Activity- and pharmacology-dependent modulation of adult neurogenesis in relation to Alzheimer’s disease

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Chapter 3: Distinct structural plasticity in the hippocampus and amygdala of the middle-aged common marmosets (Callithrix jacchus)

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Abstract:

Adult neurogenesis in the primate brain is generally accepted to occur primarily in two specific areas; the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of the lateral ventricles. Hippocampal neurogenesis is well known to be downregulated by stress and aging in rodents, however there is less evidence documenting the sensitivity of neuroblasts generated in the SVZ. In primates, migrating cells generated in the SVZ travel via a unique temporal stream (TS) to the amygdala and entorhinal cortex. Using adult common marmoset monkeys (Callithrix jacchus), we examined whether i) adult-generated cells in the marmoset amygdala differentiate into doublecortin-positive (DCX+) neuroblasts, and ii) whether lasting changes occur in DCX-expressing cells in the DG or amygdala when animals are exposed to 2 weeks of psychosocial stress.

A surprisingly large population of DCX+ immature neurons was found in the amygdala of these 4-year-old monkeys with an average density of 163,000 DCX+ cells per mm$^3$. Co-labeling of these highly clustered cells with PSA-NCAM supports that a subpopulation of these cells are migratory and participate in chain-migration from the SVZ to the amygdala in middle-aged marmosets. Exposure to 2 weeks of isolation and social defeat stress failed to alter the numbers of BrdU+, or DCX+ cells in the hippocampus or amygdala when evaluated 2 weeks after psychosocial stress, indicating that the current stress paradigm has no long-term consequences on neurogenesis in this primate.

Keywords: Adult neurogenesis, Stress, Nonhuman primate, Doublecortin, PSA-NCAM, Aging
Abbreviations:
subventricular zone (SVZ)
subgranular zone (SGZ)
Neural stem cells (NSCs)
Dentate gyrus (DG)
Granule cell layer (GCL)
rostral migratory stream (RMS)
Bromodeoxyuridine (BrdU)
Temporal horn of lateral ventricles (tLV)
Temporal stream (TS)
Doublecortin (DCX)
polysialylated neural cell adhesion molecule (PSA-NCAM)
Basal Medial (BM)
Basolateral Ventral Medial Amygdala nucleus (BLVM)
Basolateral Intermediate Amygdala nucleus (BLI)
Entorhinal cortex (EC)
Ventral Cortical Amygdaloid Nucleus (VACo)
Medial (Me)
Lateral amygdala (La)
Introduction:

Adult neurogenesis occurs in at least two specific locations in the brains of adult mammals: the subventricular (SVZ) and subgranular zones (SGZ) located in the lateral ventricles and hippocampal dentate gyrus respectively. These zones are unique, specialized micro-environments that support the production of new cells. They contain neural stem cells (NSCs) with the capacity to proliferate, migrate, and differentiate into adult, functional neurons. Neuronal differentiation of adult-generated cells represents a novel form of structural plasticity with considerable relevance for olfaction and cognition [1, 2]. This process can be identified through markers such as Doublecortin (DCX), a microtubule associated protein found selectively in immature, migratory neurons. DCX is required for the proper migration of immature neurons [3, 4] and is considered an endogenous marker for adult neurogenesis [5, 6].

In the hippocampus, immature neurons migrate from the SGZ into the granule cell layer (GCL) of the dentate gyrus (DG) where they mature into granule neurons. Similarly, neuroblasts born in the SVZ migrate tangentially towards the olfactory bulb along the rostral migratory stream (RMS)[7, 8], an area that has recently also been identified in human brain [9]. Migratory, DCX-positive (+) neurons present in the primate SVZ were found to co-express polysialylated neural cell adhesion molecule (PSA-NCAM), identifying a migratory pathway to the striatum [10]. In parallel with other markers of neurogenesis, DCX expression in the rodent hippocampus decreases strongly with age and is reduced after stress as well [11-15].

A distinct, less well characterized migratory stream exists in primates allowing neuroblasts to migrate directly to the amygdala, an area associated with emotional memories [16]. The temporal stream (TS) has only been described in rhesus and squirrel monkeys, however due to structural homology it is suspected to exist in other primates. A cell fate study combined with migration of lipophilic dye identified a stream of cells extending from the temporal horn of the lateral ventricles (tLV) to the amygdala and piriform cortex [17]. The contribution of cells found in the TS to the amygdala has yet to be quantified.

Here, we studied neurogenesis in the hippocampus, amygdala and associated cortex of four-year-old, middle-aged marmoset monkeys using BrdU and DCX immunocytochemistry and further established the
migratory nature of the DCX+ immature neurons using co-labeling for PSA-NCAM. Secondly, although stress and stress hormone exposure reduces neurogenesis in the DG of young rodents and monkeys [14, 18, 19], it was not known whether psychosocial stress affects the DG and amygdala to the same extent in this primate species. In rodents, reductions in neurogenesis after psychosocial stress are long lasting and can persist despite a normalization of cortisol levels [20, 21]. Stress has also been shown to reduce neurogenesis in adult primates [19, 22]. We thus studied whether isolation and social defeat stress altered neurogenesis (DCX+) and newborn cell survival (BrdU+) in a lasting manner in the marmoset DG and amygdala and studied their brains following an additional 2 week recovery and resocialization period in order to mitigate acute effects and allow a normalization of the initial cortisol rises.

**Materials and Methods**

**Stress exposure in adult marmosets**

All experimental procedures were approved by the Animal Experimentation Committee of the Dutch Institute of Applied Scientific Research (TNO-DEC # 2148). All aspects of animal care are described in Standard Operating Procedures in agreement with the current guidelines of the European Community. Ten 4-year-old adult male (n=5) and female (n=5) common marmosets (*Callithrix jacchus*) were subjected to a 6-week experiment. All monkeys were bred and raised at the Biomedical Primate Research Centre (BPRC), Rijswijk, The Netherlands, and randomized to stress (n=5, 3:2 male: female) and control groups (n=5, 3:2 male: female) prior to the start of the study. The stress protocol (Figure 1), a 6-week paradigm, was composed of three segments; normal housing for behavioral observation (days 0-12), isolation stress and social defeat (stress cohort only) (days 12 to 27), followed by a re-socialization period of 2 weeks (days 27-40). This design was employed in order to study whether social stress had lasting consequences on neurogenesis and structural plasticity.

During the second week of isolation, animals from the stress cohort were placed in the territory of a dominant monkey of the same sex for 10 minutes to additionally expose them to a social-defeat paradigm. Animals were observed and tested during these periods using separate read-outs including behavioral observation and body weight. Cortisol concentrations were determined in 24-hour urine
samples collected in metabolic cages. Samples were collected twice during the normal housing, once in the second week of the isolation period and once at the end of the experimental period directly out of the bladder during euthanasia. 25 µls of the urine sample were applied in antibody-coated tubes in duplicate using commercial radioimmunoassays (Corti-Cote) according to standard procedures (MP Biomedicals Europe, Illkirch France). Subsequently, 0.5 ml of “cortisol tracer solution” with $^{125}$I-cortisol was applied, which binds in competition with the urine cortisol. After incubation of 45 minutes at 37°C the liquid was removed. This was followed by separation of bound and unbound radioactivity and counting of bound radioactivity to determine cortisol concentration.

**Figure 1: Timeline for BrdU injection during a 6-week study.** Marmosets were observed for 2 weeks. Prior to beginning the stress component all animals were injected with 5-bromo-2’-deoxyuridine (BrdU). Stress cohort animals were housed in isolation for one week followed by one week of isolation and daily social-defeat in the territory of a dominant male animal. Stress animals were then given two weeks of re-socialization before euthanization.

**Bromodeoxyuridine injection and immunohistochemistry**

To analyze whether stress exposure had affected the survival rate of newborn cells, 5-bromo-2’-deoxyuridine (10 mg/ml dissolved in 0.007 M NaOH/0.9% NaCl, at a dose of 200 mg/kg., Sigma) was injected subcutaneously before onset of isolation, i.e. at day 12. Twenty-eight days later, i.e. at day 40, which would allow sufficient time for newborn cells to undergo neuronal differentiation, animals were euthanized by decapitation under isoflurane/NO$_2$ inhalation anesthesia and blood was
collected from each marmoset after decapitation. The brains were then removed immediately. The right hemisphere was washed and placed in 4% paraformaldehyde overnight for immunohistochemistry while the left hemispheres were kept in nitrogen and used for proteomics analysis in a separate study.

Before sectioning from a dry-ice cooled tissue block on a sliding microtome, the brains were transferred to 30% sucrose in 0.1 M PBS, pH 7.4, until they sank. Brains were then cut in the coronal plane in 40 μm sections; free floating sections were washed thoroughly in 0.05 M PBS, pre-incubated with 0.3% H$_2$O$_2$, and incubated with the primary antibodies (Table 1). Antibodies were diluted in 1% BSA-0.05 M PBS, 0.5% Triton for 1 hr at room temperature and then overnight at 4°C: rat anti-BrdU (Accurate Chemical Westbury NY), rabbit polyclonal anti-GFAP (DakoCytomation, Denmark), goat anti-DCX (Santa Cruz Biotechnology), mouse PSA-NCAM (Millipore, Billerica, Massachusetts), corresponding secondary antibodies were incubated at 1:200 for 2 hrs at room temperature. ABC reagent (Vestastain Elite; Vector Laboratories, Burlingame, CA) was applied for 2 hrs at room temperature. Sections were double stained for BrdU and DCX with Diaminobenzidine + 0.08% Nickel (DAB-N; Sigma Germany) and DAB respectively leaving a black precipitate on BrdU+ nuclei and a brown precipitate on DCX+ cells.

### Table 1.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Immunogen</th>
<th>Species</th>
<th>Dilution</th>
<th>Source/ Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-BrdU</td>
<td>5-bromo-deoxyuridine</td>
<td>Rat</td>
<td>1:1000</td>
<td>Accurate Chemical CloneBU1/75 (ICR1)</td>
</tr>
<tr>
<td>Anti-PSA-NCAM</td>
<td>Viable meningococcus group B</td>
<td>Mouse</td>
<td>1:400</td>
<td>Millipore MAB5324</td>
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<tr>
<td>Anti-GFAP</td>
<td>GFAP (cow spinal cord)</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>DAKO Z0334</td>
</tr>
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</table>

### Quantification

The sampling volume of the DG and amygdala were determined by drawing contours around these regions of interest using the StereoInvestigator system (Microbrightfield, Williston, VT). In the hippocampus, DCX+ and BrdU+ positive cells were present in small numbers and all positive cells (on average 14 markers per section)
were therefore quantified manually. Markers in the DG were counted using 20x and 40x objectives in 3 mid-level hippocampal sections. BrdU+ nuclei in the amygdala were also hand-counted individually after morphological assessment again using 3 selected sections matched to the same anterior-posterior level. The amygdala ranges from approximately +5.50 through +3.64 mm Bregma as published in the marmoset brain atlas [23], also available online [24]. Sections for quantification were identified at the level of approximately +4.28 mm Bregma. To quantify DCX+ cells in the amygdala a different strategy was employed due to the presence of a very large number of cells. Quantification was completed using the StereoInvestigator system by systematically sampling regions of interest at regular intervals. Overcounting bias is avoided by the use of upper and lower guard zones, i.e. excluding cells found within 1.5 μm of the top and bottom of the dehydrated section according to standard stereological procedures.

DCX/PSA-NCAM Immunofluorescence

To establish whether DCX+ neurons were indeed migratory, co-expression of DCX and PSA-NCAM was examined using double immunofluorescence and confocal imaging. Sections were co-incubated with antibodies for DCX and PSA-NCAM and then incubated independently with Alexa-488 donkey anti-goat and Alexa-647 donkey anti-mouse (1:200 Molecular Probes, Carlsbad, California). Fluorescent signals were imaged with a Zeiss LSM 510 confocal laser-scanning microscope; emission signals from the Alexa-488 and Alexa-647 probes were assigned to green and red channels, respectively. Images obtained by immunofluorescence were not digitally manipulated in any way but were cropped to provide clearly framed images.

Antibody Characterization

The monoclonal mouse anti-PSA-NCAM detects a single band on Western blot according to manufacturer's technical information; recognizing the 180-kDa, highly sialiated form of NCAM found predominantly in embryonic brain [25]. The staining pattern we observed is identical to that described in previous reports [26, 27]. The goat polyclonal anti-DCX detects a single band at 40 kDa on Western blot of adult rat OB [5, 28]. Staining morphology for DCX+ cells was
identical to a prior publication (Oomen, et al., 2010). Rat monoclonal anti-BrdU reacts with BrdU in single-stranded DNA (manufacturer’s technical information). We observed no labeling when anti-BrdU was incubated with non-BrdU-injected rats used as a control. The morphological pattern of staining we observed was identical to that described in previous reports [29, 30].

The rabbit polyclonal anti-GFAP antibody precipitates one distinct GFAP protein from cow brain extract (manufacturer’s technical information). The antibody identifies a 50-kd band in western blot analysis of brain tissue [31]. The distribution and morphology of GFAP+ astrocytes matched previous descriptions [32].

**Results**

**Hippocampal neurogenesis**

In the dentate gyrus, BrdU+ cells were confined to the granule cell layer, SGZ, and hilus. The black immunostained BrdU+ nuclei had a round morphology making them easy to identify; only cells in the SGZ and GCL were used for quantitative analysis. DCX+ cells appeared as immature neurons or neuroblasts within the granule cell layer with a robust cytoplasmic staining. Many labeled cells had secondary and tertiary processes that were relatively short and lacked further bifurcations. These processes typically extending in a radial manner through the granule cell layer (Fig 2A-C); generally, the morphology was similar to other published images e.g. DG of rodents [5, 33]. In the DG, approximately 3-fold more DCX+ cells were observed than BrdU+ cells. BrdU: control 9910 ± 3120 (mean ± SE) cells/mm$^3$, stress cohort 6220 ± 820 (mean ± SE) cells/mm$^3$, DCX: control 32000 ± 3640 (mean ± SE) cells/mm$^3$, stress 24300 ± 3670 (mean ± SE) cells/mm$^3$. Rare double-positive BrdU/DCX cells were observed in the DG (Fig 2D); the two markers were never co-localized in the hilus. Populations of BrdU- and DCX-positive cells were not significantly correlated (Pearson 2-tailed test, R= 0.32, p = 0.08) in the marmoset DG. This corresponds with a higher number of immature neuroblasts transiently expressing DCX and a lower number of BrdU+ surviving cells.
Figure 2: DCX expression in the marmoset hippocampus. Low numbers of DCX+ cell bodies are found mainly in the first and second inner third of the granular cell layer (GCL), some of which are bipolar (left arrow in A). Their dendrites are short and their dendritic extent generally small (right arrow in A). DCX+ cells are often oriented in a radial manner and seem to undergo secondary division (arrow in B). Some DCX+ dendrites can be followed through the GCL (arrowheads in C). Double labeling for DCX and BrdU identified clear examples of newborn BrdU-positive cells and doublets (arrows in D), that rarely co-express DCX (arrowheads in D). Figure E shows an example of a BrdU+ newborn cell (left arrow) in the subgranular zone next to the hilus (H), that weakly co-expresses DCX. In the same subregion, a single labeled DCX+ cell is present with its dendrite running parallel to the GCL (arrowheads in E). Inset shows another example of a BrdU/DCX+ cell within the GCL. Figure F shows 2 DCX+ cells with well-developed dendrites (arrowhead) close to a BrdU+ newborn cell (arrow) located in the entorhinal cortex.
Neurogenic markers in the amygdala

As neural stem cells located in the lateral ventricle are known to deliver new astrocytes and neurons to the amygdala and neocortex, we analyzed coronal sections directly rostral of the hippocampus. Immunostaining for PSA-NCAM identified expression in the wall of the lateral ventricle (Fig 3A). The lateral ventricle ends adjacent to the caudal amygdala and PSA-NCAM was observed in a manner consistent with lateral migration from the SVZ (Fig 3B). In the caudal amygdala, DCX+ cells were first observed in the ventral basolateral nucleus where it borders the temporal horn of the lateral ventricle (tLV). Adjacent to the ventricle, DCX+ cells line the amygdala where it borders the hippocampus and entorhinal cortex (EC). The highest density of cells was observed closest to the ventricle and slightly more dispersed at locations further from the ventricle. In these immediate areas, no BrdU+ cells were observed.
In the midrostrocaudal level, the basolateral nucleus is subdivided into the ventral medial (BLVM) and intermediate (BLI) subregions. The high density of DCX+ cells extended through these regions bordering the EC (Fig. 4A-G). Interestingly, DCX+ neurons in the BLVM and BLI have projections extending through the ventral cortical and medial nuclei, located adjacent to the optic tract. In contrast to DCX+ cells in the GCL, the processes of these cells in the amygdala had no appreciable orientation (Fig. 4A-H). As rare DCX-expressing astrocytes have been documented in the human cortex [34], we also investigated this possibility by immunostaining sections with anti-GFAP antibody. In these areas, mature GFAP-expressing astrocytes had a uniform distribution within nuclei of the amygdala and had a cellular morphology entirely distinct from the DCX-expressing cells (data not shown).

Figure 3: Photomicrographs illustrating PSA-NCAM expression in the wall of the lateral ventricle and caudal amygdala. A-B) Immunostaining for PSA-NCAM identified expression in the wall of the lateral ventricle (LV). C-D) Immunostaining can be observed where hippocampus and temporal horn of the lateral ventricle (tLV) end near the ventral amygdala.
Figure 4: A-D. Photomicrographs illustrating the distribution of DCX+ cells over different anatomical levels of the marmoset amygdala. A) In sections immediately adjacent to the hippocampus cells reside in the basolateral and lateral amygdala nuclei. B-D) DCX+ cells are primarily found in the ventral nuclei but also in the lateral nucleus. DCX+ cells are also found in 1st and 2nd layers of the entorhinal cortex. The area between these two populations is largely devoid of DCX+ positive cells. Numerous densely clustered DCX+ cell bodies are present in the ventromedial (BLVM)
and intermediate parts (BLI) of the basolateral amygdaloid nucleus, that are bordered by the entorhinal cortex (EC). DCX+ cell bodies are further abundant in the ventral anteriocortical nucleus of the amygdala (VACo). Numerous fibers leave the BLVM and BLI and traverse the basomedial amygdaloid nucleus (BM) projecting towards the medial amygdaloid nucleus (Me) and anterior amygdaloid area (AA), where DCX+ fiber clusters are seen, likely representing fiber endings (Figure 4G). Details of projecting DCX+ fibers are shown in Figures 3 E and F. This projection is more extensive in the anterior regions of the basolateral nucleus (Figure 4A, B) than in other regions (Figures. 4C-F).

In the amygdala, the highest density of BrdU+ cells occurred in the BLVM and BLI subregions rostral of the tLV (Fig. 5). BrdU+ nuclei were more sporadically distributed throughout the other nuclei of the amygdala and were not highly clustered, or e.g. limited to the intersection between the amygdala and EC. BrdU+ and DCX+ were rarely co-localized in this region (Supplemental Fig. 1). Within the amygdala, a significant positive correlation existed for BrdU and DCX (Pearson 2-tailed test, R= 0.59, p= 0.002).

DCX+ cells were found in entorhinal cortical (EC) layers I and II (Fig 5D), BrdU+ and DCX+ cells were co-imaged in this area (Fig 5F). BrdU+ nuclei were also found in the inner layers of the EC, a subregion of the EC largely devoid of DCX+ cells. These BrdU+ cells were most often observed as a single nucleus and only occasionally seen in clusters or doublets. The DCX+ cells displayed a complex dendritic morphology distinct from the clustered amygdala population where they appeared less mature due to their close proximity to each other. DCX+ cells in the EC were present in clusters which is distinct from the organization seen in the amygdala.

In the amygdala, BrdU+ cells were found at densities of 3560 ± 530 and 2780 ± 200 cells/mm³ for control and stress cohorts respectively, DCX+ cells were found at averages of 163000 ± 56900 and 164000 ± 7590 cells/mm³cells in each amygdalar midlevel section. DCX+ cells are more than 50 times more prevalent than BrdU+ cells. These figures may underestimate the population of cells found within specific nuclei of the amygdala due to difficulty in properly delineating these highly specific areas. The DCX+ cell population can be found across nuclei of the amygdala (Fig 6); these nuclei lack histochemical markers necessary to determine their borders (personal communication with Xavier Palazzi, author of the marmoset brain atlas).
Figure 5: Photomicrographs illustrating the dense clusters of DCX+ cells and fibers in the amygdala. The highest density of BrdU-labeled cells occurred in the BLVM and BLI subregions (Figure A and B, resp.) while lower densities are found in the VACo (C). DCX+ fibers in the VACo appeared highly interconnected and have different orientations. This is in contrast to the DCX+ fibers in the BLI (arrow in 2B) or the DCX+ fibers leaving the BLVM and BLI, most of which were directed towards Me and AA (A and arrow in B; see also Fig 4A).

In addition, considerable numbers of DCX+ cells were present in the more basolateral parts of the entorhinal cortex (EC) where DCX expression was mainly confined to the outer layers I/II (Figure D). Higher magnifications show this group of cells, similar to
the VACo, to be strongly interconnected with many DCX+ dendrites touching other DCX+ cells or dendrites (Figure D-G, arrows in E). Double labeling for BrdU (black) and DCX (brown) identified the presence of newborn cells in the EC (arrows in figure F). In the lower layers III/IV, isolated cells are occasionally found. Their dendrites often have a radial orientation (Figure G, H) and frequent bifurcations (arrow in H).

**Figure 6: Anatomical representation of DCX+ cells present in the marmoset amygdala.** Rostral of the hippocampus, DCX+ cells appear closely associated to the temporal horn of the Lateral Ventricle (tLV) where the caudal amygdala begins. In midlevel sections, DCX+ cells are found as a dense band in multiple nuclei of the amygdala where it borders the entorhinal cortex (Er). This includes the BLVM, BLI, VACo, and lateral (LA) nuclei. In the rostral amygdala this band of cells occupies a smaller volume of the nuclei. The present areas of interest illustrated here are found from approximately Bregma +5.50 through +3.64 mm.

**Doublecortin and PSA-NCAM in the amygdala and entorhinal cortex**

Since DCX is typically expressed in migratory neurons, we next immunostained sections from the amygdala for PSA-NCAM. Cell adhesion is prevented when a carbohydrate such as polysialic acid binds extracellular domains of neural cell adhesion molecule, allowing cells to migrate. Our results indicated that PSA-NCAM had a very similar distribution to DCX in the amygdala. This provides evidence that DCX+ amygdala bound neuroblasts have a migratory capacity (Figure 7A-L). The BLVM and BLI have large populations of densely packed DCX+ cells, most commonly found dispersed and without a distinct orientation. Co-imaging of DCX expression and PSA-NCAM at high magnification revealed that many of the DCX+ cells are indeed tangentially contacting
PSA-NCAM sheathed axons. However, autonomous cell bundles were also observed. At lower magnification, PSA-NCAM was expressed on highly clustered DCX+ cells. While it was clear that most of the DCX+ cells expressed PSA-NCAM, some cells in the amygdala were observed without a strong overlap with PSA-NCAM.

In the entorhinal cortex DCX and PSA-NCAM were also co-imaged, indicating that these cells may have an immature and/or migratory capacity (Figure 7M-O). These cells were morphologically different from the amygdala bound cells, and typically had branched extensions. However, these processes also express PSA-NCAM perhaps indicating structural plasticity in the EC. None of the observed images of PSA-NCAM expression suggested radial migration from the amygdala to the EC.
Structural plasticity in aged marmosets
Figure 7: DCX+ cells in the adult marmoset express PSA-NCAM. A-C, D-F) A dense band of DCX+ cells co-express PSA-NCAM on extensions that run laterally in the plane in the basolateral intermediate nucleus.G-I, J-L) A diffuse band of DCX+ cells co-express PSA-NCAM on extensions that run tangentially. M-O) DCX+ cells in the piriform cortex display a more mature morphology with branched processes that express PSA-NCAM. P-R) Two immature neurons in the DG express PSA-NCAM confirming that these molecular markers of neurogenesis are found in a known neurogenic zone. Scale bars = 50 μm (A and G), 20 μm (D, J, M, and P)

Delayed influence of psychosocial stress

The stress protocol was designed to evaluate lasting rather than acute consequences of psychosocial stress on structural plasticity. RIA urine analysis indicated that the monkeys had indeed experienced stress; mean cortisol concentrations were significantly elevated during the two weeks of isolation and social defeat in the stress cohort (Fig 8A; p< 0.01 student’s one-tailed t-test) as compared to the control group for the same 6 weeks period. During the re-socialization period, there was a significant normalization in measured cortisol levels (p<0.05 student’s one-tailed t-test). When the groups were evaluated by sex, no significant differences were observed (Fig 8B).

Semi-quantitative analysis of the hippocampal DG for the presence of BrdU and DCX positive cells did not reveal significant differences in the distribution or number between stress and control cohorts (Figure 8C) (BrdU; p <0.14, DCX; p<0.16; student’s t-test). Similarly, in the amygdala, stress effect on BrdU+ and DCX+ cell populations were not significantly different, nor were obvious differences in cellular morphology observed in animals in the stress compared to control cohort (BrdU; p<0.19, DCX; p<0.49 student’s t-test).
Figure 8: Stress induction during a 2-week period is not associated with reduced neurogenesis in the dentate gyrus or amygdala. A) Urinary corticosterone levels are significantly elevated in middle aged marmosets exposed to a combined isolation and social defeat stress (student’s t-test p = 0.004). B) Although reductions were present in DCX, in neither the hippocampus nor the amygdala were significant differences found in the number of BrdU+ or DCX+ cells after stress exposure (DG: BrdU (p = 0.14), DCX (p = 0.16), Amygdala: BrdU (p = 0.18), DCX (p = 0.48)
Discussion

The main finding of this study is the presence of a surprisingly large population of DCX-expressing newborn cells in the amygdala of 4-year-old (middle-aged) common marmosets. Moreover, low numbers of adult generated BrdU+ cells and immature DCX+ neurons were found in the hippocampus, entorhinal and adjoining temporal cortex, as also seen in other primate species and as was expected for animals this age. Exposure to a 2-week period of social stress followed by a 2-week recovery period revealed no lasting effects of stress on structural plasticity in the marmoset.

Some recent studies have now documented structural plasticity and/or DCX expression not only in the DG but also in cortical and/or amygdalar regions in various species including primates, but generally not to such an extent as found here [17, 35-42]. Indeed, our data from the DG and amygdala provides quantitative evidence of a large population of neuroblasts and/or immature neurons in the amygdala when compared to a known neurogenic zone. The low numbers of newborn neurons found in the hippocampal DG are consistent with the low rates reported earlier in middle aged and aging marmosets [43], however the large population of DCX+ cells present in the amygdala of middle-aged animals was unpredicted.

Our quantitative analysis of the DG and amygdala demonstrated that structural plasticity persists in the amygdala of marmoset monkeys, consistent with a previous study in squirrel monkeys (Saimiri sciureus) that reported approximately 14 BrdU+ nuclei per amygdala section [17]; this was the result of a 50 mg/kg intravenous injection of BrdU and coronal sectioning at 40 μm. Using sections of identical thickness, we here identified approximately 30 BrdU+ nuclei and 1600 DCX+ cells in each coronal section in the amygdala of middle aged common marmosets. Importantly, the earlier study established that migratory cells from the SVZ differentiated into mature neurons in the amygdala (41% BrdU+/NeuN+). A small population of BrdU+ cells (7%) was found to be MAP-2+ in the temporal stream and amygdala 28 days after BrdU injection. Our findings are in agreement with these results demonstrating structural plasticity in the amygdala.

The staining pattern we see for DCX is consistent with earlier evidence of neurogenesis in the amygdala of New World monkeys. Previously, newborn neurons, observed as a thick band of Bcl-2
Structural plasticity in aged marmosets

expressing cells, and chains of PSA-NCAM+ cells were observed to invade the amygdala [17]. Our double immunofluorescence analysis confirms the presence of DCX+ cell bodies and projecting axons that co-express PSA-NCAM in the amygdala, thereby verifying the migratory capacity of cells in this structure. We also documented DCX+/PSA-NCAM+ cells in the first and second cortical layers of the entorhinal cortex with immunoreactivity absent in the area separating these cortical layers from the amygdala. This is consistent with findings that oligodendroglial progenitors reside in piriform cortex [42].

Our results from the DG are consistent with the known kinetics of these two markers: BrdU labels newborn cells while DCX, at least in rodents, is expressed for approximately 10 days. [3, 5, 44, 45]. We observed rare colocalization of BrdU and DCX in the DG and amygdala, a finding that demonstrates that at least some of these cells are newly generated. In the amygdala, the DCX+ population was significantly higher, approximately 50 times more prevalent than BrdU+ cells. This represents a distinctly different relationship from that found in the DG, perhaps indicating that DCX is not transiently expressed.

At this time we have no evidence that DCX+ cells in the amygdala are functionally integrated. However, based on protein expression we expect they participate in mechanisms of structural or synaptic plasticity. DCX is typically associated with cell migration due to developmental regulation necessary for cell migration [4]. However, prenatally born cells can also maintain DCX expression and an immature neuronal phenotype [35]. DCX is additionally expressed by a subset of mature neurons. In adult rat brain, differentiated neurons in select areas, including the piriform cortex, co-express DCX and PSA-NCAM [46], reviewed by Gomez-Climent [47]. These finding agree with observations that DCX expression is not limited to migratory neuroblasts alone and may be involved in other aspects of cellular orchestration of synaptic plasticity. DCX coprecipitates with adapter proteins involved with protein sorting and vesicular trafficking [48]. The formation of new neurites implies the participation of microtubules. Indeed, DCX participation in microtubule reorganization has been linked to axonal outgrowth or synaptogenesis [49]. In this respect, it is interesting to note that in a rodent model dendritic arborization was increased in neurons of the basolateral amygdala following chronic stress [50].
Amygdala-bound DCX+ cells express PSA-NCAM which strongly suggests these cells participate in chain migration, an unusual type of cell displacement where large bulks of cells are arranged in contact with axon bundles. This is in agreement with previous findings in primates [8, 22] where migrating chains of cells have been observed along the length of the entire lateral ventricle system, both in humans and nonhuman primates [9, 22, 51, 52]. Our stainings are consistent with earlier evidence of the temporal stream however verification is ideally completed with stainings in the sagittal plane.

The role of NCAM is well established as assembly of the CNS architecture depends on this transmembrane protein [53]. The extracellular domains, when bound by the carbohydrate polysialic acid (PSA), prevent cell-cell adhesion, and permit structural plasticity in the brain [54, 55]. PSA is attached exclusively to NCAM in the brain; PSA-NCAM immunoreactivity thereby identifies migratory cells exclusively. Coordinated expression of PSA-NCAM and DCX is conserved in populations of cells migrating from the SVZ [22, 56-59]. These findings have not been extended to humans yet. Migratory progenitor cells are present in the human RMS [60] but evaluation of the same molecular markers failed to identify migration through the TS to the amygdala [61].

Exposure to isolation and social-defeat stress is known to represent a severe stressor for these social animals. Indeed, a significant increase in urinary cortisol was induced during stress conditions and normalized at the time of sacrifice. Depressive-like behaviors were commonly observed until the last day of sacrifice in the stress group (not quantified systematically), however the current stress paradigm failed to significantly affect BrdU+ or DCX+ cell numbers. Despite the rise in cortisol, the nature and duration of these stressors during a 2-week exposure period may have been too mild to significantly reduce structural plasticity in a lasting manner. In rodent studies of stress on hippocampal neurogenesis or amygdalar plasticity [50], stress duration is often prolonged, i.e. applied for 21 days prior to immediate sacrifice, and frequently contains a physical component, like restraint, that was not present here. If we assume transient expression of DCX, lasting from approximately 4 to 14 days in new neuroblasts, a putative initial reduction in DCX+ cells might have normalized during the 2 weeks of re-socialization. In the current paradigm animals were sacrificed 28 days after BrdU injection. In a previous paper on
marmosets, a significant reduction in neural stem cell proliferation was reported when sacrifice occurred shortly after stress exposure [19]. In rodents, changes in neurogenesis after chronic stress [14, 18] were indeed shown to be reversible following a recovery period [12].

In conclusion, neurogenesis in the DG and amygdala of adult marmosets was found to be insensitive to a psychosocial stress paradigm that included a recovery period. Quantification of two markers of neurogenesis provide evidence there were no long-lasting changes in the population of surviving BrdU-labeled cells and DCX+ neuroblasts in each anatomical region. Whether the population of cells in the amygdala is entirely dependent on SVZ migration remains to be determined, but a large pool of migratory neuroblasts is present in the amygdala that is unlikely to be supported fully by new cell migration from the lateral ventricle. Given the similarities in distribution in other primate species, this suggests evolutionary conservation of structural plasticity outside the classic SVZ and SGZ zones. The exact functional role of these DCX+ cells in the amygdala remains to be further discovered.

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Supplementary Figure 1: DCX+/BrdU+ cells are found in the marmoset amygdala. A) DCX+ cells in the BLA with short processes B) at high magnification show nuclear BrdU+ staining. C) DCX+ cells in the VACo are more complex but similarly D) at high magnification show BrdU+ nuclei. Scale bars = 500 μm (A,C), 20 μm (B,D)

References

6 Rao MS, Shetty AK. Efficacy of doublecortin as a marker to analyse the absolute number and dendritic growth of newly generated neurons in the adult dentate gyrus. *The European Journal of Neuroscience* 2004 Jan; 19: 234-46
10 Bedard A, Gravel C, Parent A. Chemical characterization of newly generated neurons in the striatum of adult primates. *Experimental Brain Research* *Experimentelle Hirnforschung* 2006 Apr; 170: 501-12
76 | Structural plasticity in aged marmosets

15 Rao MS, Hattiangady B, Shetty AK. The window and mechanisms of major age-related decline in the production of new neurons within the dentate gyrus of the hippocampus. *Aging cell* 2006 Dec; 5: 545-58
18 Czeh B, Welt T, Fischer AK, Erhardt A, Schmitt W, Muller MB, Toschi N, Fuchs E, Keck ME. Chronic psychosocial stress and concomitant repetitive transcranial magnetic stimulation: effects on stress hormone levels and adult hippocampal neurogenesis. *Biol Psychiatry* 2002 Dec 1; 52: 1057-65
21 van Bokhoven P, Oomen, C.A.,Van Der Harst, K.E., Hoogendijk, W.J.G., Smit, A.B., Spijker, S., Lucassen P.J. The reduction in neurogenesis after social stress is long lasting and can be restored by late treatment with imipramine. *European Journal of Neuroscience* in press:
23 Palazzi X BN. The Marmoset Brain in Stereotaxic Coordinates. *Springer* 2008;
*ISBN: 978-0-387-78384-0*: 64


29 Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *The Journal of comparative neurology* 2001 Jul 9; **435**: 406-17

30 Czeh B, Muller-Keuker JI, Rygula R, Abumaria N, Hiemke C, Domenici E, Fuchs E. Chronic social stress inhibits cell proliferation in the adult medial prefrontal cortex: hemispheric asymmetry and reversal by fluoxetine treatment. *Neuropsychopharmacology* 2007 Jul; **32**: 1490-503

31 Knabe W, Kuhn HJ. Capillary-contacting horizontal cells in the retina of the tree shrew Tupaia belangeri belong to the mammalian type A. *Cell and tissue research* 2000 Mar; **299**: 307-11


33 Oomen CA, Soeters H, Audureau N, Vermunt L, van Hasselt FN, Manders EM, Joels M, Lucassen PJ, Krugers H. Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood. *J Neurosci* 2010 May 12; **30**: 6635-45


36 Tonchev AB, Yamashima T, Zhao L, Okano H. Differential proliferative response in the postischemic hippocampus, temporal cortex, and olfactory bulb of young adult macaque monkeys. *Glia* 2003 May; **42**: 209-24

37 Xiong K, Luo DW, Patrylo PR, Luo XG, Struble RG, Clough RW, Yan XX. Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: partial colocalization with mature interneuron markers. *Experimental neurology* 2008 May; **211**: 271-82

38 Gould E. How widespread is adult neurogenesis in mammals? *Nature reviews* 2007 Jun; **8**: 481-8
41 Cai Y, Xiong K, Chu Y, Luo DW, Luo XG, Yuan XY, Struble RG, Clough RW, Spencer DD, Williamson A, Kordower JH, Patrylo PR, Yan XX. Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. Experimental neurology 2009 Apr; 216: 342-56
49 Ribak CE, Korn MJ, Shan Z, Obenaus A. Dendritic growth cones and recurrent basal dendrites are typical features of newly generated dentate granule cells in the adult hippocampus. Brain research 2004 Mar 12; 1000: 195-9
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56 Nacher J, Lanuza E, McEwen BS. Distribution of PSA-NCAM expression in the amygdala of the adult rat. Neuroscience 2002; 113: 479-84


