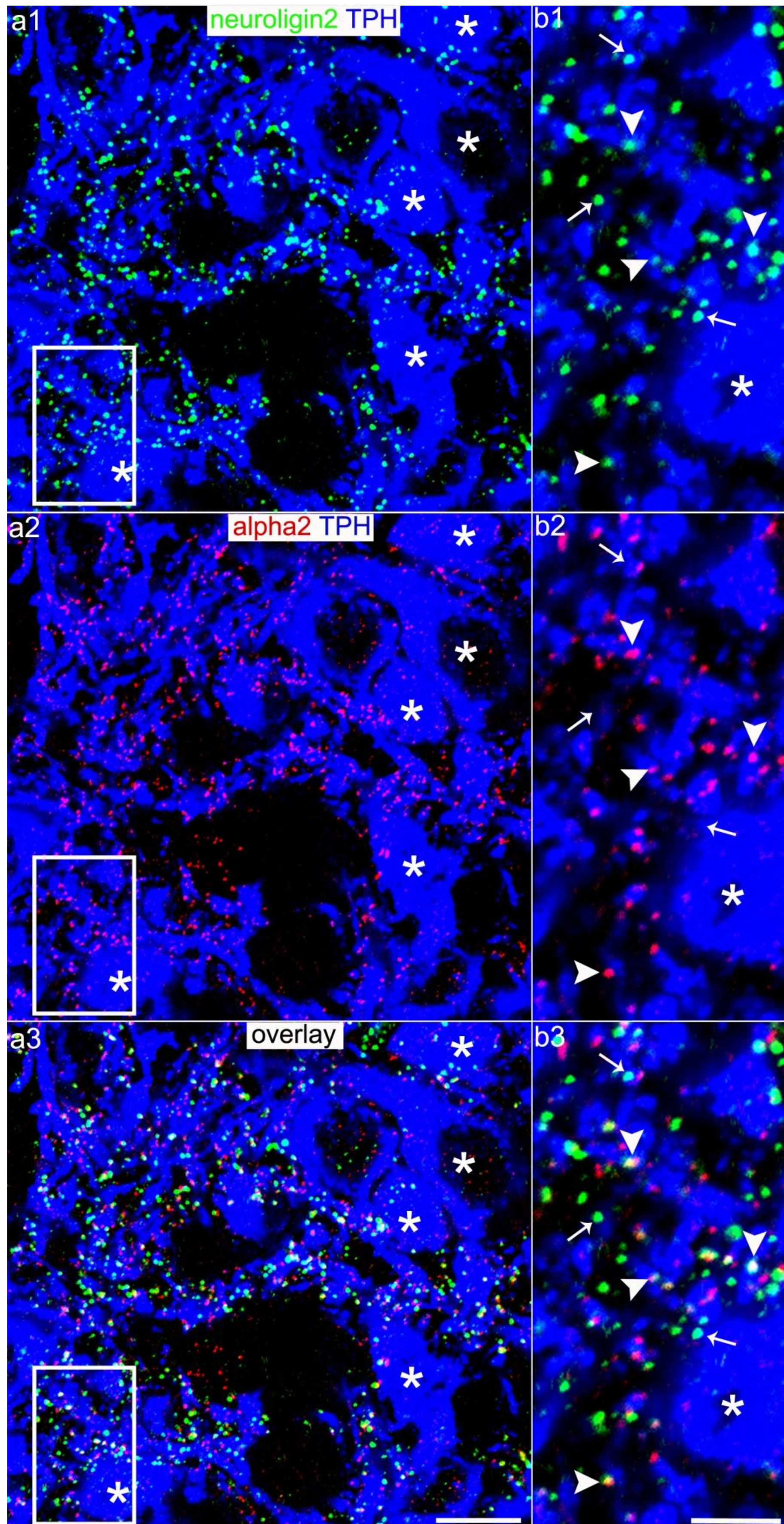

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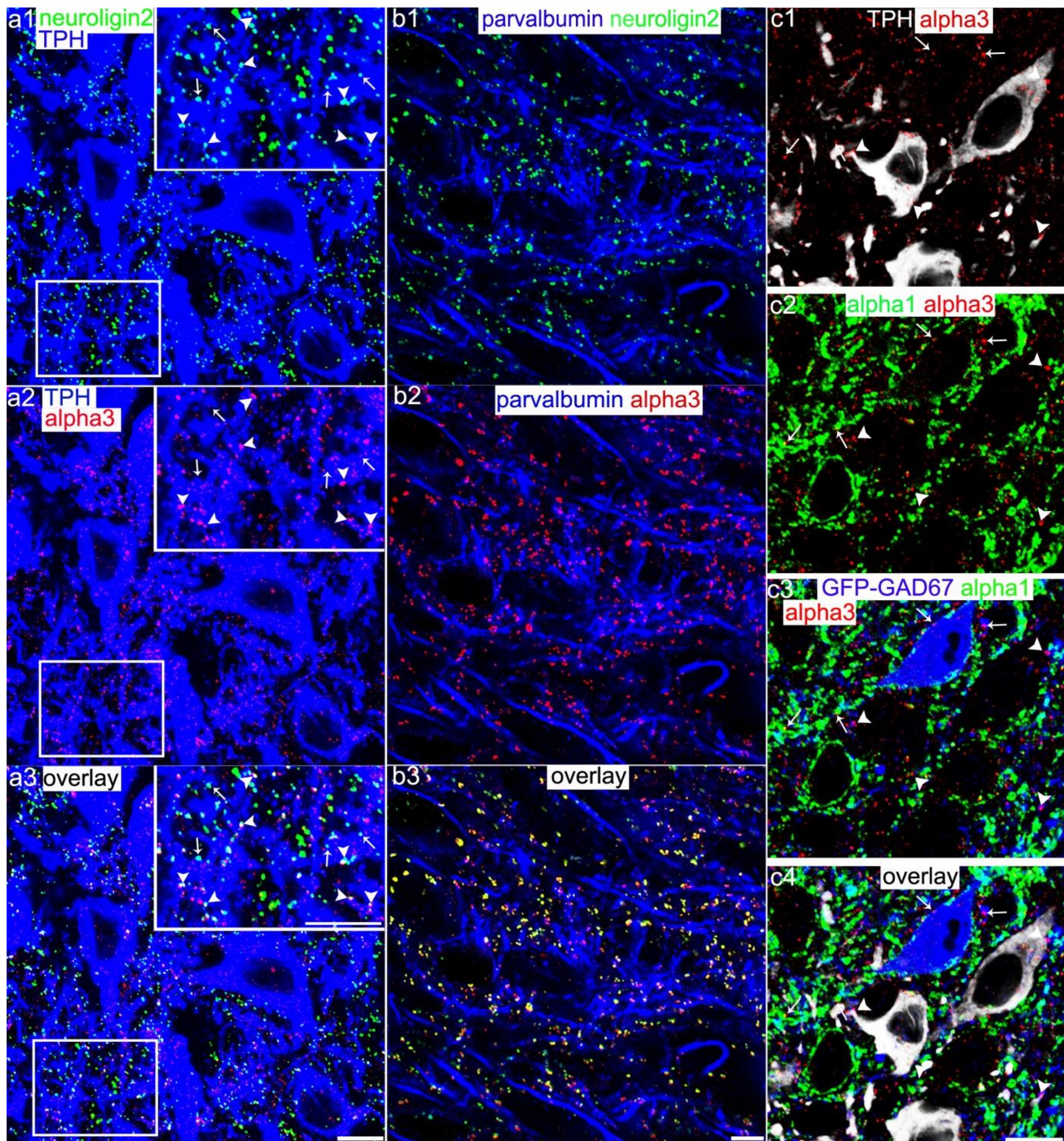

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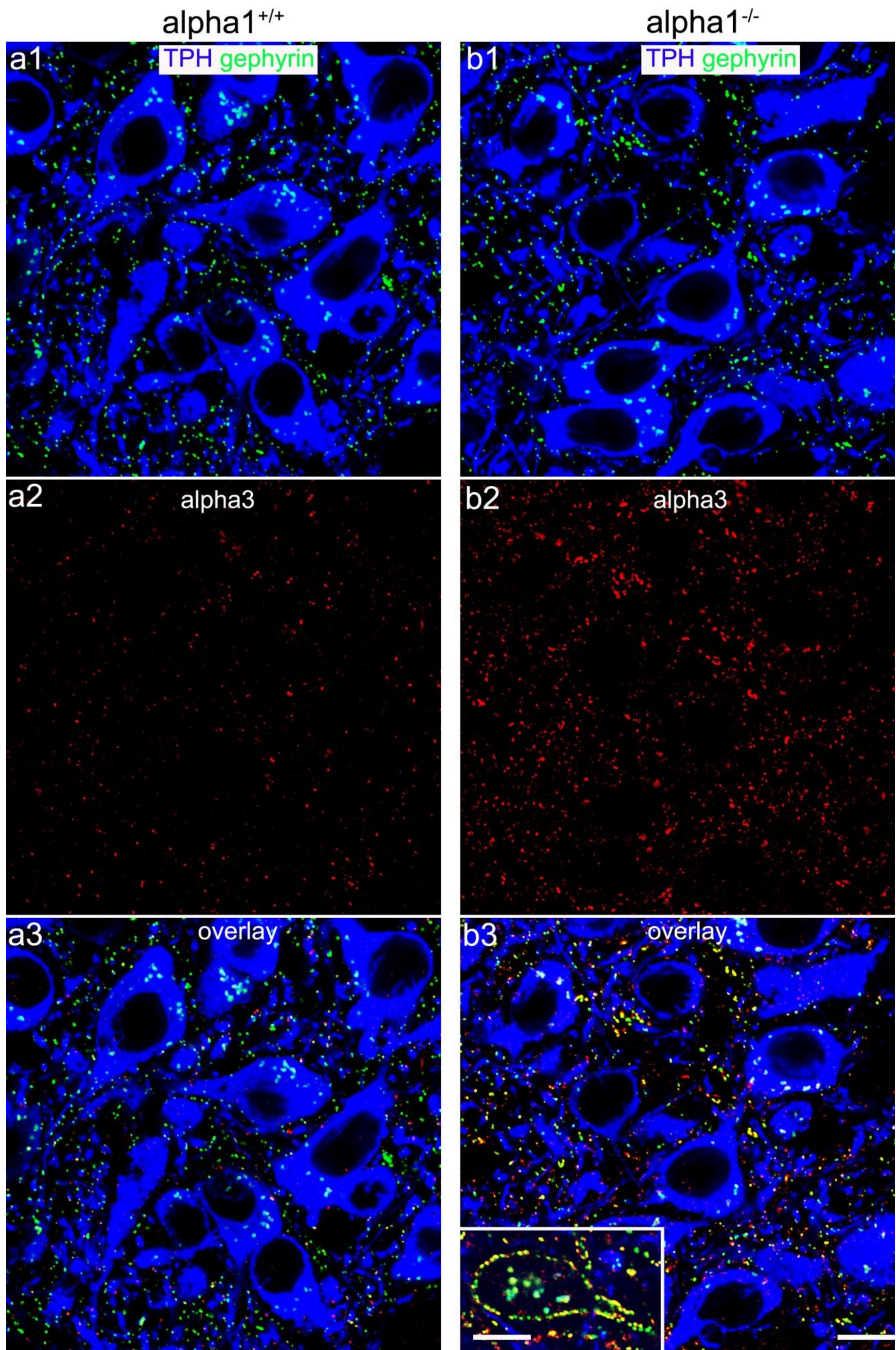

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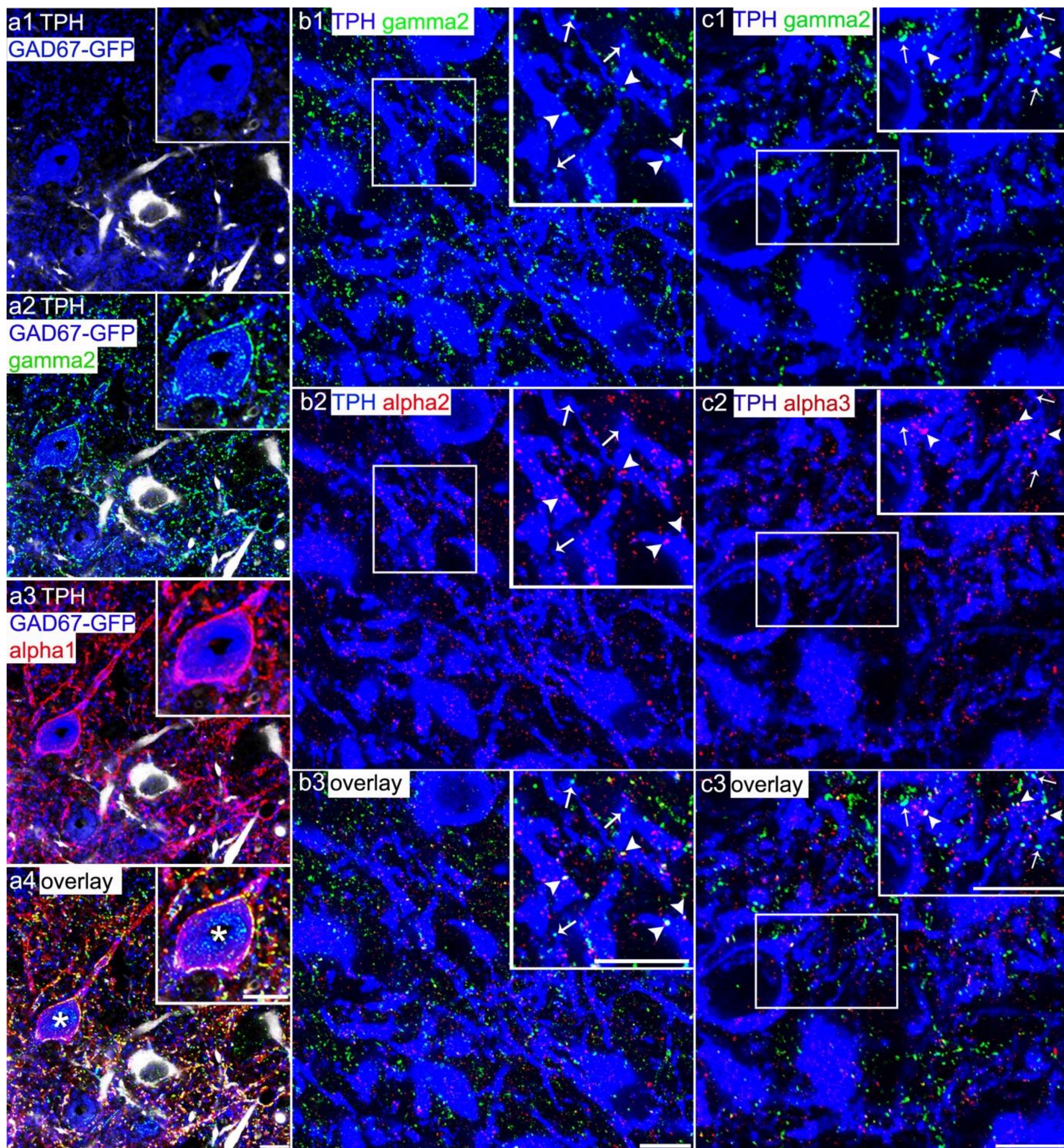

Fig8

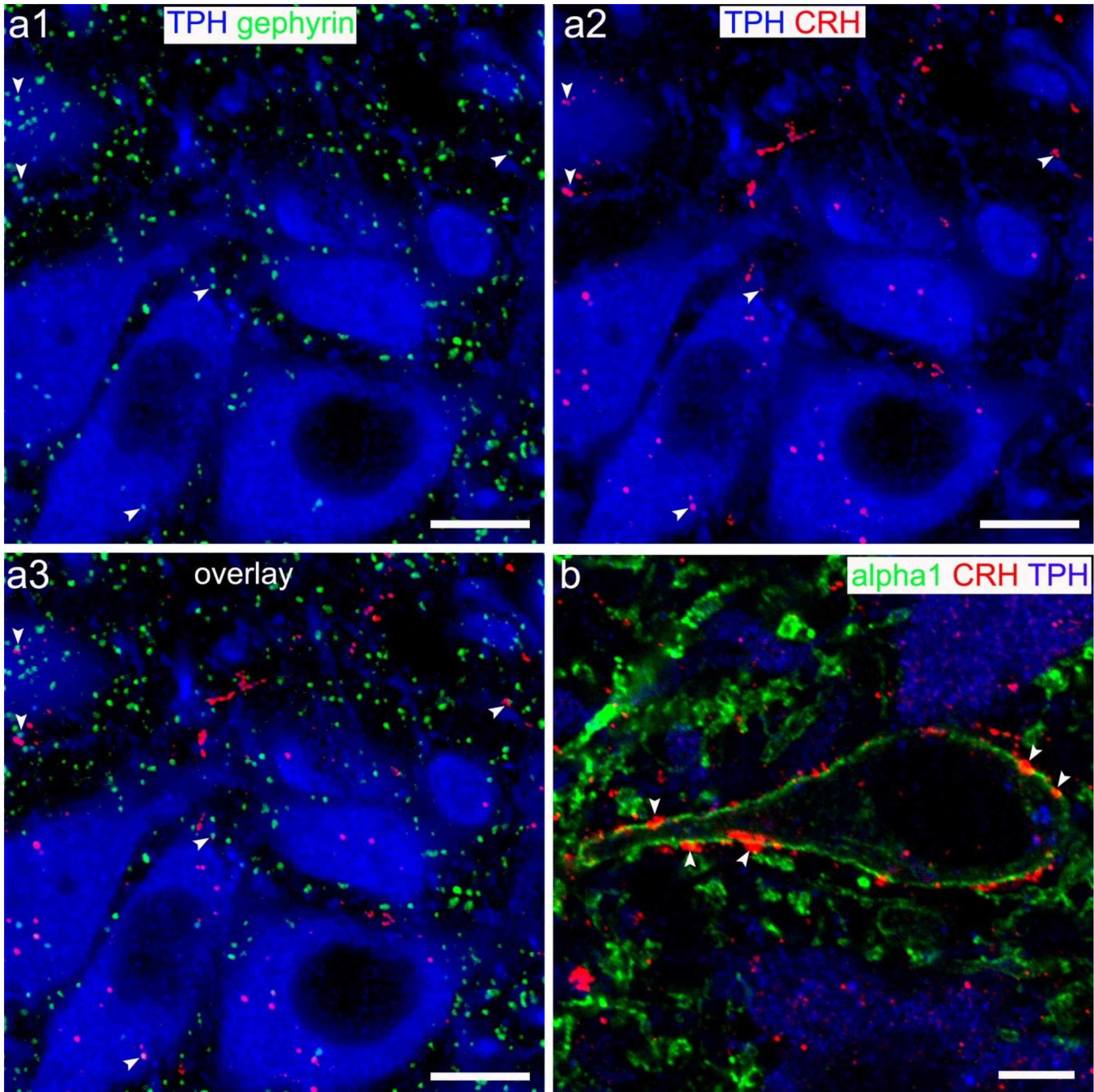

Fig9

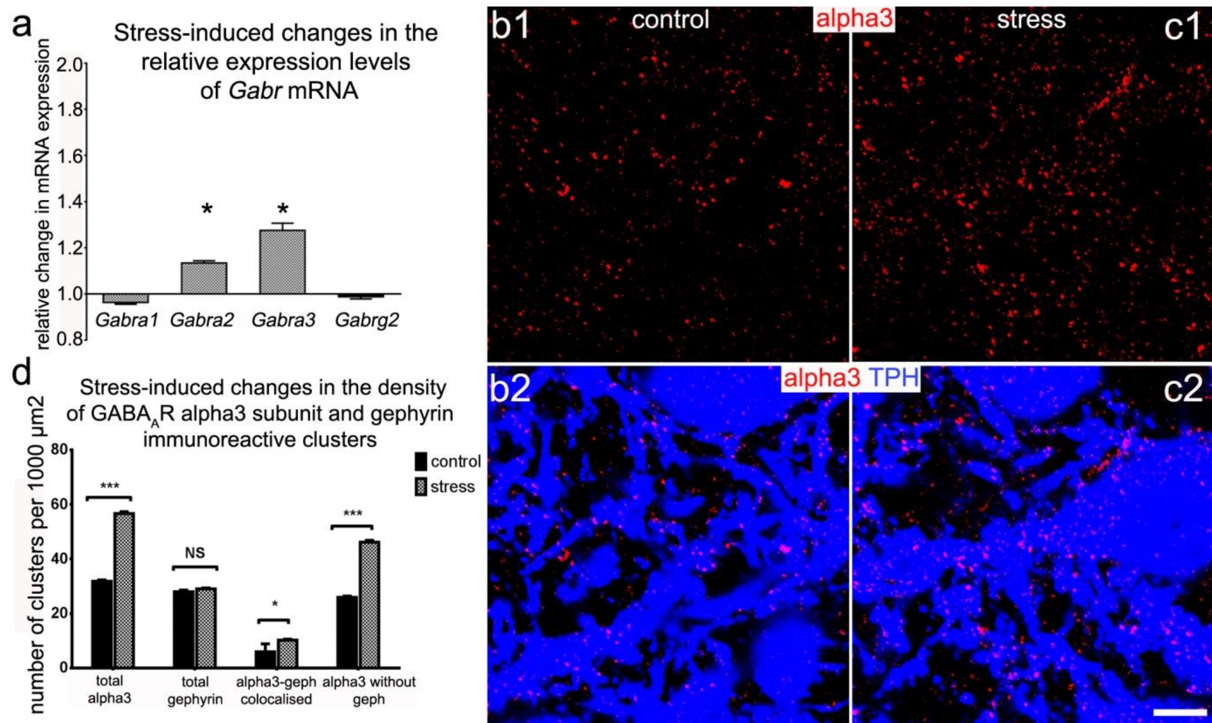

Fig10