UNIVERSITY OF CRETE

SCHOOL OF SCIENCES AND ENGINEERING

DEPARTMENT OF BIOLOGY

PhD Thesis

THE ROLE OF INHIBITION IN BEHAVIOR AND PHYSIOLOGY OF MOUSE CEREBRAL CORTEX

XANTHIPPI KONSTANTOUDAKI

HERAKLION 2014

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BY

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APPROVAL FORM

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Ευχαριστίες

Η έναρξη εκπόνησης της διδακτορικής μου διατριβής τον Ιανουάριο του 2010 χρονικά συνέπεσε με την έναρξη λειτουργίας του εργαστηρίου «Νευροφυσιολογίας και Συμπεριφοράς», αποτελώντας μία τεράστια πρόκληση που απαιτούσε υπομονή, θυσίες, αρμονική συνεργασία, επιμονή και δουλειά ώστε να καταφέραμε να παράγουμε ερευνητικό έργο, το οποίο μας κάνει υπερήφανες.

Σε αυτό το ταξίδι είχα πολλούς συνοδοιπόρους οι οποίοι με βοήθησαν πρακτικά και ψυχολογικά σε κάθε βήμα. Αυτή η δουλειά δεν είναι δουλειά ενός, είναι δουλειά πολλών και γι' αυτό θα ήταν παράλειψη να μην ευχαριστήσω όλους εκείνους που βοήθησαν για να είμαι αυτή τη στιγμή στην ευχάριστη θέση να γράφω τις ευχαριστίες της διδακτορικής μου διατριβής.

Πρώτα θα ήθελα να ευχαριστήσω την καθηγήτρια μου, δρ. Κυριακή Σιδηροπούλου, η οποία κατά τη διάρκεια της εκπόνησης της διδακτορικής μου διατριβής στάθηκε στο πλάϊ μου ως υπομονετική δασκάλα, ως μέντορας, ως συνεργάτης, ως φίλη, ως οικογένεια. Δίπλα στη Κική έμαθα να κάνω πράξη την επιστημονική σκέψη, μου μετέδωσε την αγάπη της και την πολύτιμη της γνώση για την ηλεκτροφυσιολογία, με έκανε να εμπιστευτώ τον εαυτό μου και την κρίση μου, με βοήθησε να ωριμάσω και ως επιστήμονας αλλά και ως άνθρωπος. Την ευχαριστώ για την εμπιστοσύνη της, για όλους τους δρόμους που μου άνοιξε, αλλά και για την ελευθερία που μου έδωσε τόσο στο σωστό όσο και στο λάθος.

Πολλά ευχαριστώ οφείλω και στην καθηγήτρια Δόμνα Καραγωγέως αλλά και στα μέλη του εργαστηρίου της για μία άριστη συνεργασία τόσο στο επίπεδο πρακτικής συνεισφοράς όσο και στο επίπεδο πολύτιμων επιστημονικών συζητήσεων. Πιο συγκεκριμένα, μεγάλη ήταν η βοήθεια της Δρ. Simona Tivodar, όσο και της Ζουζάνας Κούνουπα, αλλά και της Κατερίνας Καλεμάκη.

Ένα τεράστιο ευχαριστώ οφείλω και στον επιβλέποντα του διδακτορικού μου, κ Γεώργιο Χαλεπάκη. Τον κύριο Χαλεπάκη γνώριζα ως έναν εξαιρετικό καθηγητή του τμήματος από τα πρώτα έτη της φοίτησής μου στο τμήμα Βιολογίας. Με το πέρασμα των ετών αντιλήφθηκα και την μεγάλη του ανθρώπινη αξία. Η συνεισφορά του ήταν τεράστια τόσο σε επίπεδο επιστημονικών συζητήσεων όσο και όσον αφορά στην στήριξη του στο εργαστήριο μας. Δεν θα ξεχάσω τις συνεχείς παροτρύνσεις του σε κάθε μας συνάντηση για να φέρω εις πέρας σύντομα και αποτελεσματικά την διατριβή μου.

Πολλά ευχαριστώ επίσης οφείλω και στην Δρ. Παναγιώτα Ποϊράζη αλλά και στην μεταδιδακτορική ερευνήτρια του εργαστηρίου της Νάση Παπουτσή. Η συνεργασία μας ξεκίνησε από το δεύτερο έτος φοίτησης μου στο μεταπτυχιακό «εγκέφαλος και νους», το 2008 και το αποτέλεσμα της συνεργασίας αυτής έδωσε μία δημοσίευση για την οποία είμαι πολύ υπερήφανη. Πέραν τούτου, η Γιώτα συνεχίζει να με στηρίζει σε κάθε προσπάθεια πάντα με ενθουσιασμό κάτι το οποία πραγματικά με τιμάει πολύ.

Ευχαριστώ πολύ και τα υπόλοιπα μέλη της 7μελούς επιτροπής αξιολόγησης της διατριβής μου, τους κυρίους Γιώργο Παναγή, Μιχάλη Παυλίδη και Ιωάννη Χαραλαμπόπουλο.

Τα λόγια δεν φτάνουν για να ευχαριστήσω την Κλεάνθη Χαλκιαδάκη, διδακτορική φοιτήτρια του εργαστηρίου Νευροφυσιολογίας και Συμπεριφοράς. Η Κλαίρη ως μεταπτυχιακή αρχικά και ως διδακτορική φοιτήτρια τελικά στο εργαστήριο μας, έδωσε την πινελιά της για να γίνουμε ακόμα πιο δημιουργικοί. Την ευχαριστώ για την αρμονικότατη συνεργασία μας όλα αυτά τα χρόνια και της εύχομαι γρήγορα να ολοκληρώσει επιτυχώς της σπουδές της.

Όμως, πολλά ευχαριστώ οφείλω και στους φοιτητές που πέρασαν από το εργαστήριο αυτά τα χρόνια και κατανάλωσαν χρόνο και προσπάθεια δίπλα μας. Τους ευχαριστώ για την πρακτική βοήθεια που δεν θα μπορούσα να περιφρονήσω. Εύχομαι να βοήθησα έστω και ελάχιστα στο να αγαπήσουν αυτό που ονομάζουμε έρευνα, να κατάφερα να τους

μεταδώσω όλα όσα ήξερα και να καταφέρουν να πραγματοποιήσουν τους στόχους τους, όποιοι και αν είναι αυτοί. Ευχαριστώ οφείλω στον Βασίλη Κεχαγιά για τις πρώτες απόπειρες καταγραφών στην ηλεκτροφυσιολογία και για όλες τις συμβουλές που μου άφησε φεύγοντας. Ιδιαίτερα επίσης θα ήθελα να ευχαριστήσω την Γκιουλτέν Ιχτιάρ Σαλί και την Αλεξάνδρα Χοβσεπιάν, οι οποίες με μεγάλη όρεξη πέρασαν ατέλειωτες ώρες δίπλα μου στην ηλεκτροφυσιολογία και είχαν μεγάλη συνεισφορά σε πολλά από τα πειράματα του πρώτου χρόνου. Επίσης, τον Χρήστο Παπαντωνίου και τη Βάσια Σακκά οι οποίοι είχαν μεγάλη συμμετοχή και στα πειράματα της ηλεκροφυσιολογίας αλλά και στα πειράματα συμπεριφοράς. Η Ελιάννα Βασιλείου, η Νίκολα Σταθακοπούλου, η Μαίρη Βλασσοπούλου και η Στεργία Γεωργοπούλου βοήθησαν ενεργά σε κάποια από τα πειράματα του πρώτου κεφαλαίου. Όμως ενεργά και στα πειράματα αλλά και στις αναλύσεις συμμετείχαν τα περισσότερα από τα παιδιά που πέρασαν από το εργαστήριο αυτά τα χρόνια. Ευχαριστώ πολύ λοιπόν την Δήμητρα Πιπίνη, τον Βασίλη Ιωακειμίδη, την Λυδία Παυλίδη, την Ράνια Τζώρτζη, την Τίνα Κατσανεβάκη, την Μυρσίνη Βασίλα, την Κάλλια Λαμπράκη, την Ευδοκία Πάζα, την Κλαίρη Μυλοποταμιτάκη και την Μελίνα Τιμπλαλέξη, όπως επίσης τους Κώστα Πετουσάκη, Δέσποινα Κορτεσίδου, Εμμανουέλα Φοινικιανάκη, Μιχάλη Λαγκουβάρδο, Χρήστο Λυμπερόπουλο και Αναστάσιο Κόλλια.

Όσο σημαντικές αποδείχτηκαν όμως αυτές οι συνεργασίες, άλλο τόσο αλλά και πολύ περισσότερο σημαντικοί αποδείχτηκαν οι δικοί μου άνθρωποι.

Υπήρξαν αυτά τα χρόνια άνθρωποι που με στήριζαν παρακολουθώντας πάντα από τα παρασκήνια την πρόοδο της προσπάθειάς μου και ίσως ένιωθαν περήφανοι κάποιες στιγμές. Το σίγουρο είναι ότι ένα «μπράβο» τους με έστελνε στα ουράνια και μου έδινε την ώθηση να προχωράω ακάθεκτη. Ίσως δεν καταλάβαιναν καν πόσο με βοηθούσαν. Ευχαριστώ λοιπόν…

Ευχαριστώ τον Γιάννη γιατί ήταν πρόθυμος να ακούσει τους προβληματισμούς μου, να συζητήσει για τους φόβους μου και να με διαφωτίσει για όσα αγνοούσα. Ήταν μακριά πολύ, αλλά ήταν δίπλα μου και ήταν ένας από τους πιο σημαντικούς μου συνεργάτες σε αυτήν την προσπάθεια.

Σας άφησα τελευταίους γιατί σε εσάς οφείλω όλα τα ευχαριστώ αυτού του κόσμου. Μαμά, μπαμπά.. Μπαμπά, μαμά.. σας ευχαριστώ για την δύναμη που δίνουν σε κάθε μου βήμα η αγάπη σας, τα χαμογελά σας, τα μάτια σας, τα μπράβο σας και η χαρά που δεν κρύβετε ποτέ. Που κάθε μου μικρή επιτυχία για εσάς είναι σαν να σας δίνω όλο το κόσμο. Που είστε δίπλα μου φύλακες – άγγελοι να δίνετε μαζί μου την κάθε μου μάχη, που προσπαθείτε να καταλάβετε τις αγωνίες μου, που θυμώνετε με ότι με θυμώνει. Είστε η δύναμη μου σε κάθε μου αναπνοή!

Στην γιαγιούλα μου….

Θα προσπαθώ πάντα. Στο υποσχέθηκα!

Σε θυμάμαι, μου λείπεις και σε αγαπάω πολύ

List of Abbreviations

5-HT: serotonin

AC: adenylate cyclase

AC: anterior cingulate gyrus cortex

Ach: acetylcholine

aCSF: artificial cerebrospinal fluid

ADP: afterdepolarization

AGm: medial agranular cortex,

AHP: afterhyperpolarization

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

CaMKII: Ca2+/calmodulin-dependent protein kinase II

cAMP: Cyclic adenosine monophosphate

CAN: calcium-activated non-selective cation current

CB: Calbindin

Cdc42: Cell division control protein 42 homolog

cKO: conditional knock-out

CR: Calretinin

CREB: cAMP response element-binding protein

DA: dopamine

dADP: delayed afterdepolarization

DAG: diacylglycerol

DARPP-32: Dopamine- and cAMP-regulated phosphoprotein, Mr 32 kDa

EPSP: excitatory postsynaptic potential

ER: Endoplasmic reticulum

ERK 1/2: extracellular-signal-regulated kinase 1/2

FS: Fast-spiking

GABA: gamma-Aminobutyric acid

GDPs: giant depolarizing potentials

GTPases: guanosine triphosphate hydrolases

HPA: hypothalamic–pituitary–adrenal

IB: Intrinsic bursting

IC: infralimbic cortex

IS: Irregular-spiking

ISI: Inter-spike interval

LFP: Local field potential

LTD: Long-term depression

LTP: Long-term potentiation

MAP: microtubule-associated protein

MEK1/2: Mitogen/Extracellular signal-regulated Kinase 1/2

MRI: Magnetic resonance imaging

NA: noradrenaline

NMDA: N-methyl-D-aspartate

PC: prelimbic cortex

PFC: Prefrontal Cortex

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PP-1: Protein phosphatase 1

PSD: postsynaptic density

PTX: Picrotoxin

PV: Parvalbumin

Rac1: Ras-related C3 botulinum toxin substrate 1

RhoA: Ras homolog gene family, member A

RS: Regular-spiking

RSNP: Regular-spiking non pyramidal

SST: somatostatin

VTA: ventral tegmental area

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Περίληψη

Ο προμετωπιαίος φλοιός συμμετέχει σε ανώτερες γνωσιακές λειτουργίες (λειτουργίες βραχύχρονης και μακρόχρονης μνήμης) αλλά και σε συναισθηματικές λειτουργίες. Αν και η ηλικία φαίνεται να παίζει πολύ σημαντικό ρόλο και στην φυσιολογική λειτουργία του προμετωπιαίου φλοιού αλλά και στην εμφάνιση ασθενειών, λίγα είναι γνωστά για τις ηλικιο-εξαρτώμενες αλλαγές των συμπεριφορών όπου συμμετέχει ο προμετωπιαίος φλοιός αλλά και για τους υποκείμενους κυτταρικούς μηχανισμούς. Προηγούμενες μελέτες, κυρίως βασιζόμενες στην μορφολογία των δενδριτών, έχουν προτείνει ότι αυτή η ανώτερης-τάξης εγκεφαλική περιοχή παρουσιάζει καθυστερημένη ανάπτυξη, συγκρινόμενη με πρωτοταγείς αισθητικές φλοιϊκές περιοχές, η οποία διαρκεί μέχρι και την πρώιμη ενηλικίωση. Η κατανόηση της μεταγεννητικής ανάπτυξης του προμετωπιαίου φλοιού είναι κρίσιμη προκειμένου να βελτιώσουμε ριζικά την κατανόηση της εγκεφαλικής λειτουργίας και της συμπεριφοράς όσο και το υπόστρωμα εκκίνησης ολέθριων νευροψυχιατρικών διαταραχών στης οποίες συμμετέχει ο προμετωπιαίος φλοιός. Ο προμετωπιαίος φλοιός, όπως και άλλες περιοχές του φλοιού, αποτελείται από γλουταματεργικούς διεγερτικούς νευρώνες και GABAεργικούς ανασταλτικούς διάμεσους νευρώνες. Η ισορροπία μεταξύ αυτών των δύο συστημάτων απαιτείται για την ορθή λειτουργία του προμετωπιαίου φλοιού. Πρόσφατα, ο ρόλος του GABAεργικού συστήματος συσχετίστηκε έντονα με πιθανή ανισορροπία μεταξύ των δύο συστημάτων, που τελικά οδηγεί σε παθολογικές καταστάσεις. Ο στόχος μας σε αυτή τη διατριβή ήταν διπλός. Αρχικά, θελήσαμε να κατανοήσουμε καλύτερα την μεταγεννητική ανάπτυξη του προμετωπιαίου φλοιού και δεύτερον, να μελετήσουμε της αλλαγές στην συμπεριφορά του προμετωπιαίου φλοιού και στους υποκείμενους κυτταρικούς μηχανισμούς σε περιπτώσεις μειωμένης GABAεργικής αναστολής. Τα αποτελέσματα μας παρουσιάζονται σε τρία κεφάλαια.

Στο **κεφάλαιο Ι**, χρησιμοποιήσαμε μία διεπιστημονική προσέγγιση που περιελάμβανε κυτταρικές, ηλεκτροφυσιολογικές και συμπεριφορικές τεχνικές σε διαφορετικές ηλικιακές ομάδες μυών για να κατανοήσουμε καλύτερα την ανάπτυξη του προμετωπιαίου φλοιού. Σε πολλές περιπτώσεις, πραγματοποιήθηκε και η σύγκριση με άλλες φλοιϊκές περιοχές. Διαπιστώνουμε μία διαφορετική έκφραση των διάφορων τύπων δενδριτικών ακάνθων σε πυραμιδικούς νευρώνες του προμετωπιαίου φλοιού μεταξύ των μυών που ανήκουν σε διαφορετικές ηλικιακές ομάδες. Συγκεκριμένα, οι «έφηβοι» πυραμιδικοί νευρώνες (ηλικίας 40 ημερών) παρουσιάζουν την μικρότερη πυκνότητα ακάνθων, με αυξημένο ποσοστό κοντόχοντρων (stubby) ακάνθων, ενώ στις ομάδες των «νεαρών» (ηλικίας 35 ημερών) και «πρώιμων ενήλικων» (ηλικίας 60 ημερών) πυραμιδικών νευρώνων παρουσιάζεται αυξημένος αριθμός δενδριτικών ακάνθων και συγκεκριμένα μανιταροειδών ακάνθων. Αυτό το αναπτυξιακό πρότυπο παρατηρήθηκε επίσης και στις ηλεκτροφυσιολογικές μας μελέτες, στις οποίες οι ηλικιακές ομάδες των «νεαρών» και «πρώιμων ενηλίκων» παρουσιάζουν αυξημένη μακρόχρονη ενδυνάμωση (long-term potentiation (LTP)) της συναπτικής διαβίβασης σε απόκριση τετανικού ερεθισμού, ενώ η ομάδα των «εφήβων» παρουσιάζει μειωμένη μακρόχρονη ενδυνάμωση. Τέλος, οι «έφηβοι» μύες έχουν μικρότερη απόδοση σε δοκιμές που εξαρτώνται από τον προμετωπιαίο φλοιό, όπως είναι η εναλλαγή βραχιόνων με καθυστέρηση στο λαβύρινθο τύπου Τ (T-maze) και στη συμπεριφορική δοκιμή αναγνώρισης αντικειμένων με χρονική σειρά, χωρίς να παρουσιάζουν αντίστοιχες διαφορές σε δοκιμές που δεν εξαρτώνται από τον προμετωπιαίο φλοιό, όπως είναι η δοκιμή αναγνώρισης νέου αντικειμένου (novel object recognition task) και νέας θέσης αντικειμένου (object-to-place recognition task).

Στο **κεφάλαιο ΙΙ**, μελετήσαμε τον ρόλο της μειωμένης αναστολής στην φυσιολογία του προμετωπιαίου φλοιού και στην συμπεριφορά μυών, χρησιμοποιώντας το διαγονιδιακό ποντίκι Rac1 conditional knockout (Rac1 cKO) που διαθέτει ~50% λιγότερους φλοιϊκούς

διάμεσους νευρώνες εξαιτίας της απώλειας της πρωτεΐνης Rac1 από κύτταρα που εκφράζουν τον μεταγραφικό παράγονται Nkx2.1. Βρίσκουμε ότι τα ενήλικά Rac1 cKO παρουσιάζουν αυξημένη ευπάθεια σε φαρμακολογικά επαγόμενες επιληπτικές κρίσεις αλλά και αυξημένο άγχος. Σε κυτταρικό επίπεδο, τα Rac1 cKO παρουσιάζουν διαταραγμένη βραχύχρονη και μακρόχρονη πλαστικότητα (LTP) στον προμετωπιαίο φλοιό. Αλλαγές στην δενδριτική μορφολογία, όπως η μείωση των μανιταροειδών δενδριτικών ακάνθων και το μειωμένο μήκος των δενδριτών, θα μπορούσαν να υπόκεινται στη μείωση του LTP των Rac1 cKO. Σε τομές εγκεφάλου από Rac1 cKO, η ενίσχυση της GABAεργικής νευροδιαβίβασης με τη χρήση ήπιας δόσης διαζεπάμης ήταν επαρκής για την διάσωση του διαταραγμένου LTP. Τα παραπάνω ευρήματα μας οδήγησαν στην υπόθεση ότι το δίκτυο του προμετωπιαίου φλοιού των Rac1 cKO μυών παρουσιάζει μία ανισορροπία διέγερσης και αναστολής, που προκαλείται από την απορύθμιση του γλουταματεργικού συστήματος σε απόκριση της λειτουργικής μείωσης του GABAεργικού συστήματος, που τελικά έχουν ως αποτέλεσμα το αυξημένο άγχος και την ευαισθησία σε επιληπτικές κρίσεις. Μελετήσαμε επίσης και τους νεαρούς Rac1 cKO μύες που παρουσιάζουν μειωμένο άγχος και αυξημένη επαγωγή LTP μετά από τετανικό ερεθισμό, με μία υποκείμενη αύξηση του αριθμού των δενδριτικών ακάνθων σε σύγκριση με τα Rac1 Het (ετερόζυγα) ποντίκια, τα οποία χρησιμοποιούνται ως ομάδα ελέγχου. Τέλος, η οξεία ή χρόνια αναστολή του GABAεργικού συστήματος στα Rac1 Het ποντίκια με πικροτοξίνη επίσης διατάραξε το LTP στον προμετωπιαίο φλοιό. Τα αποτελέσματα μας προτείνουν ότι η κατάλληλη και επαρκής αναστολή κατά τη διάρκεια της παιδικής ηλικίας είναι κρίσιμη για την φυσιολογική ανάπτυξη των συναπτικών ιδιοτήτων αλλά και της πλαστικότητας κατά μήκος της στιβάδας ΙΙ του προμετωπιαίου φλοιού, όπως και την ανάπτυξη της φυσιολογικής συμπεριφοράς και των γνωσιακών λειτουργιών.

Στο **κεφάλαιο III**, προσεγγίσαμε υπολογιστικά, με τη χρήση ενός μοντέλου μικροκυκλώματος του προμετωπιαίου φλοιού, την επίδρασης της μειωμένης GABAεργικής αναστολής στις ιδιότητες της παραμένουσας δραστηριότητας, που θεωρείται το κυτταρικό υπόβαθρο της μνήμης εργασίας στον προμετωπιαίο φλοιό. Γι' αυτό, κατασκευάσαμε ένα μικροκύκλωμα του προμετωπιαίου φλοιού, που αποτελείται από μοντέλα πυραμιδικών νευρώνων και τα τρία διαφορετικά είδη διάμεσων νευρώνων: γρήγορης πυροδότησης (fast-spiking (FS)), κανονικής πυροδότησης (regular-spiking (RS)), και ακανόνιστης πυροδότησης (irregular-spiking (IS)). Έγινε επαγωγή της παραμένουσας δραστηριότητας στο μοντέλο-μικροκύκλωμα και αναλύθηκαν οι ιδιότητές της. Αφαίρεση ή μείωση των εισόδων από το μοντέλο FS στους πυραμιδικούς νευρώνες-μοντέλα μείωσαν σημαντικά την βιοφυσική τροποποίηση της επαγωγής της παραμένουσας δραστηριότητας, μείωσαν τα χρονικά διαστήματα μεταξύ των δυναμικών ενεργείας (interspike intervals (ISIs)), τον νευρωνικό συγχρονισμό και τις ταλαντώσεις γάμμα ρυθμού κατά τη διάρκεια της παραμένουσας δραστηριότητας. Η επίδραση στον συγχρονισμό και στις ταλαντώσεις μπορούσε να αντιστραφεί με την προσθήκη άλλων ανασταλτικών εισόδων στο σώμα των πυραμιδικών κυττάρων, αλλά σε βαθμό πέραν των επιπέδων του κυκλώματος ελέγχου. Συνεπώς, γενικά η σωματική αναστολή δρα ως βηματοδότης της παραμένουσας δραστηριότητας και συγκεκριμένα η αναστολή από FS διάμεσους νευρώνες τροποποιεί το σήμα εξόδου αυτού του βηματοδότη.

Συνολικά, τα αποτελέσματα μας συνεισέφεραν στην αναπτυσσόμενη γνώση στις λειτουργίες του προμετωπιαίου φλοιού κατά τη διάρκεια της ανάπτυξης και στους υποκείμενους μηχανισμούς που καθιστούν τον προμετωπιαίο φλοιό «την περιοχή που οι άνθρωποι αξιώνουν για την ικανότητά της να ρυθμίζει τις σκέψεις μας και τις συμπεριφορές μας».

Abstract

The prefrontal cortex (PFC) is involved in higher-order cognitive functions (short-term and long-term mnemonic functions) as well as in emotional processes. Although age seems to play a significant role in both the normal function of PFC and the emergence of disease states, very little is known with regards to the age-dependent changes of behaviors involving the PFC and the underlying cellular mechanisms. Previous studies, primarily based on dendritic morphology, have suggested that this higher-order brain region exhibits delayed cortical development compared to primary sensory cortical areas that lasts until young adulthood. Investigating the PFC postnatal development is critical to radically improve our understanding of brain function and behavior as well as of the emergence substrate of devastating neuropsychiatric disorders involving the PFC. The PFC, similar to other cortical areas, is composed of glutamatergic excitatory neurons and GABAergic inhibitory interneurons. A balance between these two systems is required for proper functioning of the PFC. Recently, the role of the GABAergic system has strongly been implicated in contributing to possible imbalances between the two main systems, ultimately leading to pathological states. Our goal in this dissertation was two-fold. Firstly, we aimed to better understand the postnatal development of the PFC and secondly, to study changes in PFC behavior and underlying cellular mechanisms in cases with reduced GABAergic inhibition. Our results are presented in three different chapters.

In **Chapter I,** we used a multidisciplinary approach including cellular, electrophysiological and behavioral techniques in different age groups of mice in order to better understand the PFC development. In many cases, a comparison to other cortical areas was made. We find a differential expression of distinct types of dendritic spines in pyramidal neurons of PFC between different age groups of mice. In particular, 'adolescent' pyramidal neurons (40 days old) exhibit the lowest spine density measured, with an increased percentage of

stubby spines, while the 'juvenile' (35 days old) and 'young adult' pyramidal neurons (60 days old) have increased number of spines, and particularly of the mushroom type. This developmental pattern was also observed in our electrophysiological studies, in which the 'juvenile' and 'young adult' age groups exhibit increased long-term potentiation (LTP) of synaptic transmission in response to tetanic stimulation, while the 'adolescent' age group exhibits decreased LTP. Finally, 'adolescent' mice perform poorer in PFC-dependent tasks, such as the delayed alternation in the T-maze and the temporal order object recognition task, without exhibiting differences in non-PFC-dependent tasks, such as the novel object and objet-to-place recognition tasks, compared to young adult mice.

In **Chapter II,** we studied the role of decreased inhibition in PFC physiology and in mouse behavior, using the Rac1 conditional transgenic mouse (Rac1 cKO) that displays ~50% less cortical interneurons due to the loss of the Rac1 protein from Nkx2.1-expressing cells. We find that the adult Rac1 cKO exhibit increased susceptibility to pharmacological-induced epileptic seizures as well as increased anxiety. At the cellular level, Rac1 cKO mice exhibit impaired short-term plasticity and LTP in response to tetanic stimulation within the PFC. Changes in dendritic morphology, such as reduced mushroom-type spines and reduced dendritic length, could underlie the decrease in LTP of the Rac1 cKO mice. In Rac1 cKO brain slices, up-regulation of GABA-receptor-mediated neurotransmission using a mild dose of diazepam was sufficient to rescue the impaired LTP. The above findings led us to further hypothesize that the PFC network of Rac1 cKO mice exhibit an imbalance of excitation and inhibition, caused by deregulation of the glutamatergic system in response to functional reductions of the GABAergic system, which ultimately result in increased anxiety and vulnerability to epileptic seizures. We also studied the juvenile Rac1 cKO mice, which exhibit decreased anxiety and increased LTP induction after tetanic stimulation with an underling increase in the number of dendritic spines compared to juvenile Rac1 Het

(heterozygous) mice, used as control. Finally, acute or chronic inhibition of the GABAergic system in Rac1 Het mice with picrotoxin also impaired the LTP in the PFC. Our results suggest that proper inhibition during the juvenile period is critical for the normal development of synaptic properties and plasticity within layer II of PFC, as well as for the development of the normal behavior and cognitive functions.

In **Chapter III**, we undertook a computational approach to study how reductions in GABAergic inhibition in a PFC microcircuit model affect the properties of persistent activity, considered the cellular correlate of working memory function in PFC. To this end, we constructed a PFC microcircuit, consisting of pyramidal neuron models and all three different types of interneurons: fast-spiking (FS), regular-spiking (RS), and irregular-spiking (IS) interneurons. Persistent activity was induced in the microcircuit model and its properties were analyzed. Removing or decreasing the FS model input to the pyramidal neuron models greatly limited the biophysical modulation of persistent activity induction, decreased the ISIs (inter-spike intervals), neuronal synchronicity and gamma-power oscillations during persistent activity. The effect on synchronicity and oscillations could be reversed by the addition of other inhibitory inputs to the soma, but beyond the levels of the control network. Thus, generic somatic inhibition acts as a pacemaker of persistent activity and FS specific inhibition modulates the output of the pacemaker.

Overall, our results contributed to the growing knowledge on PFC functions during development and their underlying mechanisms that render the PFC “the region that humans prize for its ability to regulate our thoughts and behaviors”.

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"The brain is a tissue. It is a complicated, intricately woven tissue, like nothing else we know of in the universe, but it is composed of cells, as any tissue is. They are, to be sure, highly specialized cells, but they function according to the laws that govern any other cells. Their electrical and chemical signals can be detected, recorded and interpreted and their chemicals can be identified; the connections that constitute the brain's woven feltwork can be mapped. In short, the brain can be studied, just as the kidney can."

David Hubel, "The Brain"

GENERAL INTRODUCTION

1.0. General Introduction

1.1. Genes and environment

The dilemma *'heredity or environment' (nature vs. nurture)* was formulated and delivered in the first half of the 19th century by Francis Galton. He argued that the personality and characteristics of each human are determined by the genome, and he became a founder of the discipline of eugenics, a discipline that proposes to allow the control of the evolution of humanity (Galton, 1907). In direct opposition to the views of Galton were the behaviorists' views, according to which the genetic material plays no role in shaping the personal characteristics, but the environment is solely responsible. Arguing about the above two extreme positions often occurs even today as a "barren trench warfare", but it is now clear that the downgrading of the importance of heredity and environment in the organization of brain and behavior counter to documented scientific knowledge (Παπαδόπουλος, Γ.Χ. Καραγωγέως, Κούβελας, & Τριάρχου, 1998).

The first arguments offered by Neurobiology to tackle the above dilemma have a numerical basis. The human brain consists of about 10^{11} nerve cells, which are interconnected by at least 10^{14} synapses (Kandel, Schwartz, Jessell, Siegelbaum, & Hudspeth, 2000). Since the total number of genes in the human DNA is calculated around 100.000, it is evident that the existing genetic information is not sufficient to predetermine the position, characteristics and synaptic interactions of all nerve cells. Thus, the genome is not possible to code for the complete and accurate profile of the brain (Παπαδόπουλος, Γ.Χ. Καραγωγέως et al., 1998).

Studies using cell lines show that the effect of genetic information on neuronal phenotype is limited. The *'induction'* phenomenon shows that neighboring cells and tissues exhibit decisive influence on the fate of a cell. Changing the environment of a growing nerve cell (in cell cultures or after transplantation) can lead to a change in the phenotype of the cell (e.g.

of the neurotransmitter release). Therefore, genes form a coarse pattern of neuronal organization, in which the developing neuronal population, under the complicated influence of environmental factors (molecules in the extracellular environment or even sensory-social experiences), will find their way. This is proposed to be achieved by fine-tuning an initial 'rough draft' of the genetically guided formation of neural networks through selective reinforcement or withdrawal of neural connections. Therefore, it has become clear that both of these factors together partake in the development of brain and behavior. Genes influence the environment of development and the environment of development affects the output of genetic information. We cannot override either the genes or the environment. We are totally biological and totally environmental at the same time (Παπαδόπουλος, Γ.Χ. Καραγωγέως et al., 1998).

1.1.1. The role of experience in brain development

The capabilities of an adult organism, such as fine movements, cognitive abilities, fine sensory sensation, do not occur in the newborn, but are rather developed gradually through constant exercise. The genetic program ensures for the newborn fully developed sensory organs and a nervous system whose billions of nerve cells have already been organized into individual systems serving specific functions. However, the formation and use of the capabilities offered by the nervous system depend on the experiences of the organism. Under this influence, the developing brain is gradually sacrificing its initial multipotency in trying to achieve the adult specificity and accuracy in selected behaviors.

During the formation of the complex neural networks, the developing brain is facing the challenge to achieve two seemingly contradictory goals. On one hand, neuronal connections must be precise to allow for proper brain function and, on the other hand, they have to be

able to adapt according to the changing needs of the developing organism. The consequences of the environmental influences, particularly during the postnatal brain development, are critical for the formation of the adult fully developed neural connections. Visual information, sounds, touch, smells, tastes, speech and posture are stimuli-experiences that result in the induction and amplification of specific neural pathways and the degradation of others. The effect of normal or abnormal experience on the developing brain, and thus behavior changes with age. There are several experimental models showing that the normal development and function of brain regions were affected dramatically by the deprivation of normal experience during certain *'critical periods'* after birth.

Classic examples showing the experience-dependent effects on the developing brain and resulting behavior during these early and time-restricted periods include the effect of imprinting on birds (Reik & Walter, 2001), the chirping of birds (Marler & Peters, 1981) and the formation of ocular dominance columns in the primary visual cortex of the cat and primates (Hubel & Wiesel, 1959, 1965; LeVay, Wiesel, & Hubel, 1980; Stryker & Harris, 1986) (Figure 1.1.). Ocular dominance columns are subregions in the mammalian visual cortex that respond preferentially to input from one eye or the other resulting in a striped pattern across the surface of the striate cortex (V1) (Stryker & Harris, 1986). Studies have shown that early sensory experiences are necessary for the proper formation of connections from neurons in the sensory organs to neurons in the cortex. Specifically in the primary visual cortex, Hubel and Wiesel showed that absence of visual experience alters the connectivity and function of cortical neurons (Hubel & Wiesel, 1959, 1965; LeVay et al., 1980). In their effort to study the formation of cataracts in childhood, they closed the eyelids of the one eye during the first months of life in cats and monkeys, and then, when they once again opened eyelids, they measured the sensitivity of the nerve cells at different 'stations' alongside the main visual pathway. They found that while the cells of the retina

and the lateral geniculate nucleus react normally to visual stimuli in both eyes, almost all cells of the primary visual cortex permanently react only to visual stimuli in the eye whose eyelids were not sutured. This means that if one eye is closed or not functioning properly because of a defect, such as cataracts, for a specific postnatal period, the eye will stay blind forever. In contrast, visual deprivation in adulthood does not create any problem in the nerve cells of the visual cortex. Therefore, there is a "critical period" or "sensitive period" after birth when the formation of ocular dominance columns may be modified by activity dependent plasticity.

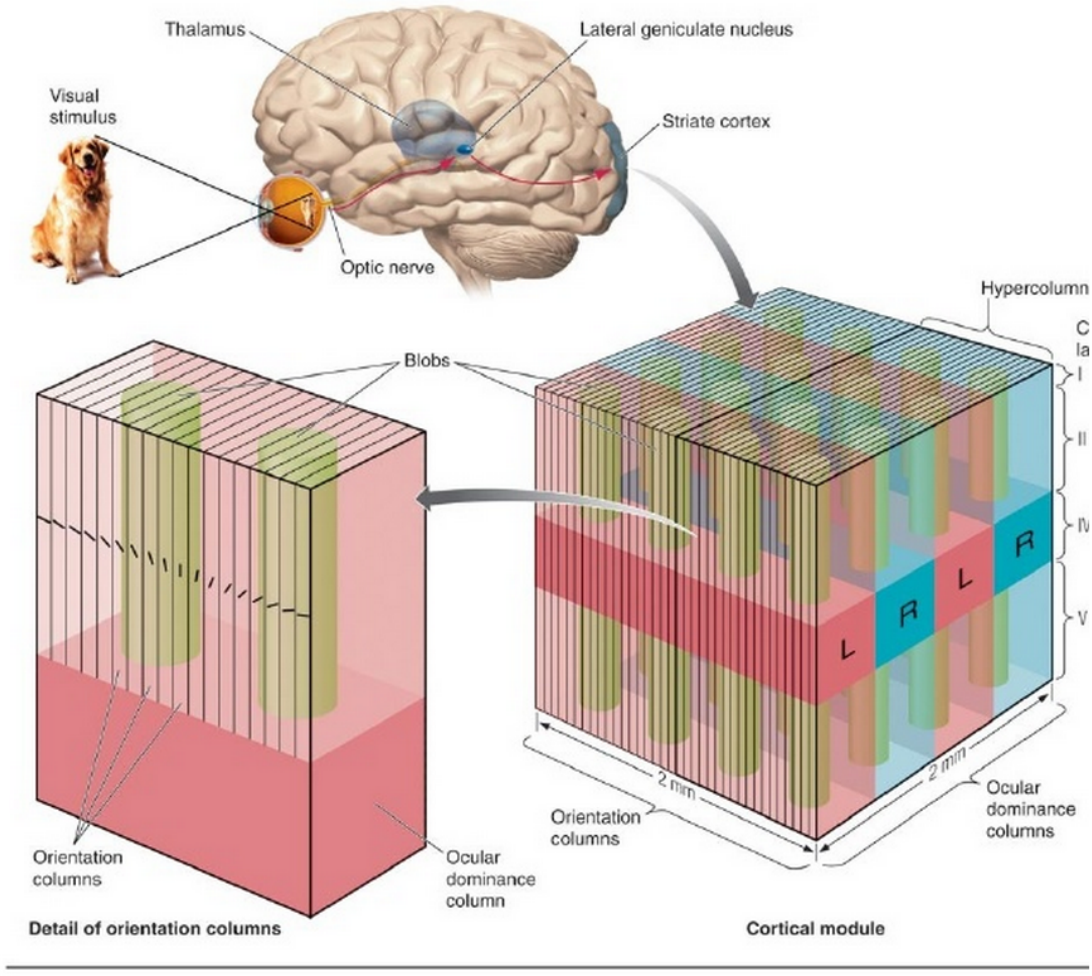

Figure 1.1. *Ocular dominance columns. The stripe shaped regions of the primary visual cortex that lie perpendicular to the orientation columns are called ocular dominance columns*

and they are innervated by input from the lateral geniculate nucleus (LGN) into layer IV. A hyper-column contain a full set of orientation and ocular dominance columns

1.1.2. Relationship between neuronal activity and organization of neural circuits

The visual information from both eyes enters the visual cortex in the form of electric waves alongside the cortical afferent fibers. The effect of visual deprivation, after closing one eye during the critical period, in the development of ocular dominance columns, is probably due to the fact that the electrical activity from the closed eye are significantly reduced. This was further demonstrated by experiments using intraocular tetrodotoxin (a chemical that blocks all neuronal activity) injections inhibiting the transmission of action potentials along neuronal axons (Stryker & Harris, 1986). Inhibition of the electrical activity in the retinal ganglion cells after injection of tetrodotoxin, only during a specific time window, resulted in alteration of the size of ocular dominance columns. Therefore, the formation of columns depends on the existence of neuronal activity, which cannot be random. It needs to be time and location specific. The development of normal binocular vision presupposes synchronized activity of the fibers of each eye and slightly asynchronous activity of the fibers of the two eyes.

The example of the developing visual system allows us to conclude that the neural networks that characterize the adult brain are formed in two main phases. In the first, which is largely genetically guided, axons reach their cellular targets and, using *'molecular drivers',* form a coarse topographic plan of synaptic connections. During the second phase, the experience, in the form of selective neuronal activity, modulates the already established neural networks through cooperation and competition of axons and retrograde enhancement of active axons, an accurate synapse-wiring network. Thus, the role of early experience is to sculpt and specify the developmental plan that has been prepared by

genetic factors. The final organization of the adult brain, and therefore the behavior of the adult, results from the interaction of innate hereditary and environmental factors. The degree of environmental influence on the brain changes with age and is usually more important during the early postnatal period, particularly for the sensory functions of the brain. However, the ability of the brain to adapt to different experiences, that is the plasticity of the brain , continues well even during adulthood.

The specific temporal window during which the brain is most sensitive to the effects of environmental experience is known as a critical period. This critical period is thought to exist during the early postnatal period, for example the critical period for the development of binocular vision extends from three months to three years for human children (Siegler, DeLoache, & Eisenberg, 2003), six months for monkeys (Hubel, Wiesel, & LeVay, 1977), or 28 days for mice (Gordon & Stryker, 1996). Sensory deprivation during this period deprives individuals the opportunity to exploit the full potential of the brain. However, there are indications that the critical postnatal period is not synchronous for all regions of the brain and consequently for the various aspects of behavior.

1.2. Cortical structure

The central nervous system consists of the brain and the spinal cord. The brain lies rostral to the spinal cord and can be divided into 3 main parts: the rhombocephalon (the medulla, pons, midbrain - termed as the brain stem – cerebellum), diencephalon, and the cerebral hemispheres or telencephalon. The cerebral hemispheres are the largest part of the human brain; consist of the cerebral cortex - a thin outer layer of the cerebral hemispheres -, the white matter (corpus callosum) and three deep-lying structures: the basal ganglia, amygdala, and hippocampal formation. These brain areas process different sensory, motor,

perceptual, and cognitive functions. The cerebral cortex is divided into four major lobes – frontal, parietal, temporal, and occipital - and is responsible for much of the planning and execution of actions in everyday life as it is concerned with cognition. Phylogenetically, humans have the most elaborate cerebral cortex. The cerebral cortex, also known as the neocortex, is organized into layers and columns (Figure 1.2.). Columns – the fundamental computational modules of the cortex - run from the white matter to the pial surface, traversing the layers. Neurons within a column (1mm diameter) tend to have similar response properties, as they form a local processing network. The main input to the neocortex comes from the thalamus. However, different parts of the neocortex receive input from other cortical regions on both sides of the brain, as well as from subcortical brain areas, for example the hippocampus and the basal ganglia. Its output is directed to other regions of the neocortex, basal ganglia, thalamus, pontine nuclei, and the spinal cord. These complex input-output relationships are efficiently organized in the orderly layering of cortical neurons; each layer contains different neurons and different efferent and afferent fibers. Most of the neocortex contains six layers, numbered from the outer surface (pia mater) of the cortex to the white matter (Kandel et al., 2000).

1.2.1. Layers

Layer I, the molecular layer, is occupied by the dendrites of neurons located in deeper layers and afferent axons. Layers II and III contain mainly small pyramidal shaped cells. Layer II, the external granular cell layer, is one of two layers that contain small spherical neurons. Layer III is called the external pyramidal cell layer (an internal pyramidal cell layer lies at a deeper level). The neurons located deeper in layer III are typically larger than those located more superficially. The axons of pyramidal neurons in layers II/III project

locally to other neurons within the same cortical area, as well as, to other cortical areas, thereby mediating intracortical communication. Layer IV contains a large number of small spherical neurons and thus is called the internal granular cell layer. It is the main recipient of sensory input from the thalamus and is most prominent in primary sensory areas. Layer V, the internal pyramidal cell layer, contains mainly pyramidal-shaped cells that are typically larger than those in layer III. Pyramidal neurons in this layer give rise to the major output pathways of the cortex, projecting to other cortical areas and to subcortical structures. The neurons in layer VI are fairly heterogeneous and thus this layer is called the polymorphic or multiform layer. It folds in the white matter that forms the deep limit of the cortex and carries axons to and from areas of cortex. Although each layer of the neocortex is defined primarily by the presence, absence, and packing density of distinctive cell types, each layer also contains the dendrites of specific cortical neurons. Layers I through III contain the apical dendrites of neurons that have their cell bodies in layers V and VI, as well as in layers II and III, whereas layers V and VI contain the basal dendrites of neurons with cell bodies in layers III and IV, as well as in layers V and VI. The inputs to a cortical neuron thus depend on the location of its dendrites and cell body. The thickness of individual layers and the details of their functional organization vary throughout the cortex. Korbinian Brodmann, in 1909, used the relative prominence of the layers above and below layer IV, cell size, and packing characteristics to divide the cerebral cortex into 47 regions, known as Broadman areas (Kandel et al., 2000).

1.2.2. Columns

Neurons in the neocortex are often organized functionally into columns of cells that run from the white matter to the pial surface, thus traversing the layers. Each column is a

fraction of a millimeter in diameter. In rodents, a neocortical column of about 0.3 mm in diameter contains roughly 7.500 neurons (100 neurons in layer I, 2.150 in layer II/III, 1.500 in layer IV, 1.250 in layer V and 2.500 in layer VI). Neurons within a column tend to have very similar response properties, probably because they form a local processing network. Each column comprises a computational module with a highly specialized function. The larger the area of cortex dedicated to a function, the greater the number of computational columns that are dedicated to that function. The highly discriminative sense of touch in the fingers is a result of the large area of cortex dedicated to processing somatosensory information from the fingertips (Kandel et al., 2000).

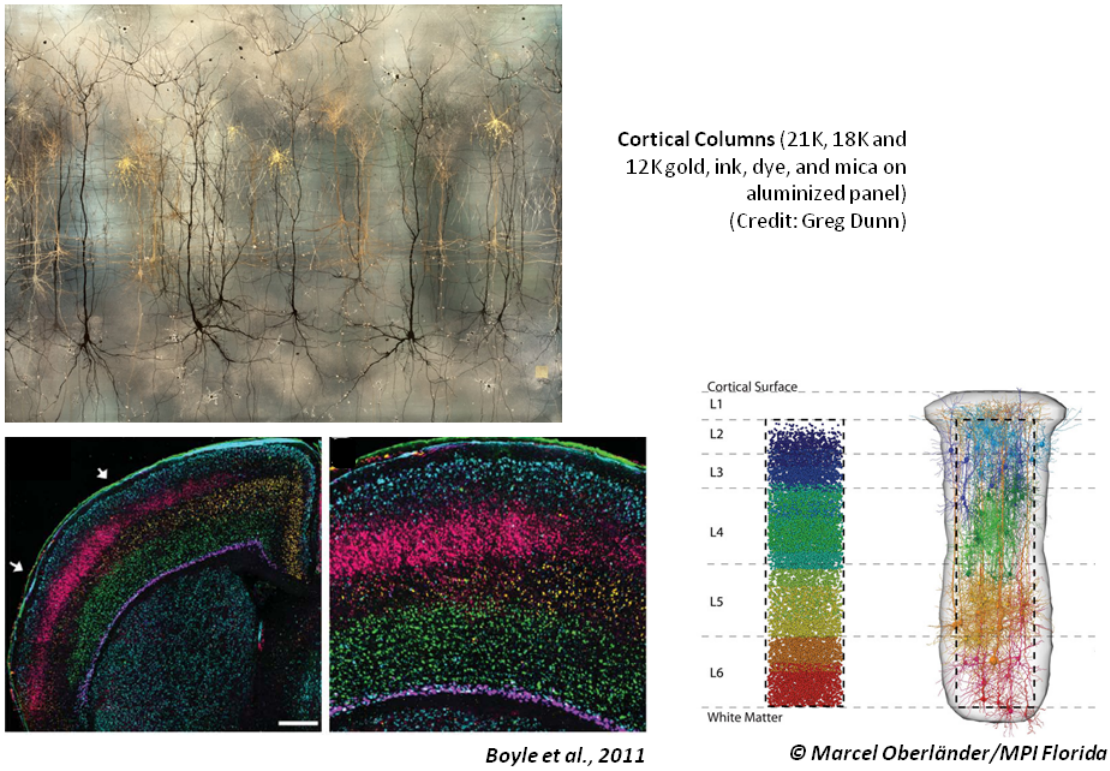

Figure 1.2. *Cortical columns and cortical layers. Every cortical region is organized in layers and columns. Specifically, they follow a six-layer organization within each of them a distinct neuronal type or even a connection with other cortical or subcortical regions appears. In layer V there are only pyramidal neurons connecting with subcortical regions, while within layer II only pyramidal neurons connecting with other cortical regions. Cells within a single column consists a microcircuit sending and receiving cognate information.*

1.3. Cortical Neurons

Cortical neurons have a variety of shapes and sizes. A student of Santiago Ramón y Cajal, Raphael Lorente de Nó, used the Golgi staining procedure to identify more than 40 different types of cortical neurons based only on the distribution of their dendrites and axons (Larriva-Sahd, 2002). Generally, cortical neurons, as elsewhere, can be broadly defined as either principal (projection) neurons or local interneurons (Figure 1.3.). Principal or projection neurons typically have pyramid-shaped cell bodies, and are referred as pyramidal neurons. They are located mainly in layers III, V, and VI and release the amino acid glutamate as their primary transmitter from their axon terminals. Glutamate then binds to its receptors, which include both ionotropic receptors (AMPA (2-amino-3- (3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid) and NMDA (N-methyl-D-aspartate)) and metabotropic glutamate receptors, at the post-synaptic neuron. The action of glutamate is excitatory (Spruston, 2008). Local interneurons have axons that remain within the same region where their cell body is located and use the neurotransmitter γ-aminobutyric acid (GABA) at their axon terminals. Upon release, GABA binds to either ionotropic (GABAa) or metabotropic receptors (GABAb) on the post-synaptic neuron and exerts an inhibitory effect at the post-synaptic neuron. The cortical interneurons constitute 20% to 25% of all cortical neurons and are distributed throughout all layers. Interneurons may receive inputs from the same sources as the pyramidal cells, as well as from the pyramidal cells within the region that are found (Markram et al., 2004).

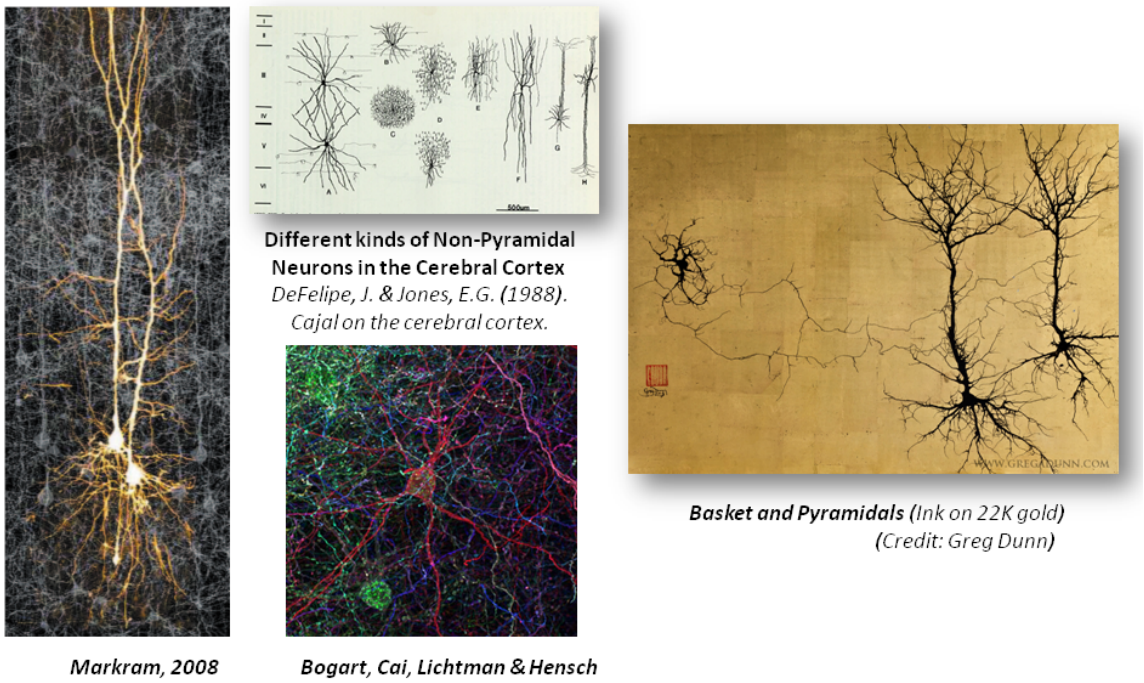

Figure 1.3. *Pyramidal neurons and interneurons. There are two types of neurons in the cortex; pyramidal neurons and interneurons. Pyramidal neurons comprise the 70-80% of neuronal cells in the cortex and they provide excitatory action releasing glutamate as a neurotransmitter. Interneurons are inhibitory neurons releasing GABA as a neurotransmitter and they comprise the 20-25% of cortical neurons.*

1.3.1. Pyramidal Neurons in the cortex

Pyramidal neurons are found in the cerebral cortex, the amygdala and the hippocampus, but not the spinal cord, the olfactory bulb, the striatum, the midbrain or the hindbrain (Pestronk, 1997; Spruston, 2008). The morphological features of pyramidal neurons are characteristic. The pyramidal neurons have a triangular shaped soma and each pyramidal neuron has a single axon that typically emanates from the base of the soma, which then branches along its length, until its target region. The dendritic tree of a pyramidal neuron has two distinct domains: the basal and the apical dendritic tree, which descend from the base and the apex of the soma, respectively. The basal dendritic tree is composed of multiple, relatively short dendrites that extend radially from the soma. The apical dendritic

tree is composed of a single apical trunk, which emerges from the apex of the soma. Several smaller, oblique side branches emanate along its length at various angles, while at the top, the several, thinner dendritic sections form the apical tuft. There is great variability concerning the complexity of the apical side branches and the apical tuft among different pyramidal neurons of different layers, cortical regions and species (Gao & Zheng, 2004; Kasper, Larkman, Lübke, & Blakemore, 1994; Spruston, 2008).

Beside the morphological features, there is a variation in some other properties of pyramidal cells, such as the endogenous neuronal excitability, which affects the evoked electrical firing patterns in response to input signals. The firing pattern is formulated by spike trains, which are sequences of action potentials. The action potential is a transient (1 ms) reversal in the polarity of neuron's transmembrane potential, which then moves from its point of initiation, down the axon, to the axon terminals. In a series of elegant experiments Hodgkin and Huxley, together with Bernard Katz, discovered that the action potential results from transient changes in the permeability of the axon membrane to sodium (Na^+) and potassium (K^+) ions (Barnett & Larkman, 2007). The classification based on these firing patterns in response to depolarizing current pulses and the characteristics of the action potentials have revealed two main types of pyramidal neurons: Regular Spiking and Intrinsic Bursting neurons (Connors & Gutnick, 1990; D. A. McCormick, Connors, Lighthall, & Prince, 1985).

Regular Spiking (RS) pyramidal neurons: their response consists of individual, long-lasting action potentials that emerge at a regular rate, upon continuous stimulation of the neuron. In some cases, the train of action potentials exhibits adaptation, meaning that the action potentials emerge at an increasingly longer time. Each spike is usually followed by a complex set of intrinsically generated afterhyperpolarizations (AHPs) and afterdepolarizations (ADPs) (Connors & Gutnick, 1990).

Intrinsic bursting (IB) pyramidal neurons: these neurons respond initially with two action potentials that occur within a few milliseconds, a pattern known as burst, and then with action potentials that are spaced regularly. Within a burst, each successive spike usually declines in amplitude, presumably because sustained depolarization inactivates sodium conductances (Connors & Gutnick, 1990).

This heterogeneity in the electrical properties of neurons is the result of the concerted action of different combinations of ion channels of the membrane and the differences in the morphology of neurons. The specific pattern of electrical behavior for each type of neuron is actually the end product of a series of other - unique for its type - properties, such as the combination of ion channels expressed, the quantity of each ion channel expressed, the post-translational modifications of these molecules, and their distribution on the membrane (Markram et al., 2004).

1.3.2. Cortical Interneurons

As mentioned above, cortical interneurons comprise the remaining 20–25% of neocortical neurons, they are mostly inhibitory, they use GABA (γ-aminobutyric acid) as their transmitter and they have diverse morphological, physiological, molecular and synaptic characteristics (Cauli et al., 1997; Javier DeFelipe, 2002; Gupta, Wang, & Markram, 2000; Y Kawaguchi & Kubota, 1997; Somogyi, Tamás, Lujan, & Buhl, 1998; A M Thomson & Deuchars, 1994) (Figure 1.4.). They can be differentiated from the pyramidal neurons based on the following: a) Interneurons target different subdomains of pyramidal neurons or other interneurons, i.e. their dendrites, axon, soma (Javier DeFelipe, 1997; Tamás, Somogyi, & Buhl, 1998), while pyramidal neurons primarily target the dendrites of their target neurons. b) Most mature interneurons have aspiny dendrites, while pyramidal

neurons have spiny dendrites (Markram et al., 2004). c) The axons of cortical interneurons do not typically project down the white matter to contact distant brain regions, but they usually arborize within a cortical column and can project laterally across columns, hence, their identification as 'local circuit neurons' (Letinic, Zoncu, & Rakic, 2002). d) The somata of the interneurons can receive both excitatory and inhibitory synapses (Markram et al., 2004).

The morphological characteristics of the body, the dendrites and the axons of the interneurons are characterized by great diversity. The dendritic morphology is the most variable characteristic and so it is not possible to consistently determine the type of the cell. The anatomical identity of an interneuron can be more accurately determined through the morphology of axonal branches, since interneurons appear to be specific in relation to the different parts of neurons (axon/soma/basal or apical dendrites), the individual layers of a cortical column, but also the different columns that they target. The relative percentages of representation of different kinds of interneurons vary both between species and between brain areas and cortical layers (Javier DeFelipe et al., 2003). Based on their morphology therefore interneurons can be classified into a plurality of subtypes, some of them are: Basket Cells, Chandelier cells (Ch), Martinotti cells (M), Bipolar Cells, Double bouquet Cells (DB), and Bitufted Cells.

Basket Cells comprise the 50% of all inhibitory interneurons and specialize in targeting the somata and proximal dendrites of pyramidal neurons and interneurons (Gilbert, 1994; Markram et al., 2004; Y. Wang, Gupta, Toledo-Rodriguez, Wu, & Markram, 2002). They have a basket-like appearance around pyramidal cell somata and they typically express the two calcium-binding proteins; Parvalbumin (PV) and Calbindin (CB).

Chandelier Cells are axon-targeting interneurons (Javier DeFelipe, 1997; Markram et al., 2004; Somogyi et al., 1998) with chandelier like appearance that seems to become

progressively more refined in "higher" species. They typically express the calcium-binding proteins; Parvalbumin (PV) and Calbindin (CB).

Martinotti cells project their axons towards layer I, inhibiting the tuft dendrites of pyramidal neurons, while they also provide the only source for cross-columnar inhibition via layer I (Javier DeFelipe, 2002; Markram et al., 2004; Y. Wang et al., 2004). They target the most distal, the proximal, the perisomatic dendrites and the somata. They always express Somatostatin (SOM), but they never express Parvalbumin or VIP (vasoactive intestinal polypeptide).

Bipolar cells are small cells with narrow bipolar or bitufted dendrites. They can be excitatory by releasing only VIP, or inhibitory by releasing mainly GABA. They contact only a few cells, mainly on the basal dendrites of pyramidal neurons and they typically express Calretinin (CR) and VIP (Markram et al., 2004).

Double bouquet cells have a bitufted dendritic morphology and they are dendritic-targeting cells. They express CB, they have the unique tendency to express CR and CB together and can also express VIP or cholecystokinine (CCK), but not PV, SOM or NPY (neuropeptide Y) (J DeFelipe, Hendry, Hashikawa, Molinari, & Jones, 1990).

Bitufted cells are similar to bipolar and to double bouquet cells. They are dendritic-targeting and they can express CB, CR, NPY, VIP, SOM, CCK, but not PV (Markram et al., 2004; Somogyi et al., 1998).

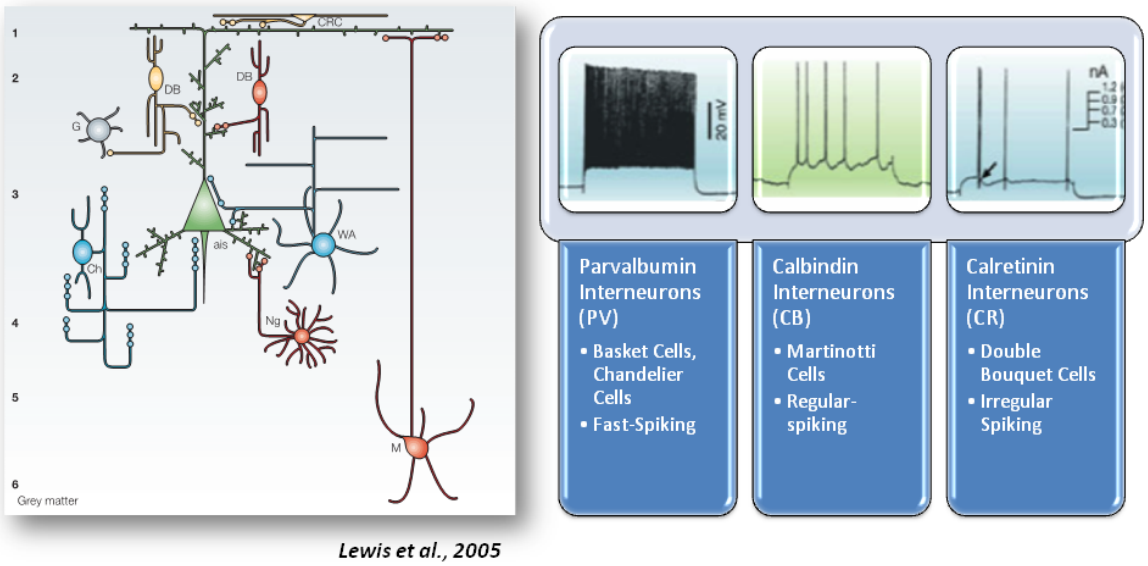

Figure 1.4. *Classification of interneurons. Interneurons are characterized by a great diversity regarding their electrophysiological, molecular, biochemical and morphological features rendering their classification in distinct groups a difficult task. However, there is a widely accepted classification according to calcium-binding proteins that they express; Parvalbumin, Somatostatin and Calretinin. Parvalbumin interneurons (PV) fire high frequency action potentials projecting on pyramidal somata providing the main inhibitory action in the cortex. Calbindin interneurons (CB) fire regularly, similarly to the majority of pyramidal neurons and they project to distal dendrites of pyramidal neurons. Finally, Calretinin interneurons (CR) fire irregular action potentials and they project in distal pyramidal dendrites or even on other interneurons providing disinhibitory action.*

Another variable characteristic of interneurons, which is provided to identify their subtype, is their specific pattern of expression, especially in terms of calcium binding proteins (CaBPs), which are involved in intracellular signaling by changing the cytosplasmic calcium levels. These are calretinin (CR), parvalbumin (PV), and calbindin (CB), and each is expressed in a different but partly overlapping subset of interneurons. On the other hand, an equally important biochemical marker is the protein somatostatin (SST), an inhibitory hormone-peptide that regulates the endocrine system and affects neural transmission and cell proliferation. Somatostatin with three other neuropeptides (vasoactive intestinal peptide - VIP, cholecystokinin - CCK and neuropeptide Y - NPY) may also be used for the

identification of partially overlapping categories of interneurons (Cauli et al., 1997; Y. Kubota, Hattori, & Yui, 1994).

Regarding their electrophysiological properties, all inhibitory interneurons were originally described as "fast-spiking" (Connors & Gutnick, 1990). Subsequent studies, however, revealed that non-pyramidal cells exhibit a wide variation in their firing properties, with the possibility of adaptation. The following firing patterns have been described among others:

Fast spiking (FS) interneurons fire individual fast spikes that usually last less than 0.5 ms, because of a more rapid rate of repolarization, and each spike is cut short by a deep, relatively brief AHP. Another property which characterizes the FS cells is that they undergo little or no adaptation during prolonged intracellular current pulses, sustaining spike frequencies of at least 500 - 600 Hz for hundreds of milliseconds (ms) (Connors & Gutnick, 1990) and they exhibit relatively negative resting potential and low input resistance compared with other cell types (Cauli et al., 1997).

Late spiking (LS) interneurons, discharge with a considerable delay after a depolarizing step. These cells were found in layers II/III and V.

Low threshold spiking (LTS) cells, also known as "burst - spiking non pyramidal cells" (BSNP), are located primarily in layer V and respond to hyperpolarizing pulses of low intensity (near the threshold) with burst-like discharges.

Regular spiking non-pyramidal cells (RSNP), are located in layers II/III & V and are characterized by long lasting action potentials and plasticity of their firing frequencies.

Irregular spiking (IR) cells, are also found in layers II/III & V and fire an initial burst of action potentials followed by irregularly spaced action potentials (Yasuo Kawaguchi, 1995; Markram et al., 2004).

Therefore, it is evident why the problem of classifying and naming neurons has been a topic of debate for over 100 years. Nevertheless, a satisfactory consensus remains to be reached,

even for restricted neuronal populations such as the GABAergic interneurons of the cerebral cortex. Over the past two decades, the amount of morphological, molecular, physiological and developmental data has grown rapidly, making classification harder rather than easier. A consistent neuronal classification and terminology will help researchers to manage this multidisciplinary knowledge, and is needed for specialists in neuroscience subfields to establish and maintain effective communication and data sharing (Ascoli et al., 2008). A recent study found a significant correlation between the ion-channel genes expressed in specific interneurons and its electrical phenotype, providing a coefficient of correlation for each gene with respect to the value of each electrophysiological parameter. This correlation map revealed many candidate genes that underlie different electrical properties and a cluster analysis of co-expression revealed three main classes of ion-channel expression, which mapped around the three calcium-binding properties (PV, CB, and CR). The PV cluster includes: HCN2[1], KV3.1[2], KV1.2[3], KV1.6[4], KV1.1[5], PV, KV3.2[6], HCN1[7], KVβ1[8] and Caα1A[9], that complement each other to generate the high-frequency discharge, the CB cluster includes: CB, Caβ4[10], HCN3[11], KV1.4[12], Caα1G[13], Caβ1[14], HCN4[15], KV3.3[16] and Caβ3[17], that generate bursting discharges and finally the CR cluster includes:

[1] *hyperpolarization activated cyclic nucleotide-gated potassium channel 2*
[2] *potassium voltage gated channel, shaw-related subfamily, member 1*
[3] *potassium voltage-gated channel, shaker-related subfamily, member 2*
[4] *potassium voltage gated channel, shaker related subfamily, member 6*
[5] *potassium voltage-gated channel, shaker-related subfamily, member 1*
[6] *potassium voltage gated channel, Shaw-related subfamily, member 2*
[7] *hyperpolarization-activated cyclic nucleotide-gated potassium channel 1*
[8] *potassium voltage-gated channel, shaker-related subfamily, beta member 1*
[9] *calcium channel, voltage-dependent, P/Q type, alpha 1A subunit*
[10] *calcium channel, voltage-dependent, beta 4 subunit*
[11] *hyperpolarization-activated cyclic nucleotide-gated potassium channel 3*
[12] *potassium voltage-gated channel, shaker-related subfamily, member 4*
[13] *calcium channel, voltage-dependent, gamma subunit 1*
[14] *calcium channel, voltage-dependent, beta 1 subunit*
[15] *hyperpolarization activated cyclic nucleotide-gated potassium channel 4*
[16] *potassium voltage gated channel, Shaw-related subfamily, member 3*
[17] *calcium channel, voltage-dependent, beta 3 subunit*

SK2[18], KV3.4[19], CR and Caα1B[20], that generate accommodating discharges (Toledo-Rodriguez et al., 2004).

1.4. Cortical connectivity

1.4.1. Synaptic inputs onto distinct domains

Pyramidal neurons receive synaptic input at the soma, the axon and the dendrites. Particularly excitatory synapses are formed onto specialized protrusions of the dendrites, called dendritic spines. The dendrites of pyramidal cells receive signals from thousands of excitatory synapses via glutamate receptors. The primary glutamate receptors are the ionotropic ones: AMPA and NMDA. Inhibitory synapses are activated by the binding of GABA (gamma-aminobutyric acid) to different types of receptors: GABAb or GABAa receptors. Glutamatergic synapses are usually found in neuronal dendrites, while GABAergic synapses are found primarily at the soma. Usually, proximal dendrites receive excitatory inputs from local sources (collaterals in the same area or from an adjacent area) whereas the distal apical tuft receives inputs from more distant cortical and thalamic locations. The complexity of the dendritic tree allows for multiple modes of synaptic integration to take place, that allow coincidence detection, signal amplification, temporal integration depending on the relative behavioral condition (Sidiropoulou, Pissadaki, & Poirazi, 2006). All synaptic inputs are subject to modification by a number of other neuromodulatory signals, such as acetylcholine, dopamine, serotonin and norepinephrine (Arnsten, Wang, & Paspalas, 2012).

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[18] *potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2*
[19] *potassium voltage gated channel, Shaw-related subfamily, member 4*
[20] *calcium channel, voltage-dependent, beta 1 subunit*

1.4.2. Structure and function of dendritic spines

The dendritic spines are the sites where primarily excitatory synaptic contacts between neurons occur (Figure 1.4.). The number of spines represents a minimum estimate of the number of excitatory synaptic inputs onto a neuron, which varies considerably in their size and shape (Sorra & Harris, 2000) in different cortical regions and species (Ballesteros-Yáñez, Benavides-Piccione, Elston, Yuste, & DeFelipe, 2006; Elston & DeFelipe, 2002; Spruston, 2008).

Spine structure

Ramon y Cajal discovered dendritic spines, in 1888, using the Golgi-Cox staining technique. After decades of studies it is now known that dendritic spines are dynamic structures of neurons, due to the rapid generation, degeneration and change of their shape (Benavides-Piccione, Ballesteros-Yáñez, DeFelipe, & Yuste, 2003).

Dendritic spines are protrusions of the cell membrane of neurons in vertebrates and invertebrates (Nimchinsky, Sabatini, & Svoboda, 2002) and human brain possesses more than 10^{13} spines, which are located in specific types of neurons, including pyramidal neurons of cerebral cortex and hippocampus, in medium spiny neurons in basal ganglia and in Purkinje cells of the cerebellum. They consist of a head (~0.001-1 μm^3 in volume), linked with the neuron by a thin neck (Kasai, Fukuda, Watanabe, Hayashi-Takagi, & Noguchi, 2010). Higher numbers of dendritic spines are observed in higher order brain regions (Nimchinsky et al., 2002), whereas in several mental disorders, dendritic spines are found decreased in numbers and in shape (Kasai et al., 2010). Regarding the developmental stage, and particularly for the dentate gyrus and the CA1 area of the hippocampal formation, it seems that spines do not show any age-dependent decrease (Bohlen, 2009).

More than 90% of excitatory synapses end up in these sub-cellular compartments which control local cell-signaling mechanisms and, this is the reason why, dendritic spines are specialized structures of synaptic transmission (Yuste & Bonhoeffer, 2004). They are the result of continuous network activity and not of a single memory event. In the absence of network activity, there is no formation of new dendritic spines, but degeneration or conversion into filopodia of the existing ones may be observed. In the absence of spines, neurons can retain synapses by afferent fibers, which abut on dendrites, producing strong synaptic potentials. However, without spines, neurons are less capable to cope with strong synaptic potentials, which can lead to cell death (Segal, 2010). Dendritic spines are also important for increasing the possible connections between adjacent dendrites for the formation of a highly connected network (Yuste, 2011).

Concerning the hippocampal formation, it has been shown that dendritic spines represent the main position of excitatory synapse formation in pyramidal neurons of CA1 region (Bohlen, 2009; Megías, Emri, Freund, & Gulyás, 2001). A very important subregion of the spines, located in the spine head, is the so-called postsynaptic density (PSD) area. PSD consists of various proteins, which are interconnected via specific interactions. These proteins include neurotransmitters' receptors and their scaffold proteins, signaling molecules, ion channels, cytoskeleton components (Sheng & Hoogenraad, 2007) and other organelles, necessary for proper synaptic function and plasticity. Moreover, apart from the PSD, spine membrane contains specific subregions for endocytosis and exocytosis (Blanpied, Scott, & Ehlers, 2002; Bosch & Hayashi, 2012).

On the other hand, the spine neck mediates the calcium (Ca^{2+}) compartmentalization in the head in mushroom and thin type of spines of pyramidal neurons in hippocampus. Spine neck dimensions are crucial concerning the calcium concentration. In particular, thin and long necks seem to retain more calcium in head, compared to short and fat necks.

The local calcium increase modulates signaling pathways which strengthens or weakens synapses (Bourne & Harris, 2007).

The protrusive shape of spines from cytosol is based on the cytoskeleton, mainly consisting of F-actin filaments, which support structurally and provide a mechanism for vesicular trafficking (Bosch & Hayashi, 2012). In addition, PSD proteins and F-actin filaments together with many small GTPases (RhoA, Rac1 and Cdc42) regulate spines' morphology and the synapse creation molecularly (Lai & Ip, 2013).

Dendritic spine morphology

The common dendritic spine consists of a bulbous head, which connects with the dendrite via a thin neck. The most common terminology categorizes dendritic spines into 3 groups, based on the head size and neck.

Mushroom spines consist of a large head, with an extensive PSD, supported by a long, thin neck. Thin spines have a smaller head, which includes PSD, and they have a thin, long neck, too. The third category - stubby spines - does not present a neck or a PSD region. There is also, the filopodium category that is considered to be a kind of dendritic spines. Filopodia are thin, long, with no head or PSD protrusions of the cytoplasmic membrane (Bohlen, 2009; Knyihár-Csillik, Rakic, & Csillik, 1985). These structures are highly mobile and flexible and can be stable for minutes up to hours. Due to their intense motility, they are ideal for exploring the space among dendrites for the establishment of new synapses (Yoshihara, De Roo, & Muller, 2009). Especially in hippocampus, there are also the so-called branched spines, which consist of multiple heads, protruding from a mutual neck (Bohlen, 2009). Experiments have shown that dendritic spine morphology (size and shape) does not

depend on the distance from the soma (Arellano, Benavides-Piccione, Defelipe, & Yuste, 2007).

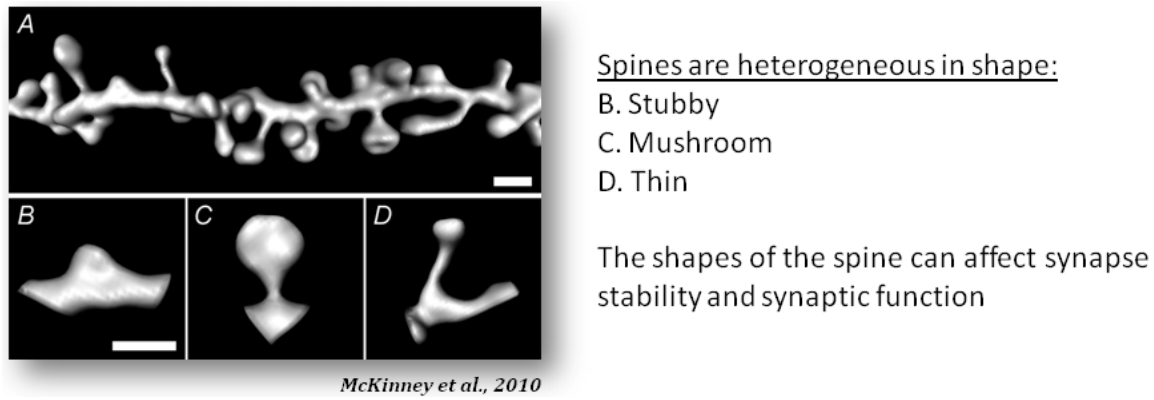


Figure 1.4. *Dendritic spines. Excitatory synapses occur on dendritic spines, which are small protrusions from the dendritic tree. Spines are heterogeneous in shape and their morphology affects the stability and function of the synapses. They are grouped in stubby spines, which are immature dendritic spines, mushroom spines, which are involved in long-term potentiation, and thin spines that are involved in persistent activity. Increase or decrease of dendritic spine density has been related to alterations in learning and memory storage abilities in pathological states or even as a normal process taking place during development.*

Spinogenesis and spine morphology conversion during development

During development, spine morphology changes, depending on the developmental stage the organism is. During early development or in immature neurons, spines are immature too, and often have the stubby shape of spines (Yuste & Bonhoeffer, 2004) and the fillopodium form (Yoshihara et al., 2009). In contrast, in the adult brain the most common types of spines are the mushroom and thin spines, which constitute the mature forms of spines, even though many stubby spines are still present in the cortex (Benavides-Piccione et al., 2003). It is also possible to detect filopodia in the mature brain, under certain circumstances, e.g. after ischemia or during neuronal regeneration (after injury) (Ostroff, Fiala, Allwardt, & Harris, 2002; Yoshihara et al., 2009).

During maturation, the spine development in pyramidal neurons can be related to synaptogenesis, since it has been proposed that spines are formed on the dendrite, after or

before the synapse formation by a neighboring dendrite. There are three main models for spinogenesis: First, the *"Sotelo model"*, in which stubby spines are generated and develop a PSD, regardless the synapse existence. When a presynaptic, adjacent neuron forms a synapse on a new-formed spine, the latter matures and turns into a mature mushroom or thin dendritic spine. This model seems to be only applicable in Purkinje cells of the cerebellum.

The second model of spinogenesis is the *"Millers/Peters 'model"*, which differs from the *"Sotelo model"* and is likely applicable in different neuronal populations, such as pyramidal neurons of the neocortex and hippocampus. This model supports the idea that synapse formation is a regenerative force that forms immature, stubby synapses, which gradually develop PSD, receive new synapses and finally, turn into mushroom an thin types of spines (Yuste & Bonhoeffer, 2004).

Furthermore, there is the *"filopodia model"*, according to which new-formed filopodia, slide and attach onto adjacent dendrites, inducing PSD formation and filopodia maturation, both in morphology and function. However, this process is only 10-20% successful (Yoshihara et al., 2009).

In developing or immature neurons, newborn spines are not stable structures and present thin neck and small head, making difficult to distinguish them from filopodia. Moreover, most of these structures take part in weak and silent connections, tending to degenerate in a few hours (Bourne & Harris, 2007). The reasons of the rapid degeneration of new-formed spines (stubby) and filopodia, soon after their formation, have not been completely clarified yet. But this could be due to the inability of new spines to stabilize, as a result of insufficient neuronal activity, PSD or synaptic plasticity transduction (Ehrlich, Klein, Rumpel, & Malinow, 2007; Yoshihara et al., 2009). During this early stage of stabilization, new-born spines will develop PSD and increase their head size (Yoshihara et al., 2009). However, in

order to strengthen their stability, a strong stimulus is needed to trigger LTP induction, which will then lead to AMPA and NMDA receptors activation and production of kinases (Bosch & Hayashi, 2012). After LTP induction, F-actin levels decrease transiently, allowing polyribosomes and other synaptic plasticity proteins to be distributed again in the heads of spines that have strengthened their synapses. Shortly after these events, F-actin levels increase again and the spine head swells (Bourne & Harris, 2007; Ostroff et al., 2002). The spine swelling is related to AMPA receptors accumulation, against NMDA receptors, and the synapse conversion from silent to active forms (Zito, Scheuss, Knott, Hill, & Svoboda, 2009). Nevertheless, the spine swelling can be transient and studies suggest that their head size shows fluctuations, throughout their life (Yoshihara et al., 2009).

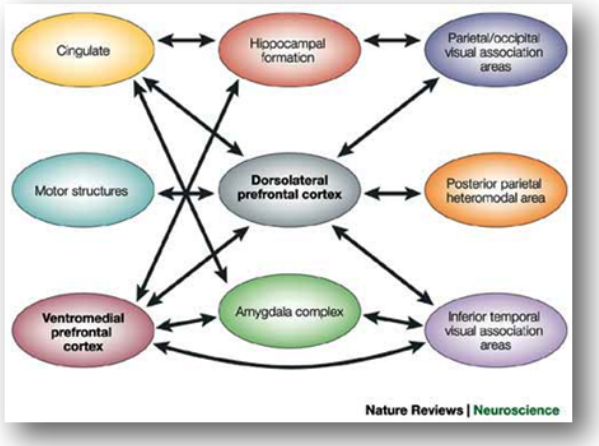
The spine swelling leads to the mature and final shape of spines, ending up in mushroom and thin types, offering more stability. It is believed that spines with larger head have a more extensive smooth endoplasmic reticulum (ER) and better calcium and Glutamate levels regulation (Bourne & Harris, 2007), producing larger synaptic potentials. Due to their stability, they can contribute more effectively in the formation of strong synaptic connections (Bourne & Harris, 2007), and for that reason, small spines represent the 'learning spines' (they are still under stabilization, creating new synapses), whereas large spines are the 'memory spines' (there are already established synapses) (Kasai, Matsuzaki, Noguchi, Yasumatsu, & Nakahara, 2003).

1.5. Prefrontal Cortex

The associative cortical region in the anterior frontal lobe of mammals' brain is often defined as the prefrontal cortex (PFC) (Figure 1.6.). There have been many attempts to define this region by using different criteria and methodology (Joaquín M Fuster, 2008).

Although there is not yet a universally accepted anatomical definition of the PFC, it can be defined anatomically, accurately for all mammals, as the part of the cerebral cortex that receives projection fibers from the mediodorsal nucleus of the thalamus. The PFC lies in front of the premotor cortex and in the front lateral surface of the limbic association cortex on the orbital and medial surfaces (Kolb et al., 2012). Gross morphological features mark certain segments of its boundary: in carnivores by the presylvian fissure, in primates by the homologs of that fissure (arcuate sulcus, inferior precentral fissure) and by the anterior curvature of the cingulate sulcus. In primates, the PFC covers about 30% of that (Miller & Cohen, 2001) and encompasses the regions 8 to 13, 24, 32, 46 and 47, according to the Brodmann' s cytoarchitectonic map (1909) (Joaquin M Fuster, 2001).

In rodents, the presence of a PFC, although controversial, is defined based on the aforementioned criterion of its connections with the mediodorsal nucleus of the thalamus (Uylings, Groenewegen, & Kolb, 2003). Although the PFC is relatively simpler compared to primates but can also be divided into distinct subregions (Uylings et al., 2003), namely, from dorsal to ventral, the medial agranular cortex (AGm), the anterior cingulate gyrus cortex (AC), the prelimbic cortex (PC) and the infralimbic cortex (IC) (Vertes, 2004). In rodents, the average PFC of the rat is composed of five layers, because of the absence of granular layer IV.



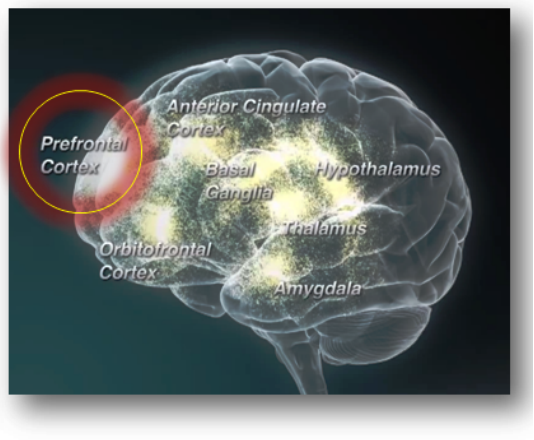


Figure 1.6. *PFC and its connectivity. PFC plays a fundamental role in executive functions presiding at them in cooperation with other brain regions. This conclusion is enhanced by PFC connectivity with a lot of brain regions. Because of its great connectivity it is thought to be on the top of the hierarchy of brain regions. PFC is located at the anterior part of the brain and it takes up a large portion of the cortex in humans.*

1.5.1. PFC Connectivity

As mentioned above, the PFC receives afferent fibers from the mediodorsal nucleus of the thalamus. Some of those thalamic projections convey influences from subcortical structures, such as the brainstem, the cerebellum and limbic structures to the PFC. The PFC also receives direct afferents from the hypothalamus, the midbrain, the amygdala, and the limbic cortex. In addition fibers from various neocortical areas implicated in sensory functions converge on the PFC. Most of the PFC connections are reciprocal: structures sending fibers to the PFC are also the recipients of fibers from it. Different subregions of the PFC have different sets of reciprocal connections. The orbital and medial PFC is primarily connected with the medial thalamus, the hypothalamus, the amygdala, and limbic and medial temporal cortex, including the hippocampus. This complex interconnected system, composed of phylogenetically old, early developing structures, is the anatomical substrate for emotional, instinctive, and affect-modulated behavior. On the other hand, the lateral PFC, is primarily connected with the lateral thalamus, the dorsal caudate nucleus, and the neocortex. This newer system of interconnected structures constitutes the substrate for executive cognitive functions and behavior (Joaquín M Fuster, 2008).

1.5.2. Functions of PFC

Functionally, the PFC is devoted to the cognitive functions that control the execution of goal-directed actions. The previous discussion of PFC connectivity supports the idea that the PFC sits at the top of the hierarchy of incoming and outgoing information streams, rendering the PFC an ideal brain area for mediating higher order functions. The PFC has actually been named the "executive center" of the brain, that is, a brain area that receives and integrates information, and subsequently makes 'decisions'. Some of these "executive functions" that the PFC underlies are the following: Focusing attention, motor planning, thought organization, problem solving, predicting behavioral consequences, temporal organization, decision-making, behavioral inhibition (also known as impulse control), rule learning, emotional regulation, long-term memory storage (Frankland & Bontempi, 2005). Most of the above higher order cognitive functions require proper functioning of the working memory system.

Working memory

Working memory is the term used for a type of memory that is active for very limited time, usually seconds (Figure 1.7). It is a system of short-term storage and processing of external and internal representations in order to link remote, in space and in time, information.

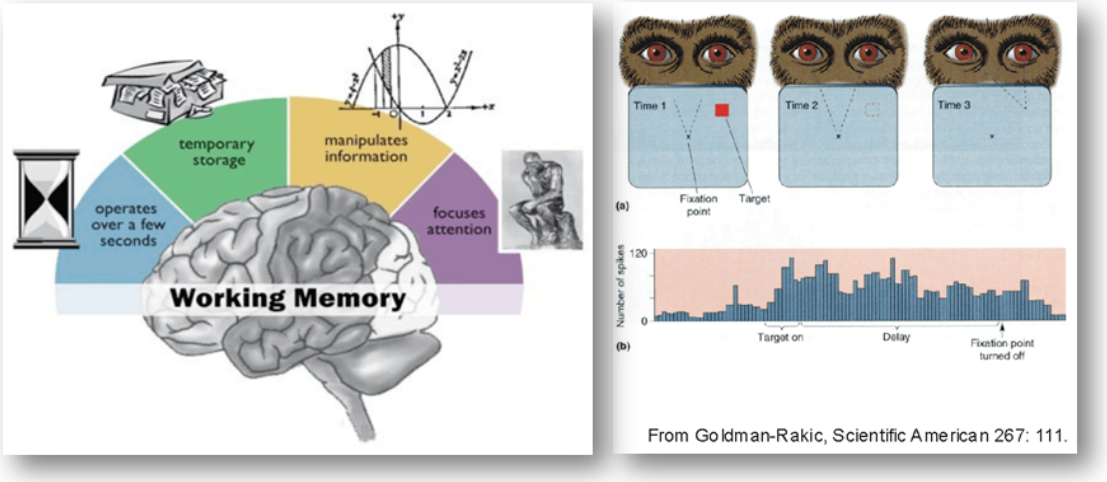


Figure 1.7. *Working memory. Working memory is a fundamental function of PFC and it is the ability to maintain information in mind without the presence of an external stimulus. It has been described as the blackboard of the brain or its RAM memory exactly because of its temporary nature and it integrates information distant in time and in place by associating them. Furthermore, it is the foundation for many PFC-dependent functions as the focus of attention, the behavioral inhibition and the decision-making. The cellular correlate supporting working memory is persistent activity. Persistent activity has been identified during recordings from PFC neurons during working memory behavioral tasks. During this kind of experiments, as described above, a visual cue is presented in the peripheral visual field of a monkey and its location must be remembered in order to turn its gaze with a saccadic movement upon signal presentation. The time period from the moment that the visual cue is out till the moment of the saccadic movement of the monkey is called delay period and during this period persistent activity has been recorded from specific cells of the PFC.*

The importance of this process has been attributed from the now classic metaphor of Baddeley (A Baddeley, Logie, Bressi, Della Sala, & Spinnler, 1986) for working memory as the 'blackboard' of the brain, where information, such as a goal or a rule, is not permanently imprinted in neural networks, but replaced quickly when no longer is useful (Patricia S Goldman-Rakic, 1995; Miller, 2000).

The importance of working memory in the context of higher cognitive functions is not necessarily obvious, perhaps because of its transient nature. However, the brain's ability to recall events without direct stimulus being present is perhaps its most flexible innate mechanism and most remarkable evolutionary achievement. The fundamental contribution of working memory in cognitive function is the perception of the existence of an object

when it is away from the visual or, in general, the aesthetic field. This process has been referred to as "representation knowledge" and is considered a basic component of abstract thinking (Patricia S Goldman-Rakic, 1995). The working memory is also mobilized in all forms of cognitive and language processing and is paramount both for the understanding and for the structure of sentences. Ultimately, this type of memory, in general, plays an important role in the processing of a wide range of complex cognitive functions such as learning, reasoning, perception, orientation of attention, decision-making, behavioral inhibition, planning of actions and creativity (Patricia S Goldman-Rakic, 1995). Long-standing evidence identify the PFC as the anatomical substrate of working memory (Joaquín M Fuster, 2008; P. S. Goldman-Rakic, 1987).

Temporal Organization

The PFC receives inputs from all other regions of the neocortex and integrates the planning and guidance of complex motor, learning, emotional and social aspects of behavior. Its fundamental and general function is the temporal organization of various activities towards biological or learning objectives. This region - especially the lateral PFC - specializes in the construction of the temporal sequence of a new series of complex, targeted actions, which have to do with behavior, reason or logic. Temporal organization of actions is based in the neural process of integrating information from time discrete stimuli, actions and action plans that will be used to perform a targeted sequence of behaviors. To perform this role, the PFC must have access to sensory, motor, mnemonic and generally all the elements that construct behavior. To better understand this mechanism, the populations of PFC neuronal populations could be seen as the cellular components of widespread cortical networks, which reflect the time structure of behavior and the relationships between its components.

The sequential temporal activation of individual sections of these networks, with a particular functional role, ultimately leads to a series of repeated actions - a temporally structured behavior. Neural networks are formed and reshaped by the experience acquired by the environmental exposure (Joaquín M Fuster, 2003).

Inhibitory control

Scientific data from the field of cognitive neuroscience converge in recent years that different subregions of the PFC contribute in various ways in inhibitory control, which is a necessary process of integrating information for the proper performance of an action (Aron, 2007; Ridderinkhof, Van Den Wildenberg, Segalowitz, & Carter, 2004). The obvious physiological purpose of inhibitory control is the suppression of all possible non-correlated internal or external stimuli that can intermingle and impede any aspect of behavior, speech or cognitive function that is in preparation (Joaquín M Fuster, 2003).

Harnishfeger (1995) (Harnishfeger, 1995) defines two aspects of inhibitory control: The first is cognitive inhibition on the suppression of cognitive elements or processes that have been activated earlier and on the resistance against the interference on sensory stimuli, emotions, unwanted memories or other processes taking place in the field of focusing attention. The other aspect is the behavioral inhibition that has to do with controlling pointless behaviors such as delaying satisfaction and overcoming internal tendencies, impulses or instinctive behavior.

In case that the distraction factors are random visual, auditory, tactile or other stimuli arriving to PFC from sensory systems the "blocking element of attention" is being activated. The action of sensory areas that process and send unwanted signals in the PFC probably is suppressed by a feedback inhibition system of orbitomedial PFC, which is part of the

broader attention control network. The orbitomedial PFC appears to have an additional role in the prevention of internal motivations' interference in the implementation of targeted actions. The biological impulses emanate from the diencephalon and the brainstem. But they are under the control of orbitomedial PFC, through anatomically identified connections towards those subcortical areas, and specifically towards the hypothalamus. Finally, another source of interference is the representations of motor actions that are irrelevant or incompatible with actions that are in the process of temporal organization before their execution. These motor habits and tendencies have been established in long-term memory, and by extension in the cortical and the subcortical network of motor systems. Their suppression is the core of the "blocking element" of motor attention (Joaquín M Fuster, 2003).

Despite the fact that the central role of the PFC in suppressing unwanted stimuli is considered confirmed, the mechanism underlying this function still remains unclear (Barbas & Zikopoulos, 2007). One mechanism recently proposed by Munakata and colleagues (2011) (Munakata et al., 2011) argues that there are two types of PFC inhibitory effects in other brain regions. The first is the directed global inhibition of whole subcortical and archicortical systems that copes with stressors, inhibits responses and suppresses memory retrieval. Two neural mechanisms support directed global inhibition; PFC excitatory projections can synapse directly onto GABAergic interneurons in the target area, as occurs in coping with stressors and PFC excitatory projections can synapse onto excitatory neurons in a region, which in turn synapse preferentially onto GABAergic interneurons in the target area, as occurs in response inhibition.

The second type of PFC inhibitory effects is the indirect competitive inhibition within cortical and subcortical regions. Within this type of inhibitory control PFC neurons directly stimulate regions associated with the processing of the desired targeted behavior. In this

way, the desired processing paths become more "competitive" than other probably uncorrelated paths and also perform an inhibitory effect on them (Munakata et al., 2011). Such indirect competitive mechanisms are often referred to theories relating to the functions of selection and attention (Desimone & Duncan, 1995).

Whatever the specific mechanism which inhibitory control depends on, the key element that makes the PFC capable of exert it is its excellent connectivity with numerous brain regions that process information from all sensory systems or others resulting through experience and stored in memory. In addition, of great importance is the direct communication of PFC with emotion-processing brain regions, since the targeted behavior is inextricably linked with motivation and its emotional context (Barbas & Zikopoulos, 2007).

Long-term memory storage

The memories of the new stimuli that are constantly received by the brain are initially under the medial temporal lobe system, including the hippocampus, which serves as a temporary storage of new memories, through their initial consolidation at the synaptic level. But as these memories mature, their consolidation depends increasingly on other cortical areas. Pharmacological and anatomical approaches, in combination with the use of transgenic models and modern imaging technologies have recently clarified that the PFC probably plays a remarkable role in remote memory processing (Maviel, Durkin, Menzaghi, & Bontempi, 2004).

Ablation or pharmacological inactivation of PFC results in disruption in recalling remote but not recent memories. Conversely, when similar interventions are waged in areas of the hippocampus the recall of recent but not of remote mnemonic events is disrupted. Based on

these data, the suggested model supports that the PFC contributes to long-term memory storage in two ways. Firstly, since the new memories are encoded in networks between the cortex and the hippocampus, the bidirectional connections of PFC with various sensory, motor and limbic structural modalities of the cortex are reinforced, allowing memories to function irrespective of the hippocampus. At this point the PFC undertakes the incorporation of information on remote memory. Secondly, the PFC is believed to regulate the activity of the hippocampus through the memory recall. The hippocampus operates normally in the processing of external stimuli from the environment. But when the incoming information matches a previously stored, remote, cortical memory, the PFC inhibits the function of the hippocampus, with direct or indirect projections to prevent unnecessary information coding. If the stimulus is present for the first time, or have been forgotten and so the incoming message does not match any stored memory, PFC does not suppress it and the hippocampus is normally employed (Frankland & Bontempi, 2005).

Emotional regulation-Trait anxiety

Anxiety is a complex emotional state associated with elevated autonomic and behavioral arousal and a sustained increase in avoidance behavior in environmental situations characterized by a level of uncertainty, unpredictability, or uncontrollability, including situations where a conflict between approach and avoidance exists (Gray, 1987; Lowry, Johnson, Hay-Schmidt, Mikkelsen, & Shekhar, 2005). Specifically, anxiety is characterized by increased attentional capture by threat-related stimuli (MacLeod, Mathews, & Tata, 1986; Williams, Mathews, & MacLeod, 1996) resulting of a hyper-responsive pre-attentive threat-detection system centered on the amygdala (Mathews, Mackintosh, & Fulcher, 1997). The physiological and behavioral arousal associated with anxiety states and anxiety-related

behaviors appears to be regulated by a distributed and interconnected system of forebrain and hindbrain structures including the septo-hippocampal system and entorhinal cortex (Gray, 1987), medial PFC (Duncan, Knapp, & Breese, 1996), basolateral amygdaloid complex (Campbell & Merchant, 2003; Spiga, Lightman, Shekhar, & Lowry, 2006) and midbrain raphe complex (N. Singewald & Sharp, 2000; Nicolas Singewald, Salchner, & Sharp, 2003). PFC mechanisms influence the top-down control of selective attention to threat (Bishop, 2009; Öhman, 2005). Trait anxiety may be characterized by impaired recruitment of prefrontal mechanisms that are critical to the active control of attention when the task at hand does not fully govern the allocation of attention. It is proposed that this deficit does not arise as a result of current or state levels of anxiety, but instead reflects an underlying trait characteristic that influences attentional processing regardless of the presence or absence of threat-related stimuli (Bishop, 2009).

1.6. Cellular mechanisms of cognition

1.6.1. Persistent activity

Persistent activity has been recorded primarily in PFC pyramidal neurons during the delay-period of delayed response tasks (J. M. Fuster & Alexander, 1971; Patricia S Goldman-Rakic, 1995; K. Kubota & Niki, 1971). This delay-related firing is thought to result from the activity of pyramidal neurons with similar spatial characteristics and to be involved in the temporary storage of information during working memory tasks. The term that has prevailed for the description of this property is ‘persistent activity’.

Since first discovered the 'persistent activity' has been identified in many other cortical and subcortical structures (Hikosaka, Takikawa, & Kawagoe, 2000), which suggests that its

induction and maintenance can be based on a mechanism that utilizes a widely extended neural circuit. It has not yet been fully clarified exactly how persistent activity is induced and configured in the neurons of the mammalian brain. However, there have been proposed several possible, not necessarily mutually exclusive, mechanisms that explain this phenomenon.

One hypothesis is that 'persistent activity' results from strong repetitive excitation between pyramidal neurons, i.e. it is induced and maintained by the mutual positive feedback within a population of neurons. Networks of pyramidal neurons in PFC are interconnected with dendritic spines and exchange excitatory postsynaptic signals via NMDA receptors, which appear to play a particularly important role in the delay-related firing (X. J. Wang, 1999).

Alternatively, 'persistent activity' is probably dependent on the inherent ionic membrane permeability or the intracellular signals. More specifically, unitary cortical neurons may exhibit persistent activity due to specific currents flowing through the membrane. For example, neurons in the entorhinal cortex exhibit a gradational depolarization after a brief stimulus. Such firing may lead to the influx of calcium cations through voltage-gated calcium channels on dendrites, causing depolarization again and thus creating a cycle of positive feedback that results in a delayed afterdepolarization (dADP). This type of persistent activity seems to depend on a calcium-activated non-selective cation current (CAN) (Egorov, Hamam, Fransén, Hasselmo, & Alonso, 2002; Fransén, Tahvildari, Egorov, Hasselmo, & Alonso, 2006; Sidiropoulou et al., 2009; X. J. Wang, 1999).

Persistent activity is stimulus selective. This means that in a spatial working memory task, for example, a particular pyramidal neuron will exhibit persistent activity, only if the stimulus is presented at a particular point in the visual field corresponding to the mnemonic field of the neuron. Despite the large number of studies that have focused on this field

recently the cellular mechanisms supporting this selectivity have not yet been clarified (Sidiropoulou & Poirazi, 2012).

1.6.2. Long-Term Potentiation

Learning could be described as the process by which new information is obtained about the environment and memory as the process by which information is maintained. It is widely accepted that the formation of memories requires activity-dependent changes in the strength of synapses between neurons involved in forming a specific memory. The activity-dependent increase in synaptic strength is a phenomenon known as long-term potentiation (LTP). Long-term potentiation could be defined as a sustained increase of excitatory postsynaptic potentials (EPSPs), following a series of repetitive, high-frequency excitatory input signals (such as tetanic stimulation) and it has been widely studied in the neural circuits of the hippocampus. At the synaptic level, the opposite phenomenon has also been observed, that is a decrease in the EPSP following long, low-frequency stimulation (Lynch et al., 2004).

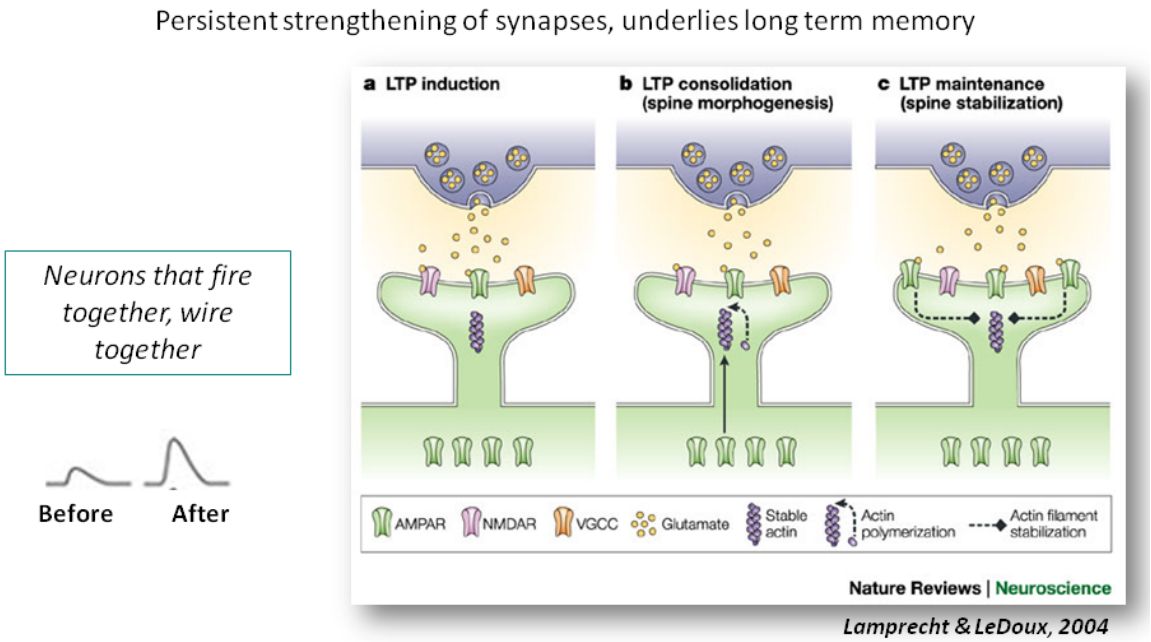


Figure 1.8. *Long-term potentiation. PFC has also been implicated in the long-term memory storage. The cellular mechanism under this function is long-term potentiation. It has been described that synapses connecting neurons that fire together get strengthened. In a synapse the firing of cell A induces a postsynaptic potential in cell B. Long-term potentiation refers to the increase of postsynaptic potential amplitude after a strong stimulus. This amplitude increase of the postsynaptic potential has to last at least half hour to days. A molecular mechanism involved in both persistent activity and long-term potentiation is the NMDA receptor, which is one of the ionotropic glutamate receptors. Its specific characteristic is that in order to be activated, membrane has to be depolarized so that the magnesium ion blocking its pore goes away.*

Basic mechanisms of LTP induction

At the cellular level, LTP is associated with the induction of gene expression during or shortly after an experience, the *de novo* protein synthesis and the creation of new synaptic connections. There are a variety of signaling pathways that, by inducing these effects and by causing both presynaptic and postsynaptic changes, allow the expression and maintenance of long-term potentiation.

One of the major paths is the following: During normal synaptic transmission of low frequency, glutamate is released from presynaptic terminals, which binds to both the NMDA

and the non-NMDA (quisqualate/kainate - Q/K) receptors located on the dendritic spines. Sodium (Na^+) and potassium (K^+) ions leak through the non-NMDA, but not through the NMDA receptor channels, since magnesium ions (Mg^{2+}) block these channels, at resting membrane potential. The flow of cations through the non-NMDA channels during a high frequency tetanic stimulation depolarizes the postsynaptic membrane and thus NMDA channels unblock. This allows the influx of calcium ions (Ca^{2+}) into the cell and increases calcium (Ca^{2+}) concentration in the cytoplasm, resulting in the activation of calcium-dependent kinases (PKC and CaMKII, which is regulated by the complex of Ca2+/calmodulin), which play a major role in changing synaptic plasticity. When long-term potentiation is achieved, the postsynaptic cell releases a retrograde messenger, which is thought to act on the presynaptic terminal kinases, to cause the continued reinforcement of glutamate release, which results in the maintenance of long-term potentiation (Kandel et al., 2000). Many studies have shown that maintenance of LTP may also be achieved by a signaling pathway that depends on the intracellular increase in the concentration of cAMP, which leads to the pre- and postsynaptic activation of protein kinase A (PKA) and ultimately to the activation of transcription factors, such as CREB inducing *de novo* synthesis of proteins. The importance of the cAMP/PKA signaling pathway appears to be greater in the late phase of LTP (L-LTP), which lasts for several hours *in vitro* and weeks *in vivo*, and holds an important place on the consolidation of spatial memory (Abel et al., 1997).

Dopamine and synaptic plasticity in PFC

In addition to the neural circuits in the region of the hippocampus, the ability to induce long-term synaptic potentiation and depression has been reported in several other brain regions. One of the first reports of LTP expression outside the hippocampus involved

projections of hippocampal neurons on prelimbic cortex *in vivo* (Doyère, Burette, Negro, & Laroche, 1993). Thereafter studies showed that LTP and LTD can also be induced in the networks of neurons in the PFC, and in the afferent pathways projecting to the PFC from the hippocampus, amygdala, thalamus and the sensory cortices (Goto, Yang, & Otani, 2010).

LTP induction in the PFC, as in hippocampus, requires activation of NMDA receptors (T. M. Jay, Burette, & Laroche, 1996), and experimental data support that activation of protein kinase A (PKA) at these synapses results in activation of CREB (Laroche, Davis, & Jay, 2000). Also, it is now widely accepted that in certain brain regions, such as the PFC, the hippocampus and neostriatum, the strength of synaptic transmission is modified by the action of endogenous modulators (neuromodulators), including the monamines dopamine (DA), noradrenaline (NA), serotonin (5-HT) and acetylcholine (ACh) (Goto et al., 2010). The best-studied regulatory pathway of modulation *in vivo* and *in vitro* is the dopamine-mediated. The studies carried out *in vivo* revealed that this pathway involves D1/NMDA synergistic effects which result in the induction of LTP, but also stimulation of D2 receptors of PFC interneurons, which down-regulate the expression of LTP in the limbic-PFC pathway. The stimulation of the D1 receptor, which is coupled to the heterotrimeric Gs protein, activates adenylate cyclase (AC) and thus the intracellular levels of cAMP are increased. Subsequent activation of PKA kinase phosphorylates specific substrates of the cytoplasm, the membrane and the nucleus, including CREB, NMDARs, AMPARs and DARPP-32 protein. The phosphorylation of the NR1 subunit of NMDA receptors is a prerequisite for the functionality of these and hence the activation of Ca^{2+}-dependent mechanisms (Snyder, Fienberg, Huganir, & Greengard, 1998). In the case of AMPARs, phosphorylation allows their placement on the membrane surface and thereby enhances the excitatory glutamatergic synaptic responses (Sun, Zhao, & Wolf, 2005), while the DARPP-32 is converted to a potent inhibitor of phosphatase PP-1 and thus CaMKII phosphorylation is

promoted, which is one of the "enzymes-keys" to induce LTP. On the other hand, increasing the intracellular levels of Ca^{2+}, apart from CaMKII, activates phosphatase RR2V or calcineurin that dephosphorylates the DARPP-32 resulting in the pause of inhibition of PP-1 and inhibition of CaMKII and the CREB. The control of the PP-1 by the DARPP-32, therefore, is a molecular switch of particular importance for regulating signal transduction through dopamine, thereby controlling the dynamic balance between the activity of kinases and phosphatases that determines synaptic strength (Thérèse M. Jay, 2003). The stimulation of D2 receptors, by contrast, has been found to attenuate the LTP induction (without inducing LTD) in hippocampal – PFC neural pathway, likely through the inhibition of the AC/cAMP/PKA signaling pathway. This effect has been considered as the basis for promoting the action of inhibitory interneurons of animals which have reached the stage of adulthood, but not pre-pubertal (Tseng & O 'Donnell, 2007). The mechanism that induces LTD *in vivo* remains unknown.

The experimental results of *in vitro* studies in brain slices, on the other hand, indicate that the mechanisms of synaptic strength change exhibit a remarkable difference in the local neural networks of PFC. Specifically, induction of LTP in this case requires a concomitant stimulation of receptors D1 and D2 (Matsuda, Marzo, & Otani, 2006), while if only one type of receptor (either D1 or D2) stimulated, causes LTD (Otani, Blond, Desce, & Crépel, 1998). The co-activation of D1 and D2 receptors starts another signaling cascade, participating the phospholipase C (PLC), the second messenger diacylglycerol (DAG), which activates protein kinase C (PKC), which in turn phosphorylates kinases MAP, as MEK1/2 and ERK 1/2. This path results in activation of CREB and induces LTP after high frequency tetanic stimulation, provided that there is a constant concentration of extracellular dopamine levels similar to those retained tonically *in vivo*, through the spontaneous firing of dopaminergic neurons (Matsuda et al., 2006).

This observed difference between *in vivo* and *in vitro* studies is partly attributable to stimulation of different synaptic ensembles. The limbic neurons in the intact brain, project mainly on the deeper layers of the PFC (layers V-VI), where greater number of D1 receptors are found. In the local networks of PFC, however, the layers that accept tetanic stimulation are mostly superficial (layers I-II), which consist of interconnecting corticocerebral fibers. In this way, a balance may be maintained in terms of information processing between local networks of the PFC and the paths that connect the PFC with limbic structures. This balance is shifted to one of those two directions, depending on the DA-dependent pathway that is activated (Goto et al., 2010).

Experimental data demonstrate the important role of the modulation of synaptic strength, through dopamine, in various aspects of the functioning of the PFC. For example, stimulation of D1 receptors in normal levels is critical for the uninterrupted performance of the animals in the spatial delayed alternation task (Seamans, Floresco, & Phillips, 1998). Both the inadequate DA or the understimulation of D1 receptors – by the neurotoxic injury of ventral tegmental area (VTA) or by the use of pharmacological D1 antagonists - and the overstimulation of D1 receptors causes significant effects on working memory performance (P S Goldman-Rakic, 1995). The dopaminergic system has also been found to be involved in information processing, in reward-associating learning, in spatial memory and in long-term memory of fear (Thérèse M. Jay, 2003).

1.7. Cortical development

The development of the nervous system is a complex series of dynamic and adaptive processes, such as, for example, changes in the white matter and the establishment of neural connections in the brain, which are predetermined by genetic, guided by epigenetic and

influenced by environmental factors (Bossong & Niesink, 2010). The developmental process starts with the formation of the progenitor cells of the nervous system - about three weeks after conception in humans - continue to occur for a very long time after birth and only completed when the person reaches the fourth decade of life (Kolb et al., 2012). In short, during the embryonic development early nerve cells are generated and then migrate to the appropriate area of the cortex. Then, after birth, they mature, they form synapses by creating neural circuits and developing glia, which produces myelin and other types of supporting cells. In the initial development phase, neurons are overproduced and trillions of neural connections are formed, resulting in gray matter expansion during early childhood. But then these networks are 'pruned' depending on neural activity: Repeated experiences and stimuli leading to neuronal firing of specific networks result in the consolidation of these circuits, and other circuits that are not used are eliminated through the process of "synaptic pruning". On the other hand, the expansion of white matter, which consists mainly of myelinated axons, may provide the structural basis required for complex cognitive functions underlied by extensive networks. Myelination increases the speed of transmission of neural signals, thereby facilitating the synchronized firing of cells belonging to the same network, but separated by a large distance (Salami, Itami, Tsumoto, & Kimura, 2003). Time periods, when this neurons and connections refinement occurs, differ significantly among different cortical areas. There appears to be a caudal-to-rostral gradient with posterior (sensory) regions peaking sooner than more anterior (PFC) ones (Kolb et al., 2012). One of the last areas to complete the maturation process is the PFC (Kolb et al., 2012).

1.7.1. Neuroanatomical changes: Synaptic pruning

During early childhood, postmortem anatomical studies and *in vivo* imaging approaches, revealed significant changes in neuronal and synaptic density (Tsujimoto, 2008). In particular, neurons of the layer III of the PFC decrease in number from 55% to 10% over the average number of adults between the ages 2 to 7 years (P. Huttenlocher, 1979) (Figure 1.9.).

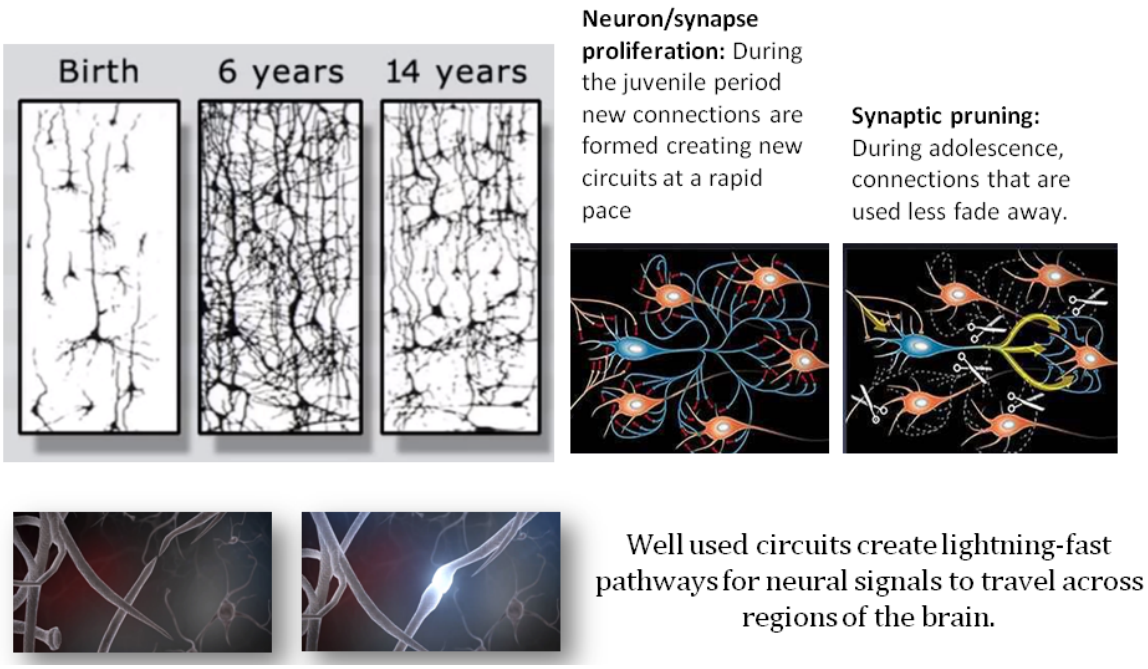


Figure 1.9. *Neuronal development. Experience, during the early stages of development, acts affecting the architecture of the developing brain. During early development neurons and synapses increase, while after a certain period and with the presence of external stimuli and experiences a circuit reorganization occurs in order to enhance the circuits that are been used and the weakening of those not used through a normal procedure called pruning, which is the synapse reduction. Pruning aims to the efficient and targeted function of neuronal circuits.*

The synaptic density reaches its maximum level at the age of 3.5 years and then at about 50% more than the average of an adult. In this age till puberty the number of synapses gradually decreases (P. R. Huttenlocher & Dabholkar, 1997). Developmental changes have

also been observed in cell morphology of PFC, including the dendritic arborization of pyramidal neurons and the significant increase in the volume of white and gray matter (Tsujimoto, 2008). Quantitative analysis of the synapses in the human cortex revealed that the density of synapses reaches its peak in early childhood, remains constant for a time period in specific brain regions, and subsequently decreases at the beginning slowly and then - during puberty - rapidly (P. R. Huttenlocher & Dabholkar, 1997). This decrease in synaptic density is a phenomenon known as synaptic pruning. The reliability of this observation is reinforced by the similar changing pattern of markers of synaptic function, such as in synaptophysin and post-synaptic density protein – 95 (PSD-95), as a function of age (Glantz, Gilmore, Hamer, Lieberman, & Jarskog, 2007).

This synaptic pruning is generally believed to be responsible for the observed reduction in the volume of gray matter of adolescents. Magnetic resonance imaging (MRI) studies in humans verify an inverted U change in the gray matter volume, with an increase in the prepubertal period followed by a reduction completed in late adolescence (Giedd et al., 1999). In contrast, the volume of white matter increases linearly throughout adolescence till the age of 22 years and shows no decrease until the individual reaches the age of 40 years old (Ge et al., 2002). These changes relate to the cortex as a whole, but in the association areas of higher cognitive functions, such as the PFC, they happen later than in all the others (Elston, Oga, & Fujita, 2009). For example, in rats the cytoarchitectonic characteristics of the rat sensorimotor cortex stabilize around 24 days, whereas those of PFC do not stabilize until about day 30 (Gourley, Olevska, Warren, Taylor, & Koleske, 2012; Van Eden & Uylings, 1985). Therefore, the finding that synaptic pruning in the PFC occurs later compared to other sensory cortices is a phenomenon that seems to occur in several animal species, suggesting, possibly, that the 'critical periods' observed during the very

early postnatal period in sensory cortex could occur at later stages during childhood and/or adolescence in the PFC.

The functional significance of synaptic pruning during adolescence could lie in the establishment of the relative balance between excitatory and inhibitory inputs, both at the cellular level and at the level of the neural network (Selemon, 2013). The main argument supporting this view is the specificity of synaptic pruning: preferably excitatory synapses are degenerated and inhibitory synapses are maintained, thus increasing slightly the ratio of inhibitory to excitatory synapses (Bourgeois & Rakic, 1993).

1.7.2. Changes in glutamate and GABA systems during development.

The phenomenon of synaptic pruning suggests that the main neurotransmitter systems in the cortex, the glutamatergic and GABAergic systems also undergo changes. Indeed, besides the structural changes, functional changes in these systems have also been found; especially during the time period that synaptic pruning occurs.

NMDA receptor changes

As mentioned previously, glutamate binds to two different types of ionotropic receptors on postsynaptic neurons: AMPA and NMDA receptors. NMDA receptors are particularly important for several 'key' physiological phenomena, such as persistent activity and LTP. NMDA receptors are tetramers, composed of at least two different types of subunits. Two NR1 subunits are expressed on all NMDA receptors. The other two subunits that comprise the tetramer could be the NR2A, NR2B, NR2C or NR3 subunits, with the first two (NR2A and NR2B) being the most abundant in the cortex. There seems to be a developmental shift in

the expression of these two subunits. During the early developmental stage, in primary sensory cortical areas and the hippocampus, NMDA receptors consist mostly of NR1/NR2B heteromers, but then the expression of NR2A subunit starts and a shift to NR1/NR2A receptors follows. Specifically, NR2B expression is highest at birth whereas NR2A in not expressed until p7 (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994). However, in the PFC, it seems that this shift towards NR2A subunits does not happen, and NR2B subunits are highly expressed even at PD60 in rodents (H. Wang, Stradtman, Wang, & Gao, 2008).

There are several functional differences between NR2A- and NR2B- containing NMDA receptors. The NR2A subunit displays lower affinity compared to the NR2B subunit for the protein kinase CaMKII (calcium/calmodulin-dependent protein kinase II), which is important for inducing LTP. This means that a higher proportion of NR2A/NR2B on mature neurons in these areas leads to increasing the threshold for induction of LTP, while creating more favorable conditions for the induction of LTD. The transition to LTD-receptive state is characteristic of cortical maturation in adolescence (Magueresse, Monyer, & Le Magueresse, 2013; H.-X. Wang & Gao, 2009). In addition, NR2B-containing receptors have slower kinetics of inactivation, hence, allowing more calcium influx during their activation (Ewald & Cline, 2009), further reducing the threshold for LTP induction and facilitating expression of persistent activity. Given the important role of PFC in working memory, the maintenance of high NR2B expression in this brain area is critical. However, the increased calcium influx through the NR2B subunits could render this brain more susceptible to excitotoxicity and apoptosis (Bossong & Niesink, 2010; Hardingham & Bading, 2003). Indeed, during maturation, PFC-mediated functions are the most susceptible ones (Lewis, Volk, & Hashimoto, 2004; Meyer-Lindenberg et al., 2002; Stockman, 2013).

GABA function changes

One of the most important developmental changes taking place during the early postnatal period is the function of the neurotransmitter GABA from having a depolarizing function during early development to having hyperpolarizing function, which constitutes its main function in the mature cerebral cortex.

As mentioned previously, GABA binds and activates two different types of receptors on the postsystnaptic neurons: the ionotropic GABAa receptor and the metabotropic GABAb receptor. GABAa receptors are chloride-permeable pentameric channels composed of an assembly of subunits from eight classes of subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3). The action of GABAa receptor activation on the postsynaptic cells depends on the extracellular and intracellular concentrations of chloride, which determine chloride reversal potential (Ecl). The extra- and intra-cellular chloride concentrations are set to a large degree by the expression of chloride cotransporters, NKCC1 and KCC2, which are, respectively, involved in chloride uptake and extrusion. Before reaching maturation, KCC2 expression in most neurons is delayed compared to NKCC1 expression. As a result, chloride accumulation in the cytoplasm and an E_{GABA} greater than the resting membrane potential occur (Rivera, Voipio, & Kaila, 2005). As a consequence, GABA has a depolarizing action in most immature neurons, at least in acute brain slices. Around the end of the first postnatal week KCC2 expression in the forebrain begins in the primary sensory cortices and switches the action of GABA to a hyperpolarizing one (Y. Ben-Ari, Khalilov, Kahle, & Cherubini, 2012; Yehezkel Ben-Ari, Cherubini, Corradetti, & Gaiarsa, 1989; Bregestovski & Bernard, 2012; Magueresse et al., 2013).

The depolarizing action of GABAa, seem to have significant and multifaceted roles in the early stages of cortical maturation. First the depolarizing GABA promotes the migration of GABAergic and glutamatergic neurons in the correct location in the cortex. Furthermore,

the onset of expression of KCC2, which is responsible for the conversion of GABA, acts as stop signal of the migration process, thus determining the final position of interneurons (Bortone & Polleux, 2009). Secondly, the excitatory GABA coordinates the formation of glutamatergic synapses in more active neurons, contributing to the balance between excitatory and inhibitory activity in the developing cortex (Magueresse et al., 2013; D. D. Wang & Kriegstein, 2011). Additionally, excitatory GABA neurons seem to participate to the wiring of immature cortical circuits among them, inducing the creation of giant depolarizing potentials (GDPs) in certain regions of the cortex (Allène et al., 2008). The GDPs of the hippocampus have been shown to enhance the immature glutamatergic synapses and are vital for the smooth transition to the next developmental stages (Mohajerani, Sivakumaran, Zacchi, Aguilera, & Cherubini, 2007).

When the contribution of GABA excitation in cortical development is completed, the GABA shift to a hyperpolarizing transmitter allows GABAergic interneurons to perform their equally important functions as inhibitory interneurons. Among these functions are included the formation of neural receptive fields, regulation of basic neuronal properties, such as the increase rate of firing frequency in response to increasing stimulation (gain) and the creation of rhythmical and synchronized oscillations of the membrane potential of neuronal populations - especially those belong to the beta (β) (14 - 30 Hz) and gamma (γ) (30 - 80 Hz) frequency range, which constitute a pacemaker of neural networks (Atallah & Scanziani, 2009; Carvalho & Buonomano, 2009; Isaacson & Scanziani, 2011).

Other changes in GABAergic intereneurons

Currently, there are no data with regards to changes of the GABAergic switch in PFC development. Based on 'synaptic pruning', a hypothesis can be made that similar changes in

GABA function will occur at a later stage in development in PFC. However, other changes are known to occur in the GABAergic system of interneurons that relate to functional properties of different interneurons subtypes as well as to the number of different subtype populations.

In the PFC, changes in the functional properties of different interneuron subtypes vary. There seems to be an increase in the proportion of FS/PV interneurons that have no NMDA currents in adulthood, compared to the adolescence period, while the RS/CB or IR/CR interneurons do not seem to undergo similar changes. In the FS/PV cells with NMDA currents, the NMDA/AMPA ratio dramatically decreases during the adolescent period but returns to juvenile levels in adults (H.-X. Wang & Gao, 2009).

Furthermore, a recent study has shown that the levels of PV protein increase during adolescence, while the levels of the CR protein decrease, and no changes are observed in the levels of CB protein. The analysis in this study suggested that the increase in PV and decrease in the CR signal are attributable to the increase or decrease, respectively, of dendritic arborization and do not reflect changes in interneuron numbers. At the synaptic level, electrophysiological data revealed that a developmental facilitation of spontaneous glutamatergic synaptic inputs onto PV-positive/fast-spiking interneurons parallels the increase in prefrontal PV signal during the periadolescent transition. In contrast, no age-dependent changes in glutamatergic transmission were observed in PV-negative/non fast-spiking interneurons. Together, these findings emphasize that GABAergic inhibitory interneurons in the PFC go through a dynamic, cell type-specific remodeling during adolescence and provide a developmental framework for understanding alterations in GABAergic circuits that occur in psychiatric disorders (Caballero, Flores-Barrera, Cass, & Tseng, 2013).

In addition to the above, changes in neuronal excitability of PV/FS interneurons has been observed. Specifically, maturation of PV/FS interneurons is characterized by a decrease in the input resistance and hyperpolarized resting membrane potential, and an increase in firing frequency during spike trains (Goldberg et al., 2011). Furthermore, GABAa receptor kinetics are increased (i.e. they become faster, due to an increase in the expression of α1 subunit) (Doischer et al., 2008; Magueresse et al., 2013). These functional changes are hypothesized to underlie the emergence of gamma-rhythm oscillations (Leinekugel et al., 2002), which have been involved in mediating important cognitive functions, such as the coordination of movements, the focus of attention and working memory (Uhlhaas, Haenschel, Nikolić, & Singer, 2008).

Changes in other transmitter systems

The activity of PFC pyramidal neurons is modulated by numerous neuromodulatory systems, including dopaminergic, serotonergic, noradrenergic and cholinergic afferent neurons. The developmental changes that characterize these systems during adolescence lead to various changes of behavioral and emotional responses, while they may be responsible for some of the peculiarities of adolescent cerebral function, such as increased sensitivity to psychoactive substances (Steketee & Frost, 2003). The most notable are the changes taking place at the dopaminergic system.

Dopamine (DA) is a catecholaminergic hormone/neurotransmitter that plays a prominent role in regulating the processes of learning, motor control, reward, motives and other brain functions. DA baseline concentrations and the density of dopaminergic afferent fibers in the PFC peak early in puberty and are then reduced. On the other hand, DA rate of synthesis and recycling in subcortical regions, wherein PFC projects to, are at lower levels in early

adolescence compared to adulthood. The same pattern of fluctuation is exhibited in the levels of the dopamine D1-type receptors, one of the DA receptors that mediate the modulation of synaptic plasticity in the PFC. D1-type receptors are positively coupled to the cAMP/PKA - dependent signaling pathway, and are involved in maintaining long-term plasticity mechanisms (Huang, Simpson, Kellendonk, & Kandel, 2004).

The effects of dopamine in PFC function displays an inverted-U function, meaning that both low or excessive activation of the D1 receptor results in impaired PFC function (P S Goldman-Rakic, 1995). Therefore, the greater levels of DA and D1-type receptor expression in the PFC during the adolescent period may be partly responsible for the delay in maturation of PFC functions, which is not completed until the end of adolescence when the levels of DA decrease to more 'optimal' levels (Thérèse M. Jay, 2003). Dopaminergic pathways are also involved in mediating the effects of stressful stimuli in PFC functions, which could explain the enhanced sensitivity to stress that characterizes adolescence (Selemon, 2013). Moreover, agonists of dopamine D2 receptors begin to have a strong stimulatory effect on inhibitory interneurons only during and after the adolescence, therefore, enhancing inhibitory function upon DA release. In total, during the maturation of the brain the role of inhibition in regulating the activity of pyramidal neurons is strengthened. This fact is likely essential for the proper functioning of cortical neurons, while deviations from this relative balance and the right coordination between excitatory and inhibitory activity have been associated with many psychological disorders (O'Donnell, 2011).

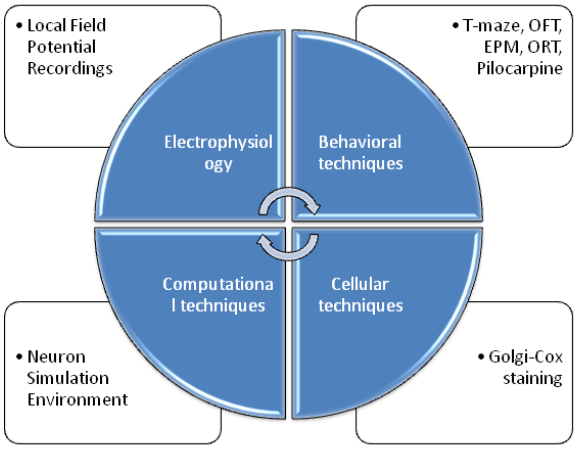
In summary, adolescence is the period during which the brain completes its maturation process and it is characterized by dramatic changes, especially in the frontal cortical areas that control more complex cognitive functions. From childhood to adolescence, the developmental process shows a shift from the increase of the volume of the brain, and the

increased number of neurons in the formation of more effective neural pathways. The main changes that result in this direction is the refinement of the inhibitory system, the improvement of the coordination between neural networks and the increasing frequency of synchronized oscillations of the membrane potential, the maturation of neurotransmitter systems and synaptic pruning, i.e. either degeneration of synapses or synaptic activation, according to their relative activation. The overall result of all these changes, in terms of the PFC, is the most targeted function and thus the improved performance of cognitive abilities related to PFC, as well as the effective control of behavior. The vast extent of these changes, however, makes adolescents vulnerable to developmental disorders that may be caused by external factors, such as stress, alcohol or psychotropic drugs. This fact is considered to be the most likely cause, for which the adolescence period coincides with the onset of many psychiatric disorders, such as schizophrenia.

Critical periods are described as specific intervals within which the development processes taking place are driven by interacting genetic and environmental factors, leading to the establishment of functional characteristics (Crews, He, & Hodge, 2007). Based on all of the above, therefore, adolescence can justifiably be regarded as a critical period for brain maturation.

1.1. Aim of the doctoral dissertation

The general aim of this doctoral dissertation is the detailed study of the age-dependent changes taking place in PFC in normal conditions and in a condition that induce pathology; decreased GABAergic inhibition in the cortex. We used a multidisciplinary experimental approach including cellular, physiological, behavioral and computational techniques in order to focus on the unique properties of PFC circuitry development and pathology. Our specific aims were:



- To examine age-dependent changes in dendritic structure of pyramidal neurons of the PFC and the effects of decreased inhibition in it, using the Golgi-cox staining technique.
- To investigate developmental changes in basal synaptic transmission, short-term and long-term synaptic plasticity and how it is modulated in conditions of decreased inhibition, using local field potential recordings.
- To study PFC-dependent and PFC-independent cognitive functions, such as working and recency memory in different age groups of mice.
- To uncover interneuron cell-type specific roles on the properties of persistent activity in a PFC microcircuit model network.

MATERIALS AND METHODS

2.0. Materials and Methods

2.1. Animals and Housing

Mice used for this thesis were born in the animal house of the Biology Department, University of Crete. Some mice were also born at the IMBB animal house and transferred to the Animal House at the Biology Department for the experiments. Animals were weaned at day 21 and housed socially in groups (3-4 per cage) with standard mouse chow and water *ad libitum*. They maintained a 12h light/dark cycle (light on at 7:00 am) with controlled temperature (21+/-1^{o} Celsius).

For the results presented in section 4, only male C57/BL6 mice were used. For the results present in section 5, the following genotypes were used for analysis: Rac1(fl/fl);Nkx2.1(+/Cre) (referred to as Rac1 cKO mice) and Rac1(+/fl);Nkx2.1(+/Cre) as Rac1 Het mice, used as control, unless otherwise noted. The generation and characterization of Rac1 cKO mice (Rac1fl/fl;Nkx2.1+/Cre) were previously described (Vidaki et al., 2012). Specifically, animals carrying a floxed allele of Rac1 (Rac1 fl/fl) (the fourth and fifth exon of the Rac1 gene are flanked with loxP sites, (Walmsley et al., 2003)) were crossed to Nkx2.1Tg(Cre) mice (Nkx2.1 transgenic Cre, (Fogarty et al., 2007)), in order to generate the Rac1fl/fl;Nkx2.1Tg(Cre) genotype, and to Lhx6Tg(Cre) mice (Fogarty et al., 2007). The ROSA26fl-STOP-fl-YFP allele was also inserted as an independent marker, to allow visualization of the Rac1 mutant (and control) neurons, via yellow fluorescent protein (YFP) expression (Srinivas et al., 2001).

All procedures were performed according to the European Union ethical standards and the IMBB and University of Crete ethical rules. In addition, all studies were consistent with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

2.2. Behavioral tests

2.2.1. Pilocarpine induced epileptic seizures

Pilocarpine injections: Adult mice (~30gr) were injected with different doses of pilocarpine to induce epileptic seizures. Mice were injected with methylscopolamine (1 mg/kg, i.p.) 30 minutes before pilocarpine injection to minimize the peripheral effects of pilocarpine (Baez, Eskridge, & Schein, 1976; Turski et al., 1983). Animals were then injected with pilocarpine hydrochloride dissolved in saline at different doses (i.p.). Control animals also received methylscopolamine but were injected with saline instead of pilocarpine. Animals were observed for 2 hours after injection with pilocarpine and different parameters were assessed, such as: stage of seizures according to Racine's scale (Lüttjohann, Fabene, & van Luijtelaar, 2009), seizure duration, time latency to seizure, and seizure frequency. The following criteria were used for scoring the intensity of seizures: Stage 1: Behavioral arrest, Stage 2: repeating movements of the face and mouth, Stage 3: Repeating movements of the head and neck, Stage 4: Neurotic tics in the front legs and sitting position, Stage 5: Clonic and/or tonic- clonic movements in either the front or back legs. Mice were usually in the rearing position leaning against the wall, Stage 6: Intense tonic-clonic movements in both front and back legs, losing balance while jumping.

2.2.2. Open Field Test

The open field test (OFT) was used to assess locomotor activity, basic exploratory behavior, which is a sampling behavior essential to stimulus exposure on spatial, contextual, and object learning tasks and anxiety-like behavior. Mice were placed in the center of an open field arena, which was a clear plexiglass chamber (45x45x45 cm). The flooring was

constructed of the same white plexiglass, and the top of the open field apparatus was open to the environment and divided into ten pre-defined areas including the center area, four corner areas, four wall areas, and one peripheral zone, using white tape. Mice were left in the chamber for 15 minutes to explore the novel environment. After 15 minutes, the animals were removed from the open field arena and returned to their home cage. The plexiglass chamber was cleaned with ethanol and baby wipes after each individual test to avoid influence from odor deposited by the previous mouse, on the next mouse (Crawley, 1999). After all mice were tested, they were returned to their home room in the animal house. Exploration by the animals was video-taped and different behavioral parameters were scored using the JWatcher software (Blumstein, Evans, & Daniel, 2006) or the ANY-MAZE software. The 15 min open-field test was divided into three blocks of 5 min. The parameters assessed included

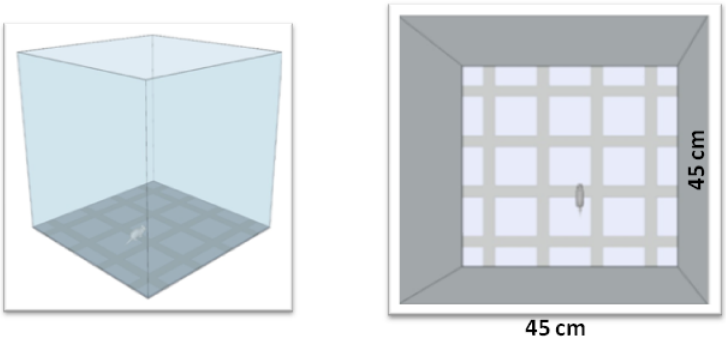


Figure 2.1. *Illustration of the open field apparatus. (Designed using Google SketchUp by X. Konstantoudaki (2010))*

1. The total number of entries and time spent in each pre-defined area of the arena. Specifically, the arena was divided into 4 different areas: a) edges, b) walls, c) periphery and d) center

2. The number of times/time spent moving,

3. The number of times/time spent in vertical/rearing posture (standing on hind legs, with or without contact with the sides of the arena) and the number of rearing episodes,

4. The number of times/time spent grooming (using paws or tongue to clean/scratch body) and the number of grooming episodes,

5. The total entries within and between each of the ten regions of the enclosure.

As thigmotaxis ratio we calculated the ratio of time spent in the extremes of the arena divided by the whole time spent in the field. The paradigm is based on the idea that mice will naturally prefer to be near a protective wall rather than exposed to a danger out in the open. Parameters as time/entries into the center of the arena, and activity within the first 5 minutes in the open field reveal some aspects of emotionality, including anxiety.

2.2.3. Elevated Plus Maze

The elevated plus maze (EPM) tests another type of anxiety, the anxiety induced by open spaces, as well as height related anxiety (Hogg, 1996).

The EPM apparatus consists of two well-lit open arms, from which the mice can see the cliff, and two closed arms. These arms emanate from a central platform that is square to form a plus shape. Our custom-made apparatus was constructed by dark metal and was an elevated (40 cm) maze consisting of four arms (30cm x 5cm each), and a center area (intersection), making the shape of a plus sign (+). The two closed arms were surrounded by 16 cm walls (closed arms), whereas the other two arms projected out of the center without walls (open arms). The apparatus was placed on a surface 65cm above the floor before the experiments.

The entire apparatus was cleaned with 70% ethanol between individual test sessions to avoid influence from odor deposited by the previous mouse, on the next mouse (Crawley, 1999). Each mouse was placed in the center box of the elevated plus maze apparatus (intersection) with its head facing the closed arm opposite to the experimenter and allowed to explore the maze freely. After 5 minutes the mouse was removed from the EPM apparatus and placed in its home cage.

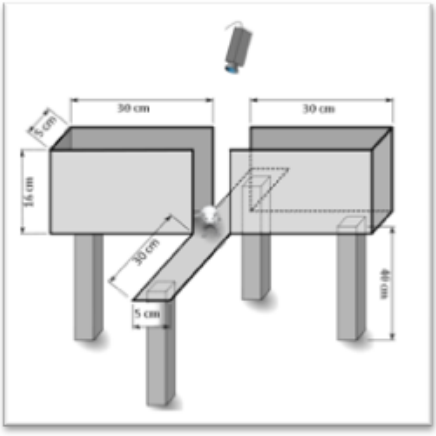


Figure 2.2. *The elevated plus maze apparatus. Elevated plus maze test used for the assessment of anxiety related behavior in mice.*

All testing sessions were recorded by a video camera that was fixed above the EPM apparatus and scored at a later time. The total time spent by the animal in the open or closed arms and the number of open or closed arms entries was assessed (Pellow, Chopin, File, & Briley, 1985). An open arm entry was defined as having all four paws into the arm of the EPM. The paradigm is based on natural aversion of mice for open and elevated areas, as well as on their natural spontaneous exploratory behavior in novel environments. Thus, normally, mice would prefer the closed arms; however, they would explore the open arms, too (Crawley, 1999). As such, confinement to the closed arms is associated with more anxiety-related behaviors (Anwar et al., 2011). The behavior of the mice was video tracked and analyzed using the JWatcher software (Blumstein et al., 2006).

2.2.4. Delayed Alternation on the T-maze

The T-maze is a T-shaped apparatus with elevated walls, in which the animal is placed in the start arm and then allowed to choose one of the two goal arms.

The delayed alternation task is a classic task used for the study of working memory (Shoji, Hagihara, Takao, Hattori, & Miyakawa, 2012). During this task, the animal needs to remember (keep in mind) the previous choice (short term memory). The simplest form of this task is the spontaneous alternation task, which is based entirely on the natural tendency of rodents to alternate their choice arm. The instinctive behavior of this alternation reflects the mood of the animal to explore the environment to find new sources of food, water, shelter or partner sites (Richman, Dember, & Kim, 1986). In these experiments any kind of reward is omitted and it is simply observed whether the mouse alternately selects where to go. The forced choice alternation task in which a reward is placed in the arms alternately provides more reliable results. If the mouse is forced (e.g. by means of a removable door) to stay for a short time in the start arm, and thus delay the arm selection, then we refer to the delayed alternation task (Deacon & Rawlins, 2006), which is the assay we used for studying the function of the working memory of two different age groups of mice.

Pre-experiment Preparation: About a week before the start of the main experiment, mice were handled on a daily basis, until they became well habituated and relaxed with the researcher and recovered their exploratory mood. This was revealed by the decreased defecation and urination observed. Then, they were subjected to food deprivation until mice reached the 85-90% of their initial weight, but also to familiarization with the food reward (pieces of chocolate cereal), to habituate the animals to its test and eliminate hyponeophagia. Mice were fed individually to ensure that one animal didn't monopolize the food with standard pellet chow. Food deprivation continued until the end of the

experiment. Mice were weighted daily to ensure that they did 't lose weight rapidly and avoid shock.

Habituation: Since T-maze is a learning and memory task, the stress of the mice, caused by the new environment should be eliminated, so mice could use their full learning and memorizing capabilities. For this reason it was necessary to apply the process of habituation to the new environment (T-maze apparatus), at least for two days. On the first day, the entire home cage group of animals that were previously food restricted was put in the maze in which food reward was dispersed, three times for ten minutes with an inter-session interval of ten minutes. The entire apparatus was cleaned with 70% ethanol between sessions. On the second day the same procedure followed, but for each individual mouse separately.

Main experimental procedure (training): Goal of this part is to 'teach' the animal that it will be rewarded if it alternates its choice between the 2 arms and not if it repeatedly chooses the same arm. Prior to commencing the procedure, the criterion point should be determined. Generally the criterion point for each trial is for the whole animal, including the tail tip, to be on the insert/goal arm. According to the criterion used in the experiments, the animal must have passed the criterion line, with his entire body, including the tail. This criterion helps to avoid errors, as mice are sometimes hesitant and change the direction of their movement.

Every day, each one of the mice in the experiment was subjected to 3 sessions of 10 trials per day (+1, the trial 0). As a trial we consider a path that makes the mouse from the original arm until the end of the choice arm. At trial 0, food is placed into both choice arms and the mouse is allowed to choose freely. In the following 10 trials, food should always be on the opposite arm from the one that was previously chosen by the mouse. Thus, when the animal enters the arm containing the food, the selection is marked as correct, and if it enters

the one that contains no reward (which happens when the mouse selects repeating the same arm) is marked as error. If the mouse does not make any arm selection within 30 seconds, we note 'omission error'. The first session ends after all the mice of the cage spent ten trials. Before entering the next animal, the maze is cleaned with ethanol for disinfection and removal of any olfactory residues, which may influence the choice of the mouse. Then follows the second and after the third session. Among the sessions a 15-minute is mediated during which again we clean the T-maze apparatus with ethanol.

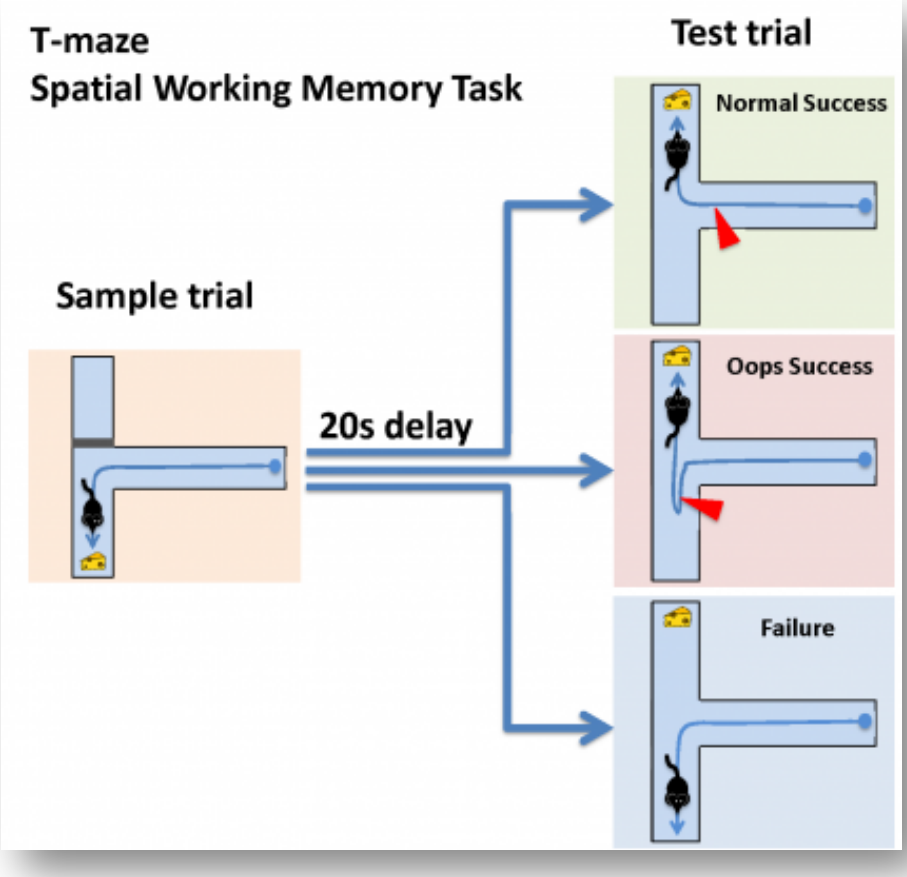


Figure 2.3. *Delayed Alternation using the T-maze in order to assess working memory function of mice. During this test, mice are trained to alternate in the two arms of the maze in order to get the reward. When succeed a delay is entered between alternations, which is increased until reaching the 20 seconds.*

Introduction of delays (testing): After mice are trained to the criterion (ie at least during the 2 of the 3 sessions of the same day, or during session 3 of one day and session 1 of the next day, to achieve 70% or more correct choices), we further tested the mice in the delayed alternation task by inserting 5, 10, 15, and 20 seconds delays consequently starting with the 5 seconds delay. This is achieved by means of a removable door that prevents the mouse to move from the beginning of the start arm. Whenever a mouse completed the predefined criterion with the given delay, longer delays were added. The experiment ends when all mice complete the predefined criterion with a 25 seconds delay and its average duration is 7-8 days.

2.2.5. Object Recognition Memory tests

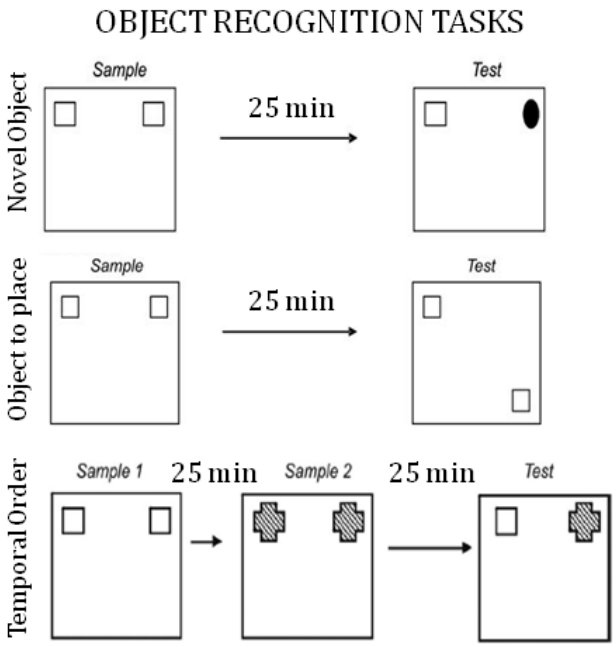


Figure 2.4. *Object recognition tasks. Cognitive functions were also examined in mice; distinct aspects of recognition memory were tested, using three types of object recognition tests: the novel object recognition during which the novel object has to be explored, the object-to-place*

during which the displaced object has to be explored and the temporal order object recognition during which the less recent object has to be explored

Object exploration was defined as the time mice spent exploring the objects by physical contact. Two different objects without ethological significance to the mice were used. In pilot works it was shown that these objects are distinguishable by the mice and that they had no per se preference for any of the objects. All animals were habituated to the open-field devoid of any objects for 10 min for three days before the beginning of the behavioral testing.

Novel object recognition

The novel object recognition memory task involved a sample trial followed by a test trial after an inter trial interval of 25 min. In the sample trial two copies of a novel object were placed in the open-field and the animals were allowed to explore the arena for 5 min. In the test trial two objects were presented in the same position – one object used in the sample trial (familiar object) and the other was a novel object. Object recognition was defined as more time engaged in exploring the novel object as compared to the familiar object (A Ennaceur, Cavoy, Costa, & Delacour, 1989). After each trial, the objects and the open field were cleaned with a 70% ethanol solution in order to remove odor cues. The behavior in this task is dominated by perirhinal cortex.

Object to place recognition

The object to place recognition memory task was carried out 3-4 days later. The animals were allowed to explore two copies of an object in the open field during the sample trial of 5

min. After an inter-trial interval of 25 min they received a 5 min lasting test trial identical to the sample trial except that two copies of the original object were present, one in the same position it had occupied in the sample trial (spatial stationary) and one in a novel position (spatial displaced). Object–place recognition memory was defined as an increase in time engaged in exploring the spatially displaced object in favor of the spatially stationary one (Dere, Huston, & De Souza Silva, 2005; A. Ennaceur & Meliani, 1992). The behavior in this task is dominated by hippocampus.

Object recognition for temporal order

The animals were exposed to the object recognition for temporal order task 3-4 days later. The object recognition task for temporal order comprised two sample trials and one test trial with an inter-trial interval of 25 min between each trial. In each sample trial the animals were allowed to explore two copies of the same object for 5 min. Different objects were used for the two sample trials. During the test trial one object from sample trial 1 (old familiar) and one object from sample trial 2 (recent familiar) were presented and the animals were allowed to explore the open-field for 5 min. The positions of objects within the two sample trials and the test trial were identical. Intact object recognition memory for temporal order was defined if the animals spent more time exploring the old familiar object compared with the recent familiar object (Mitchell & Laiacona, 1998). The behavior in this task is dominated by PFC.

2.3. Electrophysiological measurements

During our electrophysiological study we recorded local field potentials within layer 2 of PFC and barrel cortex in order to measure basal synaptic transmission, paired pulse ratios, and long term potentiation of the synapses (only in PFC).

A local field potential corresponds to the summed postsynaptic activity of neurons in the general vicinity of the extracellular recording electrode; it is a measure of primarily synchronized synaptic signals, subthreshold membrane oscillations and spike afterpotentials, while in the received signal there is a limited participation of action potentials.

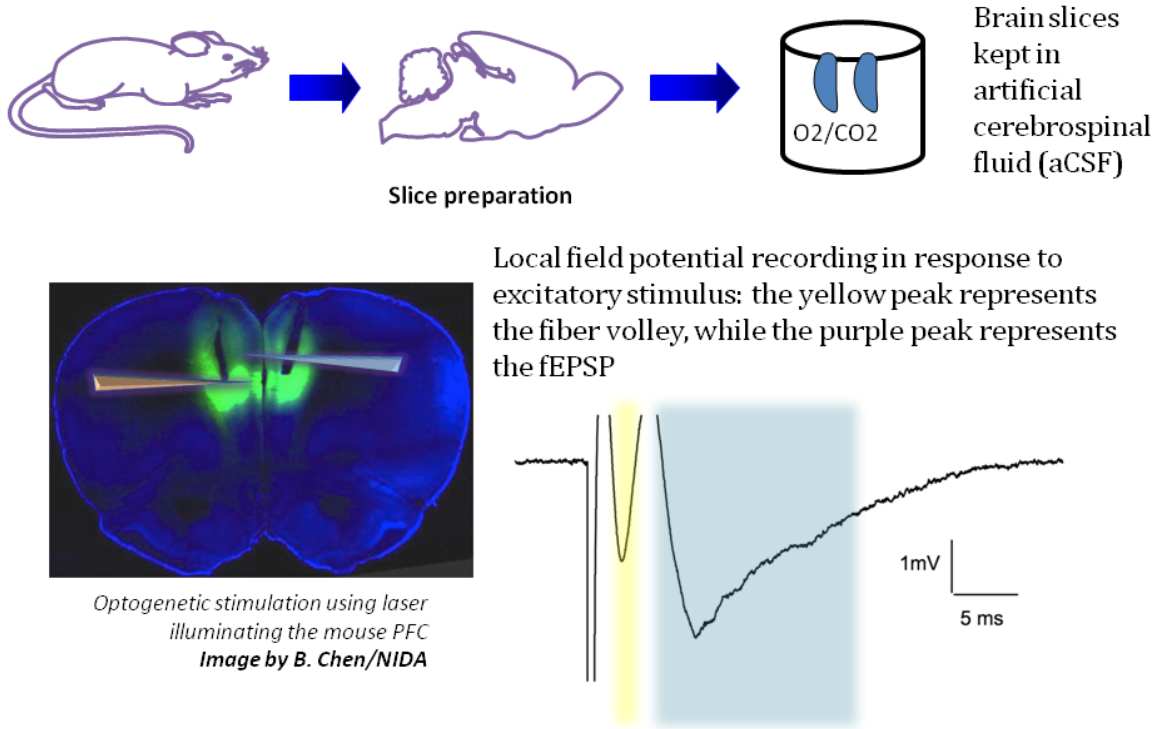


Figure 2.5. *Electrophysiological procedures. Extracellular local field potentials were measured for the study of basic synaptic properties as well as the short-term and long-term synaptic plasticity of mice. The preparation for the recordings includes: quick extraction of brain tissue, brain slicing and slice maintenance in artificial cerebrospinal fluid under continuous oxygenation. Electrodes are positioned within layer II of the prelimbic area of PFC and a representative LFP recording in response to current stimulation is composed by two*

peaks; the first peak (yellow) represents the fiber volley, while the second peak (blue) represents the fEPSP.

Electrophysiological experiments were performed using *in vitro* slice preparation. Mice were decapitated under halothane anesthesia. The brain was removed immediately and placed in ice cold, oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 1 $MgCl_2$ and 10 glucose (pH=7.4, 315 mOsm/l). The brain was blocked and glued onto the stage of a vibratome (Leica, VT1000S). 400µm thick brain slices containing either the PFC or the barrel cortex were taken and were transferred to a submerged chamber, which continuously superfused oxygenated (95% O2/5% CO2) aCSF containing (mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose (pH=7.4, 315mOsm/l) in room temperature. The slices were allowed to equilibrate for at least an hour in this chamber before experiments began. Slices were then transferred to a submerged recording chamber, which continuously superfused oxygenated (95% O_2/5% CO_2) aCSF containing (in mM): 125 NaCl, 3.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose (pH=7.4, 315mOsm/l) in room temperature.

Extracellular recording glass pipettes were pulled from borosilicate glass using a glass horizontal puller (P-97, Sutter Instruments). The pipettes were filled with NaCl (2M) were placed in layer II of prelimbic or barrel cortex (Paxinos and Watson). fEPSP responses were evoked with a stimulating electrode (Harvard apparatus) placed on layer II of prelimbic or barrel cortex, about 300µm away from the recording electrode.

Responses were amplified with the extracellular headstage of the BVC-700A amplifier (Dagan, Inc), digitized using the ITC-18 board (Instrutech, Inc) on a PC using custom-made procedures in IgorPro (Wavemetrics, Inc). The electrical stimulus consisted of a single square waveform of 100 msec duration given at intensities of 0.05-0.6 mA (depending on the brain region) generated by a stimulator equipped with stimulus isolation unit (WPI).

2.3.1. Data acquisition and analysis

Data were acquired and analyzed using custom-written procedures in IgorPro software (Wavemetrics, Inc). The field peak values of the fEPSP were measured from the minimum value of the synaptic response (4-5 ms following stimulation) compared to the baseline value prior to stimulation. The initial slope of the fEPSP was measured by fitting a straight line to the first millisecond of the fEPSP immediately following the fiber volley. Both parameters were monitored in real-time in every experiment. A stimulus-response curve was then determined using stimulation intensities between 0.05-0.3 mA. For this, 2 traces were acquired at each stimulation intensity and averaged. This is referred to as synaptic input/output relationship (stimulus intensity giving a corresponding growing EPSP). It was used to check for basal synaptic transmission to examine whether the differences observed between the different groups of mice were due to differences in basic synaptic properties. This response range was also used to determine the stimulus intensity that was applied during baseline recordings for each slice. Spontaneous activity was monitored in-between different stimulation intensities.

Baseline stimulation parameters were selected to evoke a response of 1mV. Paired pulse facilitation (PPF), a transient form of plasticity induced by presenting two closely spaced pulses of equal intensities, was also examined to check for facilitation in synaptic transmission for the different groups of mice. The paired-pulse protocol consisted of two pulses at baseline intensity separated by 100, 50 and/or 20 msec. It is assumed that if two pulses are paired, the first facilitates the second because of the residual calcium ions from the first. Data points were presented as the ratio of response to the second pulse to that of the response to the first pulse. For the long-term potentiation experiments, baseline stability was tested by recordings signals for 20 minutes using the same stimulus value, then three 1 sec tetanic stimuli (100Hz) with an inter-stimulus interval of 20 sec were

applied and finally responses were acquired for at least 50 min post-tetanus. Synaptic responses were normalized by dividing all fpeaks by the average of the 10 fEPSP fpeaks 10 min pre-tetanus and LTP was quantified as the normalized fEPSP response at the 50 min post-tetanus.

For the epileptiform activity analysis, the first derivative of the voltage response was taken and the logarithm of its histogram was plotted (Dyhrfjeld-Johnsen, Berdichevsky, Swiercz, Sabolek, & Staley, 2010). In order to study the exact synaptic mechanisms causing the differences observed between different mice groups we used: different concentrations of the NMDA receptor antagonist AP5 ((2R)-amino-5-phosphonopentanoic acid) which is known to inhibit the glutamate binding site of NMDA receptors and bicuculline which is a competitive antagonist of GABAA receptors.

2.4. Golgi-Cox staining

Mice were decapitated following spinal dislocation and the brains were isolated and placed in small glass bottles filled with Golgi-Cox solution (5% Potassium Dichromate in dH_2O, 5% Mercuric Chloride (sublimate) in dH_2O, 5% Solution of Potassium Chromate in dH_2O), which was prepared at least 5 days earlier and kept in the dark. (Golgi-Cox Staining Protocol for Neurons and Processes, Contributed by Tracey Wheeler). The tissues remained in the solution for 10 days at room temperature and on day 11 were transferred in 30% sucrose solution in dH_2O, where maintained at 4^o C until the day of the slicing. Coronal slices containing the PFC were obtained using vibrating blade microtome (VT1000S, Leica). A total of 6-8 sections 150mm thick, were obtained from each animal, starting from level 30 (The mouse Brain Coronal Atlas, 2004-2008 Allen Inst. For Brain Science). The sections

were initially floated in a solution of 6% sucrose in dH_2O and then placed in gelatinized slides, were covered parafilm and kept in a humidity chamber for one or two days.

One or two days after slicing, the process of staining followed. Briefly, after removing the parafilm, the slides were immersed for one minute in dH_2O and then incubated in 100% Ammonium Hydroxide for 15 minutes in a dark chamber and immediately after in a Kodak Fix solution for an additional of 15 minutes, with an intermediate 1 minute wash in dH_2O. Subsequently, the sections were dehydrated with a series of increasing concentrations of ethanol, cleaned in xylene for 5 minutes and covered with a solution of permount and coverslips. The slides were observed at least after one month.

Analysis

The cells analyzed were pyramidal neurons of prelimbic cortex within layers II and V. 2-5 cells were selected for each animal. No differences were observed between the two cerebral hemispheres. The neurons were initially identified with a light microscope (Leica DMLB) at 20x magnification and if they met the necessary conditions, were selected to be analyzed at higher magnification (100x). Specifically, the cells should be (1) strongly impregnated along their dendrites, and (2) in relative isolation from adjacent cells to ensure correct analysis and also carry tuft at some point of their apical dendrites. The length of the dendrites leading to the tuft, the number of secondary dendrites before the tuft and the number after that, and the total number of spines in all secondary and tertiary dendrites, were studied. Micro-photos were taken of each dendritic compartment of the cells, starting from the basal dendrites surrounding the neural body until the distal dendrites, using a camera (Leica DC 300F) connected to the microscope. For each micro-photo 6 consecutive confocal layers (distance 1-2 microns), were used, so as to create a stack of consecutive

images. The stacks were attached to generate the full image of each cell. The length of the apical dendrite, the number of the dendrites and spines of each section in each focal level were measured. Only fully impregnated projections with clear contact with the dendrites, were counted as spines (Horner & Arbuthnott, 1991). All measurements and image analysis and editing was done using the Adobe Photoshop CC, and the ImageJ softwares.

2.5. Picrotoxin-treatment experiments

Picrotoxin acts as a noncompetitive antagonist for the GABAa receptor chloride channels. It is therefore a channel blocker rather than a receptor antagonist.

Juvenile Rac1 Het mice (pd20) (3 female and 4 male mice) were given daily injections of picrotoxin (1 mg/ml – non epileptic dose) or PBS intraperitoneally in their home cages for 10 days. When they reached adulthood (>2 months old) they were tested in the open field testing and the elevated plus maze. Subsequently female mice were handled for object recognition memory tests. While brains were taken from male picrotoxin treated mice in order to measure local field potentials, within layer 2 of PFC, and within layer 2 of barrel cortex.

2.6. Statistical Analysis

Data were analyzed with repeated measures and factorial ANOVAs in Excel (version 2007, Microsoft) and SPSS (version 21, IBM). Post hoc and pairwise comparisons after the ANOVAs were conducted using Tukey's and LSD tests respectively. For single comparisons, Student's t-test was used. Data were initially examined for normality with Shapiro-Wilk's test and homogeneity of variances was assessed with Levene's test. If these assumptions

were violated values were logarithmically transformed and re-examined. In cases where normality and homoscedasticity were not achieved, non-parametric equivalent tests were used (e.g. Kruskal-Wallis). In repeated measures ANOVAs sphericity was also tested with Mauchly's test and when the assumption of sphericity was violated the Greenhouse-Geisser correction was applied. Error bars represent the standard error of mean. The level of statistical significance was set at 0.05.

CHAPTER I

ALTERATIONS IN THE PHYSIOLOGY OF PREFRONTAL CORTEX UNDERLIE THE DEVELOPMENT OF EMOTIONAL AND COGNITIVE PROCESSES ACROSS LIFESPAN

3.0. Chapter I: Alterations in the physiology of prefrontal cortex underlie the development of emotional and cognitive processes across lifespan

3.1. Introduction

As mentioned in the General Introduction, there is some evidence that the development of the PFC occurs at later postnatal stages compared to primary sensory cortices. The aim of this first chapter was to study the development of the PFC with regards to dendritic morphology, emergence of synaptic plasticity and behavioral function.

3.1.1. Development of PFC-dependent functions

In humans, adolescence is a critical period during behavioral development characterized by heightened susceptibility to anxiety disorders for some individuals (Crick & Zahn-Waxler, 2003; Paus, Keshavan, & Giedd, 2008; Petersen, 1988), potentially due to the developmental changes in gonadal and adrenal hormone production that accompany this stage of life (Hayward & Sanborn, 2002; C. M. McCormick & Mathews, 2007; Reardon, Leen-Feldner, & Hayward, 2009; L. P. P. Spear, 2000; L. Spear, 2000). Epidemiological data indicate that human adolescents are more involved in reckless behaviors than adults (Berndt, 1979) and are motivated to seek novel sensations (Arnett, 1992). Low levels of harm avoidance and anxiety are reported in association with risky activities such as hazardous driving, unprotected sex and substance use and abuse (Arnett, 1992; Maggs, Almeida, & Galambos, 1995; Thomas Ashby Wills, 1994).

The different cognitive functions mediated by the PFC are thought to be compartmentalized in distinct functional modules (Miyake et al., 2000), similar to all brain systems that are responsible for higher cognitive functions (Alan Baddeley, 1998). It is thought that in early stages of the development discrete cognitive functions are processed together in a diffuse

neural circuitry. Upon maturation, however, the PFC starts to be functionally organized in independent networks, specialized to perform a particular function. It is not necessary that these circuits are separated from each other topologically, but their neurons can be interwoven in the same brain region (Tsujimoto, 2008). In conclusion, the complex morphology and more focused neural activity engaged by PFC cells at the early childhood allow for the formation of appropriate neural networks capable of supporting specialized functional compartmentalization. This results in the improved performance of complex cognitive functions mediated by the PFC (Figure 3.0.).

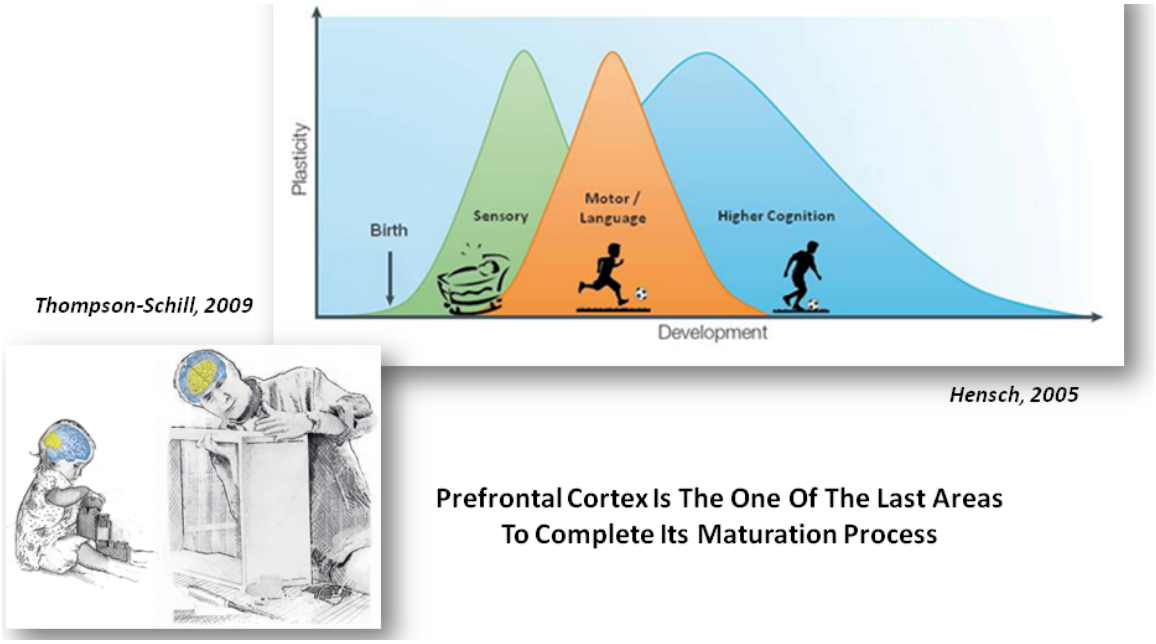


Figure 3.0. *Differential development of higher order brain regions and corresponding functions. Sensory functions mature early during development, while higher order functions (as those depending on the normal function of PFC) change during development, improve radically the first years, continue to improve during adolescence and their improvement does not stop until early adulthood, resulting in their delayed maturation. Probably this delayed maturation of executive functions is related with delayed maturation of PFC, which starts to be used in the whole of its capabilities only during adulthood.*

Adolescence is also a vulnerable period, as it coincides with the occurrence of several major psychological disorders, such as schizophrenia, depression, eating disorders and emotional

disorders (Sturman & Moghaddam, 2011). Behaviorally, rodents show behavioral changes during the adolescent period much like humans. For example, adolescent rodents display increased social interaction (Primus & Kellogg, 1989) and risk taking behavior (Laviola, Macrì, Morley-Fletcher, & Adriani, 2003; Stansfield & Kirstein, 2006). They usually show more playful and affiliative behavior (Laviola, Adriani, Terranova, & Gerra, 1999) as well as spontaneous novelty seeking (Adriani, Chiarotti, & Laviola, 1998; Palanza, Morley-Fletcher, & Laviola, 2001).

The adolescent period in rats has been defined as starting around p28 (L. P. P. Spear, 2000), coinciding with rising circulating gonadal hormone levels in both sexes (Gabriel, Roncancio, & Ruiz, 1992). Around p35–p40, females exhibit vaginal opening and irregular ovarian cycling, while testosterone levels rise gradually in males (Gabriel et al., 1992). During late adolescence (p46–p59) (Tirelli, Laviola, & Adriani, 2003), females exhibit regular cycles, and males are capable of producing fertile sperm (Gabriel et al., 1992; Tentler et al., 1997). By p60, rats are usually considered to be fully sexually mature. However, the male testes continue to develop into young adulthood (Knorr, Vanha-Perittula, & Lipsett, 1970), and testosterone levels peak around p70 before falling to adult levels (Zanato, Martins, Anselmo-Franci, Petenusci, & Lamano-Carvalho, 1994). During the adolescent period, the hypothalamic–pituitary–adrenal (HPA) axis undergoes maturational changes in rodents. For instance, juvenile rats exhibit a hyper-responsiveness of the HPA axis and a prolonged production of corticosterone following stress compared to adults, and adolescence is likely to be a period during which the developing stress axis is highly sensitive to perturbations (Susan L. Andersen, 2003). Given that developmental changes in both gonadal and adrenal hormone production have been hypothesized to underlie the increased susceptibility of adolescents to anxiety disorders in human beings (Hayward & Sanborn, 2002), a fuller

understanding of the ontogeny of anxiety-like behavior in male and female rodents will provide the basis for future investigations of the link between hormones and anxiety.

Relatively few studies have compared the behavior of adolescent and adult rodents on traditional behavioral tests of anxiety-like responses, and such studies have produced conflicting results. In the open field, adolescent rodents have been reported to exhibit either higher levels (Arakawa, 2005; Bronstein, 1972; Philpot & Wecker, 2008; Stansfield & Kirstein, 2006) or lower levels (Candland & Campbell, 1962) of locomotor exploration than adults, and, in the EPM, adolescents have been reported to spend either more time (Doremus-Fitzwater, Varlinskaya, & Spear, 2009) or less time (Doremus, Brunell, Varlinskaya, & Spear, 2003) on the open arms than adults. Other studies have failed to find age differences in performance on these pieces of apparatus (Arakawa, 2005; Hefner & Holmes, 2007). In general, age differences in anxiety-like behavior have not been the main focus of these studies, methodological differences between studies are likely to have contributed to the conflicting results, and most studies have used only one behavioral test (Doremus, Varlinskaya, & Spear, 2004; Doremus-Fitzwater et al., 2009; Slawecki, 2005).

Our aim in this section was to study the cellular, physiological and behavioral changes that relate to PFC in an age-dependent manner, focusing primarily on the adolescence and early adulthood period.

3.2. Results

In order to study the age-dependent changes on PFC function, we conducted morphological, physiological and behavioral experiments. Specifically, we analyzed the dendritic morphology of PFC pyramidal neurons using the Golgi-Cox staining procedure, we measured basal synaptic transmission and emergence of synaptic plasticity with field

recordings, and we studied mice in PFC-dependent behavioral tasks, such as the delayed alternation in the T-maze, the temporal order object recognition task and the intersession habituation to a novel environment in compared to non PFC-dependent tasks, such as the novel object, the object-to-place recognition tasks and the intra-session habituation to a novel environment. Only male C57J/B6 mice where used for all experiments.

3.2.1. Dendritic morphology of PFC pyramidal neurons changes as a function of age

We began our study on the age-dependent changes on the PFC by analyzing the dendritic morphology of PFC pyramidal neurons in an effort to fill our knowledge gaps, primarily during the adolescent period. To this end, using the Golgi-Cox staining procedure, we analyzed the dendritic morphology of PFC pyramidal neurons in mice of 5 different age groups: PD20, PD35, PD40, PD50, PD60 and PD90. We will refer to PD20 and PD35 age groups as 'juvenile', to PD40, PD50 age groups as 'adolescents', to PD60 as 'young adults' and to PD90 as 'adults'. The quantitative analysis of Golgi-cox stained pyramidal neurons of PFC revealed a significant effect of age in total spine density (One-way ANOVA, $p<0.05$). In particular there is a decrease in the total spine density in 'adolescent' mice that lies between two peaks of increased dendritic spine density in the 'juvenile' and 'young adult' mice (Fig. 3.1A). Total spine density during PD40 is significantly smaller compared to PD20, PD35 and PD60 (Tukey post hoc test, $p<0.05$). PD20 total spine density tends to differ with PD50 and PD60 (Tukey post hoc test, $p<0.1$). PD35 differs significantly with PD50 and PD90 (Tukey post hoc test, $p<0.05$). PD50 differs significantly with PD60 (Tukey post hoc test, $p<0.05$). PD60 differs with all age groups significantly except PD35 (Tukey post hoc test, $p<0.05$) (Fig. 3.1A). Furthermore, we performed analysis of the different types of spines, that is, the stubby, thin and mushroom type spines. This more detailed analysis of the different type of

spines measures in each age group revealed that during adolescence there is a significant effect of age (One-way ANOVA, $p<0.05$). Specifically, thin spine density of the age group PD20 differs significantly with PD40, PD50, PD60 and PD90. PD35 differs significantly with PD40, PD50 and PD90. PD40 differs significantly with PD60. PD60 differs significantly with all age groups except from PD35. PD90 and PD50 are also significantly different in their thin spine density (all significances: One-Way ANOVA, Tukey post hoc test, $p<0.05$). Similarly, mushroom spine density is also influenced by age (One-way ANOVA, $p<0.05$). PD60 pyramidal neurons mushroom spine density differs significantly from all age groups of mice except from PD35 pyramidal neurons. PD35 differs significantly with PD40, PD50 and PD90. PD20 also differs significantly with all groups except PD35 (all significances: One-Way ANOVA, Tukey post hoc test, $p<0.05$). Stubby spine density doesn't differ significantly across development (One-Way ANOVA, $p>0.1$) (Fig. 3.1B). Furthermore, the percentage analysis of different types of spines in each age group revealed that the relative percentage of stubby immature spines decreases during the 'juvenile' and 'early adult' period, while it is increased during the 'adolescent' and 'adult' periods. On the contrary, the percentage of mushroom and thin mature spines has the exact opposite trajectory as it is relatively increased during the 'juvenile' and 'early adult' period, and decreased during the 'adolescent' and 'adult' period (Fig. 3.1D). As a conclusion, we find that there is a biphasic change in the spine density of PFC pyramidal neurons with a peak during the 'juvenile' and 'early adult' period and a trough during the 'adolescent' and 'adult' period.

We also assessed additional morphological characteristics. The number of basal dendrites doesn't change throughout development (Kruskal-Wallis Test, $p>0.1$) (Fig. 3.2A). The number of secondary dendrites seems to be affected by age although not significantly (Kruskal-Wallis Test, $p<0.1$) (Fig. 3.2B). There are no significant changes for the apical dendritic length (Fig. 3.2C), the apical dendritic diameter (Fig. 3.2D), the total secondary

dendritic length (Fig. 3.2E) and the average length of secondary dendrites (Fig. 3.2F) (One-way ANOVA, $p>0.1$). Nevertheless, there is a significant effect of age on secondary dendritic diameter (One-way ANOVE, $p<0.05$). Particularly, PD40 pyramidal neurons have significantly decreased dendritic diameter than PD50, while PD50 and PD60 differ significantly with PD90 (Tukey post hoc test, $p<0.05$) (Fig. 3.2G).

We also calculated how the average dendritic volume of pyramidal neurons changes with age and whether it is correlated with the dendritic spine density. We found that during the two peaks of increased synaptic density (i.e. PD35, PD60), the dendritic volume is larger (Fig. 3.3). The opposite happens during adolescence (PD40, PD50) where the dendritic volume is relatively decreased (Fig. 3.3A-B). Therefore, the increased dendritic spine density corresponds correlated with increased dendritic volume of pyramidal neurons.

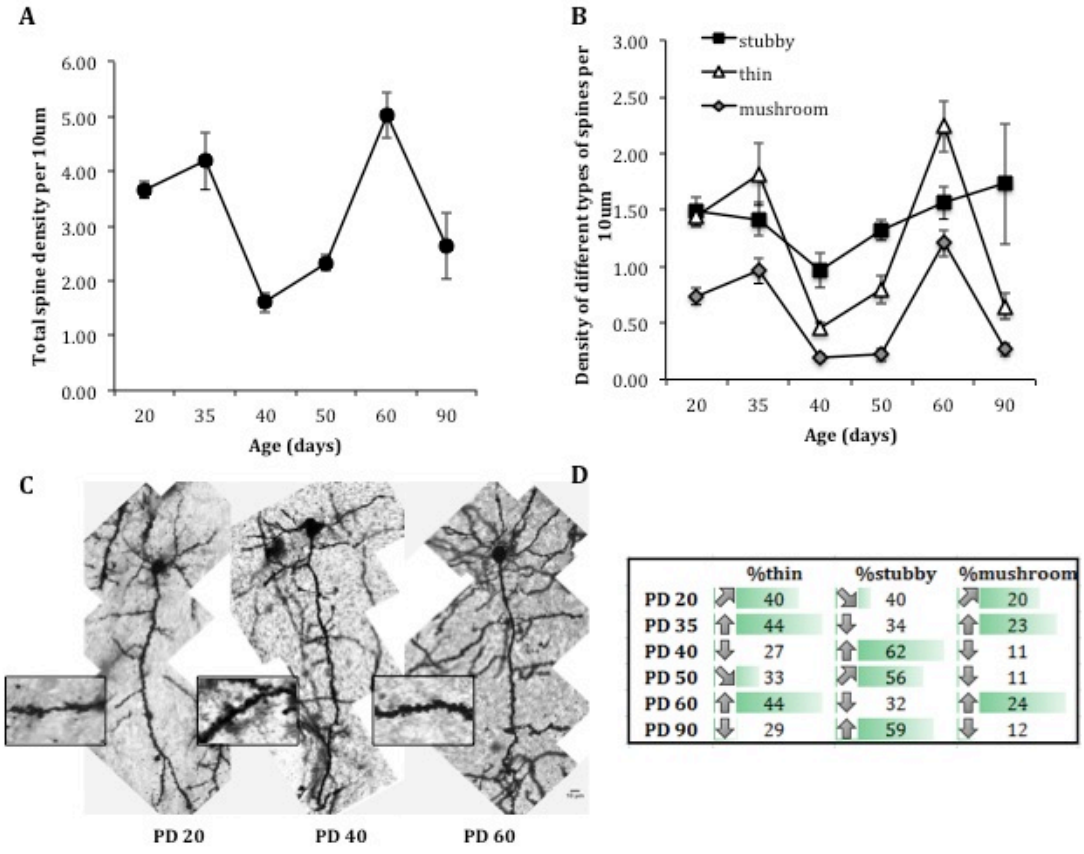


Figure 3.1. Age-dependent alterations in dendritic spine density of pyramidal neurons of the PFC.

***A**. Plot presenting the age-dependent alterations of total spine dendritic density of pyramidal neurons of the PFC. Total spine density during PD40 is significantly smaller compared to PD20, PD35 and PD60 (Tukey post hoc test, p<0.05). PD20 total spine density tends to differ with PD50 and PD60 (Tukey post hoc test, p<0.1). PD35 differs significantly with PD50 and PD90 (Tukey post hoc test, p<0.05). PD50 differs significantly with PD60 (Tukey post hoc test, p<0.05). PD60 differs with all age groups significantly except PD35 (Tukey post hoc test, p<0.05). **B**. Plot presenting the age-dependent alterations of the dendritic density of each type of spine in pyramidal neurons of the PFC. Thin spine density of the age group PD20 differs significantly with PD40, PD50, PD60 and PD90. PD35 differs significantly with PD40, PD50 and PD90. PD40 differs significantly with PD60. PD60 differs significantly with all age groups except from PD35. PD90 and PD50 are also significantly different in their thin spine density (all significances: One-Way ANOVA, Tukey post hoc test, p<0.05). Similarly, mushroom spine density is also influenced by age (One-way ANOVA, p<0.05). PD60 pyramidal neurons mushroom spine density differs significantly from all age groups of mice except from PD35 pyramidal neurons. PD35 differs significantly with PD40, PD50 and PD90. PD20 also differs significantly with all groups except PD35 (all significances: One-Way ANOVA, Tukey post hoc test, p<0.05). Stubby spine density doesn't differ significantly across development (One-Way ANOVA , p>0.1). **C**. Representative stacked images of pyramidal neurons of PFC taken by Golgi-cox stained slices of PD20, PD40 and PD60 mice. Secondary dendritic segments in magnitude. **D.** Table presenting the relative percentages of the different spine type in each age group tested. The relative percentage of stubby immature spines decreases during the high plasticity periods, PD35 and PD60, while it is increased low plasticity periods (PD40, PD50 and PD90). In contrast, the percentage of mushroom and thin mature spines has the exact opposite trajectory as it is relatively increased during PD20, PD35 and PD60, and decreased during PD40, PD50 and PD90.*

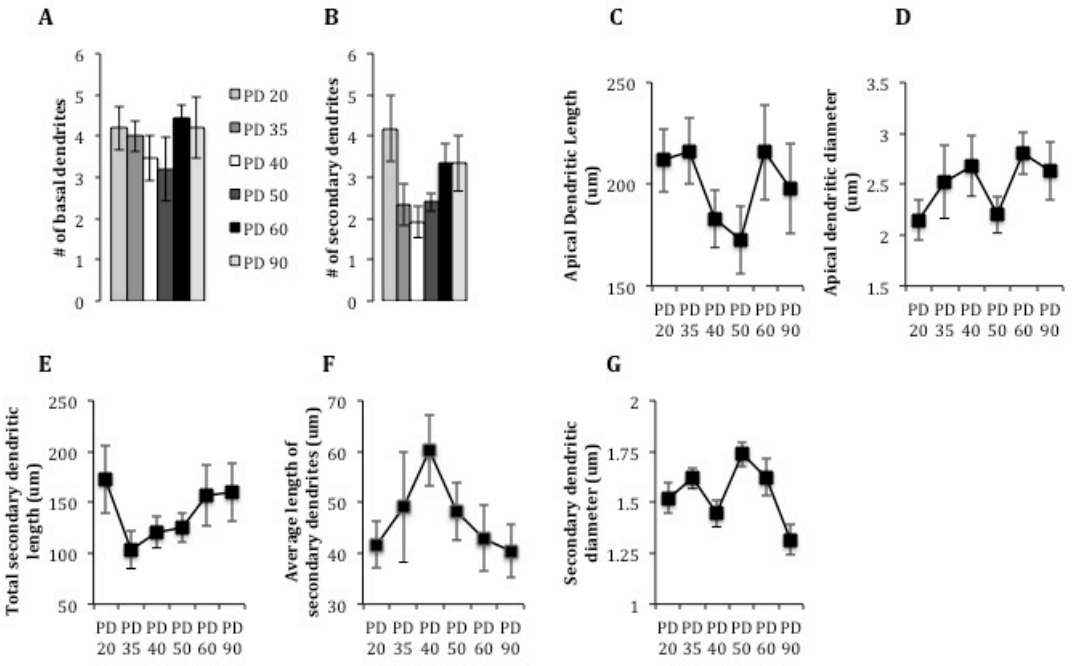


Figure 3.2. Age-dependent changes in the dendritic morphology of pyramidal neurons of the PFC

A. *The number of basal dendrites doesn't change throughout development (Kruskal-Wallis Test, p>0.1)* ***B.*** *The number of secondary dendrites seems to be affected by age although not significantly (Kruskal-Wallis Test, p<0.1)* ***C.*** *There are no significant changes for the apical dendritic length (One-way ANOVA, p>0.1).* ***D.*** *There are no significant changes for the apical dendritic diameter (One-way ANOVA, p>0.1).* ***E.*** *There are no significant changes to the the total secondary dendritic length (One-way ANOVA, p>0.1).* ***F.*** *There are no significant changes to the average length of secondary dendrites (One-way ANOVA, p>0.1).* ***G.*** *There is a significant effect of age on secondary dendritic diameter (One-way ANOVE, p<0.05). Particularly, PD40 pyramidal neurons have significantly decreased dendritic diameter than PD50, while PD50 and PD60 differ significantly with PD90 (Tukey post hoc test, p<0.05).*

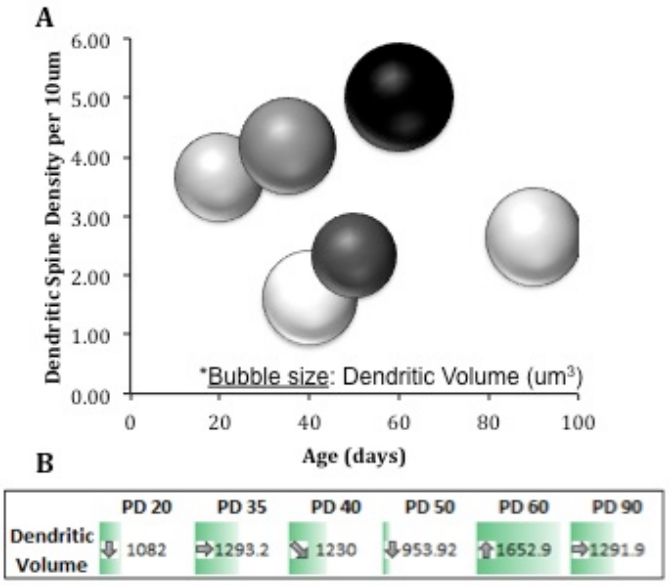


Figure 3.3. Age-dependent changes in dendritic volume as a function of age.
A. *Plot showing the effect of dendritic volume to the spine density as a function of age. The increase of dendritic spine density corresponds to increased dendritic volume of pyramidal neurons. The opposite happens during adolescence (PD40, PD50) where the dendritic volume is relatively decreased (bubble size represent the size of the dendritic volume)* ***B.*** *Dendritic volume changes as a function of age.*

3.2.2. Effect of age on LTP induction

Numerous studies have associated the dynamic structural changes of spines with the probability for induction of LTP. Although this relationship between spines and LTP is quite established on hippocampus (De Roo, Klauser, Garcia, Poglia, & Muller, 2008), this is not the

case for PFC. LTP in the PFC is not extensively studied, so the mechanisms underlying its induction are not very clear and in many cases they implicate other neurotransmitter systems (Goto et al., 2010). We performed an age dependent study of LTP induction following tetanic stimulation in four age groups of mice, namely the PD35, PD40, PD60 and PD120, in order to determine whether the emergence of LTP changes with the animal age as well as to identify a possible association between the spine densities with LTP induction in PFC. Furthermore, in an effort to compare the LTP in the PFC to other primary sensory brain regions, we also performed the same LTP induction protocol in barrel cortex (BC), in the same age groups of mice. Evoked fEPSPs were recorded in layer II of either PFC or BC. LTP was induced following tetanic stimulation (100 Hz) in both brain regions.

In PFC, tetanic stimulation induced LTP that lasts at least 30min after tetanus, for all age groups of mice, except the PD40 (adolescent) group. Time has a significant effect on post-tetanic fEPSPs (Repeated Measures ANOVA, $p<0.05$). PD40 and PD60 groups, corresponding to the groups with lower and higher LTP respectively, tend to differ (Tukey post hoc test, $p<0.1$). In PD60 group (young adults), LTP of 40% is induced, while in PD40 group (adolescents) no LTP is induced. Furthermore, in the PD35 (juvenile mice) and PD120 (adult) groups induce LTP of 35-40% post-tetanus (Fig 3.4A). In sum, during adolescence (PD 40) there is a decrease in LTP induction, while two distinct periods of increased synaptic plasticity are observed one on PD 35 and the other on PD 60 (young adulthood) (Fig. 3.4A). These results correlate well with the decreased synaptic density during 'adolescence' and increased 'synaptic density' during the 'juvenile' and 'early adult' period. More experiments are required to better understand how the LTP correlates with the synaptic density during the adult period.

In barrel cortex a different pattern of plasticity is followed across postnatal development. It seems that there is no period of decreased plasticity, as in all groups tested LTP of at least

50% is induced. Time has a significant effect on post-tetanic fEPSPs (Repeated Measures ANOVA, $p<0.05$). Furthermore, there is a significant interaction between "time" and "age" on LTP induction (Repeated Measures ANOVA, $p<0.05$), while the effect of "age" alone is statistically not significant on LTP induction (Repeated Measures ANOVA, $p>0.1$). In post-hoc comparisons, the PD60 group tends to differ from the PD40 group (Tukey post hoc test, $p<0.1$), and differs significantly from the PD35 group (Tukey post hoc test, $p<0.05$) (Fig. 3.4B).

In order to compare directly the developmental trajectory of LTP induction between the two brain regions, we plotted the average post-tetanic normalized fEPSP of each age group for both areas. The factor "brain region" has a significant effect on average post-tetanic fEPSPs (2-way ANOVA, $p<0.05$). PD40 and PD60 differ significantly between PFC and BC (Tukey post hoc test, $p<0.05$). We observe that while in PFC there are two peaks of increased plasticity with a peak of decreased plasticity lying between, in BC there is only one peak of increased plasticity, while there is not a peak of decreased plasticity among the age groups tested, indicating that such a group should precede the age of 35 postnatal days (Fig. 3.4C). These results are in line with the hypothesis of delayed maturation of PFC compared to primary sensory areas, as BC.

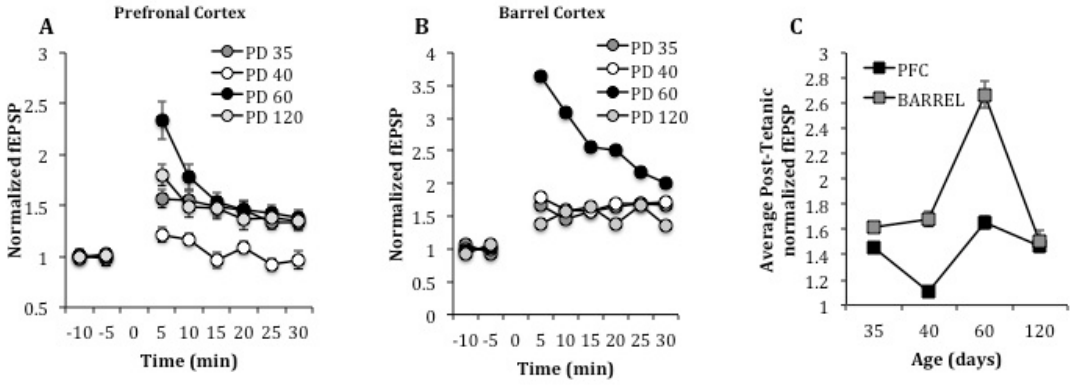


Figure 3.4. Age-dependent changes in LTP induction within PFC vs. barrel cortex
A. *Plot showing the normalized fEPSPs recorded within layer II of PFC five minutes before the tetanic stimulation and thirty minutes after the tetanic stimulation in averaged 5min time*

bins for four age groups of mice. Time has a significant effect on post-tetanic fEPSPs (repeated Measures ANOVA, p<0.05). PD40 and PD60 groups, corresponding to the groups with lower and higher LTP respectively, tend to differ (Tukey post hoc test, p<0.1). In PD60 group (adults), LTP of 40% is induced, while in PD40 group (adolescents) no LTP is induced. PD35 (juvenile mice) and PD120 groups induce LTP of 35-40% post-tetanus. ***B.*** *Plot showing the normalized fEPSPs recorded within layer II of barrel cortex five minutes before the tetanic stimulation and thirty minutes after the tetanic stimulation in averaged 5min time bins for four age groups of mice. In all groups tested LTP of at least 50% is induced. Time has a significant effect on post-tetanic fEPSPs (repeated Measures ANOVA, p<0.05). PD40 and PD60 groups, tend to differ (Tukey post hoc test, p<0.1), PD 60 and PD35 groups differ significantly (Tukey post hoc test, p<0.05).* ***C.*** *Plot showing the average post-tetanic normalized fEPSP of each age group for both areas. PD40 and PD60 differ significantly (Tukey post hoc test, p<0.05). In PFC there are two peaks of increased plasticity with a peak of decreased plasticity lying between, in barrel cortex there is only one peak of increased plasticity, while there is not a peak of decreased activity among the age groups tested.*

3.2.3. Basal synaptic transmission age-dependent alterations

We also measured the basal synaptic transmission in brain slices from the different age-groups of mice. In PFC, we find that there is a significant linear effect of stimuli in fEPSP responses (repeated measures ANOVA, $p<0.05$), while there is a significant interaction between "stimulation intensity" and "age" in fEPSP responses (repeated measures ANOVA, $p<0.05$). Furthermore, fEPSP responses of PD40 and PD35 are significantly increased compared to PD120 in response to 0.1 mA stimulation (One-way ANOVA, $p<0.05$) and fEPSP responses of PD40 are significantly increased compared to PD120 in response to 0.2 mA stimulation (One-way ANOVA, $p<0.05$) (Fig. 3.5A). In general, the largest fEPSP responses are observed in the PD40 group, while the smallest fEPSP responses are observed in the PD120 group. In BC, we find that there is a significant linear effect of stimuli in fEPSP responses (repeated measures ANOVA, $p<0.05$), while "age" doesn't have an effect in fEPSP responses (repeated measures ANOVA, $p<0.05$) (Fig. 3.5B). In order to compare directly the developmental trajectory of basal synaptic transmission between the two brain regions, we plotted the average fEPSP responses to a 0.15 mA stimulus of each age group for both areas.

The factor "brain region" has a significant effect on fEPSP responses (2-way ANOVA, $p<0.05$), while the effect of "age" alone is statistically not significant on fEPSP responses (2-way ANOVA, $p<0.1$). However, in PFC, "age" has a significant effect (One-way ANOVA, $p<0.05$), as PD40 tends to differ with PD60 (Tukey post hoc test, $p<0.1$), PD40 differs significantly with PD120 (Tukey post hoc test, $p<0.05$) and PD35 tends to differ with PD120 (Tukey post hoc test, $p<0.1$). In BC there are not significant effects of any factor on fEPSPs (One-way ANOVA, $p>0.1$) (Fig. 3.5C). Therefore, in our experiments, basal synaptic transmission is increased in the PFC compared to BC, while there seems to be age-dependent changes in basal synaptic transmission in the PFC, there are no such differences in BC.

We also studied short-term plasticity in the PFC and BC at the different age groups, using the paired-pulse stimulation protocol, which provides an indication of transmitter release properties. We applied paired-pulses of increasing frequency within layer II of PFC and BC, aiming to uncover possible age-dependent alterations in short-term synaptic plasticity. There is a significant interaction of "frequency" and "age" in paired-pulse ratios of PFC (repeated measures ANOVA, $p<0.05$). In PD120 there is a significant effect of "frequency" on paired-pulse ratios of PFC (One-way ANOVA, $p<0.05$), with paired-pulse ratios in response to 20Hz being significantly increased than those in response to 20Hz frequency (Tukey post hoc, $p<0.05$) (Fig. 3.5D). In Barrel Cortex we do not detect any significant effect of "age" or "frequency" on paired pulse ratios (repeated measures ANOVA, $p>0.1$) (Fig. 3.5F).

In order to compare directly the developmental trajectory of short-term synaptic plasticity between the two brain regions, we plotted the paired-pulse ratios in response to an average paired-pulse stimulation of 20Hz of each age group for both areas. The factor "brain region" has a significant effect on paired-pulse ratios (2-way ANOVA, $p<0.05$), while there is

a significant difference between the two brain regions in all age groups tested, except PD120. In PFC, there is a significant effect of "age" (One-way ANOVA, $p<0.05$), with the PD120 group being significantly different compared to all other age groups tested (Tukey post hoc test, $p<0.05$) (Fig. 3.5G)

In sum, the developmental trajectory of paired-pulse responses on PFC presents alterations during different developmental stages. Specifically, during adolescence (PD40), paired-pulse potentiation is diminished compared to the other developmental stages, while in BC, short-term plasticity is stable across development.

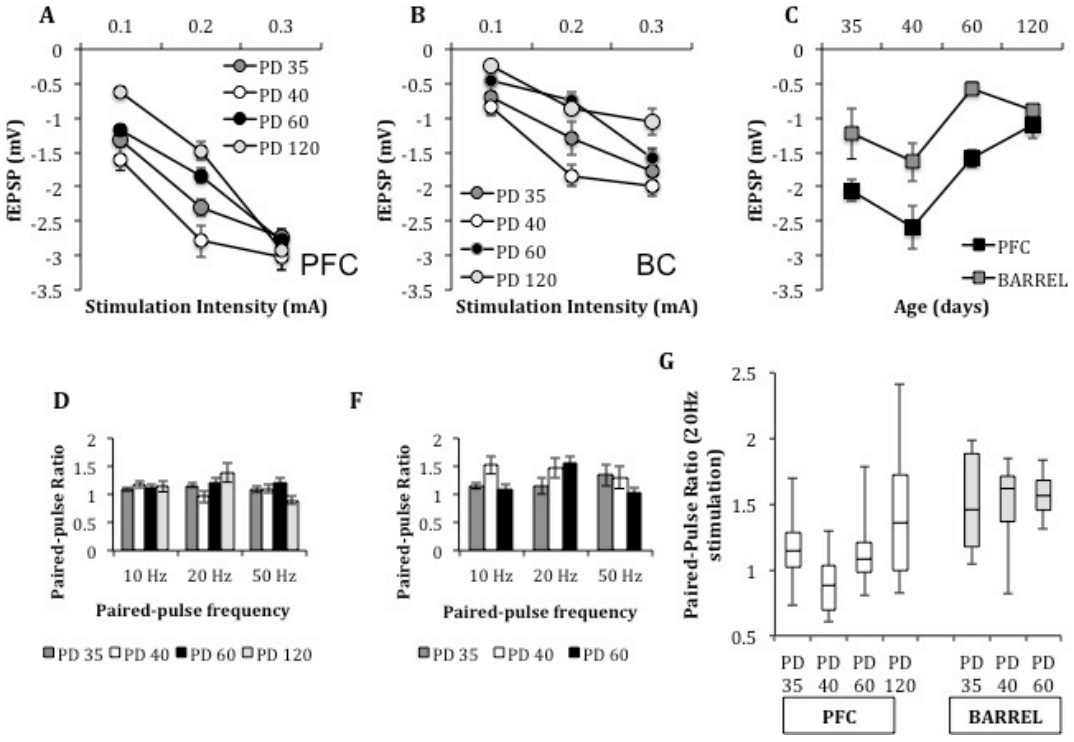


Figure 3.5. Age-dependent changes in basal physiological properties of PFC vs. Barrel Cortex

A. *Plot of fEPSP responses of PFC in response to increasing stimulation intensities in different age group of mice. fEPSP responses of PD40 and PD35 are significantly increased compared to PD120 in response to 0.1 mA stimulation (one-way ANOVA, Tukey post hoc test, $p<0.05$) and fEPSP responses of PD40 are significantly increased compared to PD120 in response to 0.2 mA stimulation (one-way ANOVA, Tukey post hoc test, $p<0.05$).* ***B.*** *Plot of fEPSP responses of barrel cortex in response to increasing stimulation intensities in different age groups of mice. There is a significant linear effect of stimuli in fEPSP responses (repeated measures ANOVA, $p<0.05$),*

while "age" doesn't have an effect in fEPSP responses (repeated measures ANOVA, p<0.05). ***C.*** *Plot of the developmental trajectories of basal synaptic transmission of PFC and Barrel cortex (fEPSP responses to a 0.15 mA stimulus). PD40 tends to differ with PD60 (Tukey post hoc test, p<0.1), PD40 differs significantly with PD120 (Tukey post hoc test, p<0.05) and PD35 tends to differ with PD120 (Tukey post hoc test, p<0.1). In Barrel Cortex there are not significant effects of any factor on fEPSPs (One-way ANOVA, p>0.1). fEPSP responses in adolescent mice (PD 40) are increased compared to adult (PD 60) mice.* ***D.*** *Plot of paired-pulse ratios in response to increasing frequency paired-pulse stimulations in PFC of different age groups of mice. There is a significant interaction of "frequency" and "age" in paired-pulse ratios of PFC (repeated measures ANOVA, p<0.05). There is no significant effect of "frequency" in PD35, PD40 and PD60 slices (one-way ANOVA, p>0.1). In PD120 there is a significant effect of "frequency" on paired-pulse ratios of PFC (One-way ANOVA, p<0.05), with paired-pulse ratios in response to 20Hz paired-pulse stimulation, being significantly increased than those in response to 20Hz frequency paired-pulse stimulation (Tukey post hoc test, p<0.05)* ***F.*** *Plot of paired-pulse ratios in response to increasing frequency paired-pulse stimulations in barrel cortex of different age groups of mice. Not significant effect of "age" or "frequency" on paired pulse ratios (repeated measures ANOVA, p>0.1)* ***G.*** *Plot of the developmental trajectories of paired-pulse ratios of PFC and Barrel cortex (paired-pulse ratios in response to 20Hz paired pulse stimulation). The factor "brain region" has a significant effect on fEPSP responses (2-way ANOVA, p<0.05), while there is a significant difference between the two brain regions in all age groups tested, except PD120 (Tukey post hoc test, p<0.05). In PFC, there is a significant effect of "age" (one-way ANOVA, p<0.05), while PD120 is significantly different compared to all other age groups tested (Tukey post hoc test, p<0.05)*

3.2.4. Impaired working memory in adolescents compared to adult mice

In order to understand whether the changes in dendritic morphology and long-term potentiation we observe particularly during adolescence also translate to changes in behavior, we performed PFC-dependent and not PFC-dependent behavioral tasks in mice. We used two different age groups of mice for the behavioral experiments: a) the 'adolescent' group (PD40-50) and the 'young adult' group (PD60-80). In order to test the working memory of mice, which is a PFC-dependent function, we used the delayed alternation procedure in the T-maze. In this test, mice need to alternate the arms they choose to enter in the T-maze in order to receive a food reward. Once mice are successful in the alternating procedure (meaning they reach a performance criterion of 70% correct trials within a session), then delays are introduced in the alternation procedure. In each

delay, once mice reach the performance criterion, the delay between the trials is increased (from 5 to 20 sec). Adolescent mice, like adult mice, could perform the delayed alternation task and reach a performance criterion of 70% correct trials within a session.

However, adolescent mice required a greater number of sessions to reach the performance criterion at different delays (Fig. 3.6A) and performed poorer (Fig. 3.6B) overall compared to adult mice, particularly in the early part of the test. The factor "age" has also a significant effect (repeated measures ANOVA, $p<0.05$). Within all delays significant differences occur between adolescent and adult mice (repeated measures ANOVA, $p<0.05$). Specifically, adolescent mice need significantly increased number of sessions to reach criterion during the first two delays in the T-maze (One-way ANOVA, $p<0.05$) (Fig. 3.6A).

Furthermore, adolescent mice show decreased percent of correct choices during the different delays in the T-maze. The factor "age" tends to have an effect (repeated measures ANOVA, $p<0.1$), particularly in the first delay of the 5'' (One-way ANOVA, $p=0.11$) (Fig. 3.6B).

Finally, we measured the number of maximum sequential errors performed by the two groups of mice tested in the delayed alternation in the T-maze, as an index of perseveration. We find that adolescent mice exhibited a significantly increased average of maximum sequential errors in the test (One-way ANOVA, $p<0.05$), indicating an increased perseverance phenotype (Fig. 3.6C).

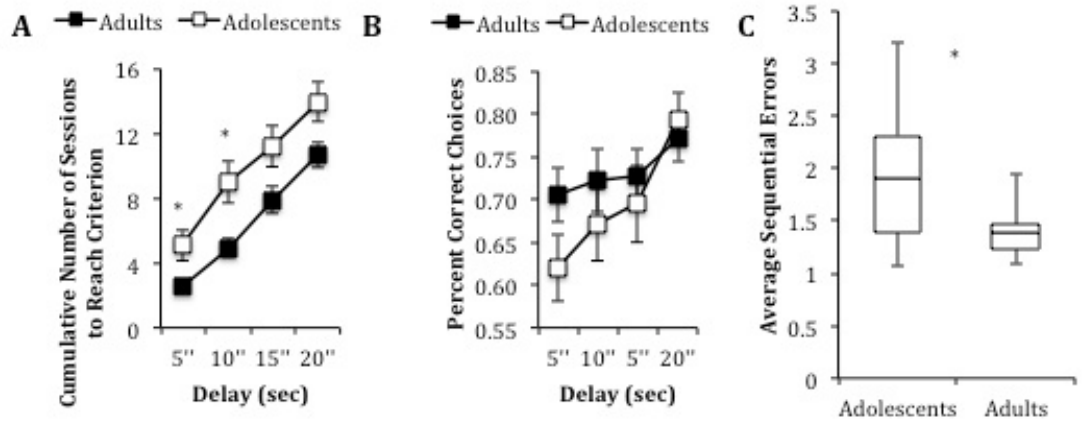


Figure 3.6. Impaired working memory in adolescents compared to adult mice
A. *Plot of the cumulative number of sessions needed to reach criterion for adolescent and adult mice. There is a significant linear effect of "delay" on the cumulative number of sessions to reach criterion (repeated measures ANOVA, $p<0.05$) and a non-significant interaction of the factors "delay" and "age" (repeated measures ANOVA, $p=0.071$). Within all delays significant differences occur (repeated measures ANOVA, $p<0.05$). The factor "age" also has a significant effect (repeated measures ANOVA, $p<0.05$). Adolescent mice need significantly increased number of sessions to reach criterion during the first two delays in the T-maze (one-way ANOVA, Tukey post hoc test, $p<0.05$).* ***B.*** *Plot of the percentage of correct choices during the increasing delays of the test for adolescent and adult mice. Adolescent mice show decreased percent of correct choices during the different delays in the T-maze. There is a significant linear effect of "delay" on the number of correct choices (repeated measures ANOVA, $p<0.05$) and a non-significant interaction of the factors "delay" and "age" (repeated measures ANOVA, $p<0.1$). The factor "age" appears to have an effect although it is not statistically significant (repeated measures ANOVA, $p<0.1$), particularly in the first delay of the 5 seconds (Tukey post hoc test, $p=0.11$).*
C. *Box-plot of the sequential errors performed by the two groups of mice tested in the delayed alternation in the T-maze. Adolescent exhibited a significantly increased average of sequential errors in the test (one-way ANOVA, $p<0.05$).*

3.2.5. Adolescent mice perform poorly in the Temporal Order Object Recognition Memory task, but not in the Novel Object or Object-to-Place Recognition Memory tasks

We performed three different types of object recognition tasks in 'adolescent' and 'young adult' mice. Object exploration was defined as the time spent exploring the objects by physical contact. Two different objects without ethological significance to the mice were

used in each one of the three different object recognition tests that were conducted. In pilot experiments it was ensured that these objects were distinguishable by the mice and that they had no per se preference for any of the objects. Furthermore, we tested whether mice had any object or location preference in the field during the trials, to exclude any bias from our results.

In the object recognition test with temporal order, testing the temporal integration ability of mice (PFC-dependent-function), there is a significant effect of "object" (2-way ANOVA, $p<0.05$) and the interaction of "object" and "age" in the time exploring the two objects (2-way ANOVA, $p<0.05$). There is not a significant difference in the time adolescents spent exploring the recent and the old familiar object (Tukey post hoc, $p>0.1$), while adult mice explore significantly more the old familiar object compared to the recent familiar object (Tukey post hoc, $p<0.05$), indicating that adults can differentiate better than the adolescent mice the least recent object (Fig. 3.7A). During the object-to-place recognition test there is a significant effect of both the factor "age" and the factor "object" (2-way ANOVA, $p<0.05$). In particular, both age groups of mice spent significantly more time exploring the displaced object compared to the time spent exploring the stationary object (Tukey post-hoc, $p<0.05$). Furthermore, adolescents spent significantly more time exploring the displaced object compared to the adult mice (Tukey post-hoc, $p<0.05$) (Fig. 3.7B). During the novel object recognition test there is not a significant effect of factor "age" in the exploration of the objects (2-way ANOVA, $p>0.1$), while there is a significant effect of the factor "object" (2-way ANOVA, $p<0.05$), as both age groups of mice explore the novel object significantly more than the familiar one (2-way ANOVA, $p<0.05$) (Fig. 3.7C).

We also calculated the discrimination index for the three object recognition memory tests performed by the two age groups of mice by dividing the time spent exploring the old familiar to the time spent exploring the recent familiar object (for the temporal order

recognition test), the time spent exploring the displaced to the time spent exploring the stationary object (for the object-to-place recognition test), the time spent exploring the novel to the time spent exploring the familiar object (for the novel object recognition test). We find that there is a significant effect of the type of object recognition memory test performed (2-way ANOVA, $p<0.05$). Further analysis showed that there is a significantly improved discrimination ratio when performing the novel object recognition task compared to the temporal order recognition task (Tukey post hoc, $p<0.05$), while the discrimination ratio during the object-to-place recognition task is not significantly different with neither of the two other tests used (Tukey post hoc, $p>0.1$). Furthermore, during the novel object recognition task adults and adolescents have similar discrimination indexes (Tukey post hoc, $p>0.1$), as during the object-to-place recognition test (Tukey post hoc, $p>0.1$). However, during the temporal order object recognition task the two age groups of mice have different discrimination indexes, although not significantly different (Tukey post hoc, $p=0.115$). Finally, adults seem to have similar discrimination indexes in all three tests used (Tukey post hoc, $p>0.1$), while adolescent mice tend to have decreased discrimination index in the temporal order recognition task compared to the other two -not PFC-dependent- tests used (Tukey post hoc, $p<0.1$) (Fig. 3.7D).

In addition, we calculated the average exploring index of all tests by dividing the time spent exploring objects to the time spent exploring the environment. We find that adolescent mice explore the objects more compared to adult mice, as they have a significantly increased exploring index (One-way ANOVA, $p<0.05$). Increased exploratory behavior is a typical behavior for this time period of development (L. P. P. Spear, 2000) (Fig. 3.7E).

In conclusion, adolescent mice have a poorer performance in the object recognition with temporal order test, but not in the novel object or object-to-place recognition tasks, suggesting that adolescent mice have not yet established the proper interactions between

the medial PFC, hippocampus and perirhinal cortex which are necessary for the temporal order recognition memory (Warburton & Brown, 2010).

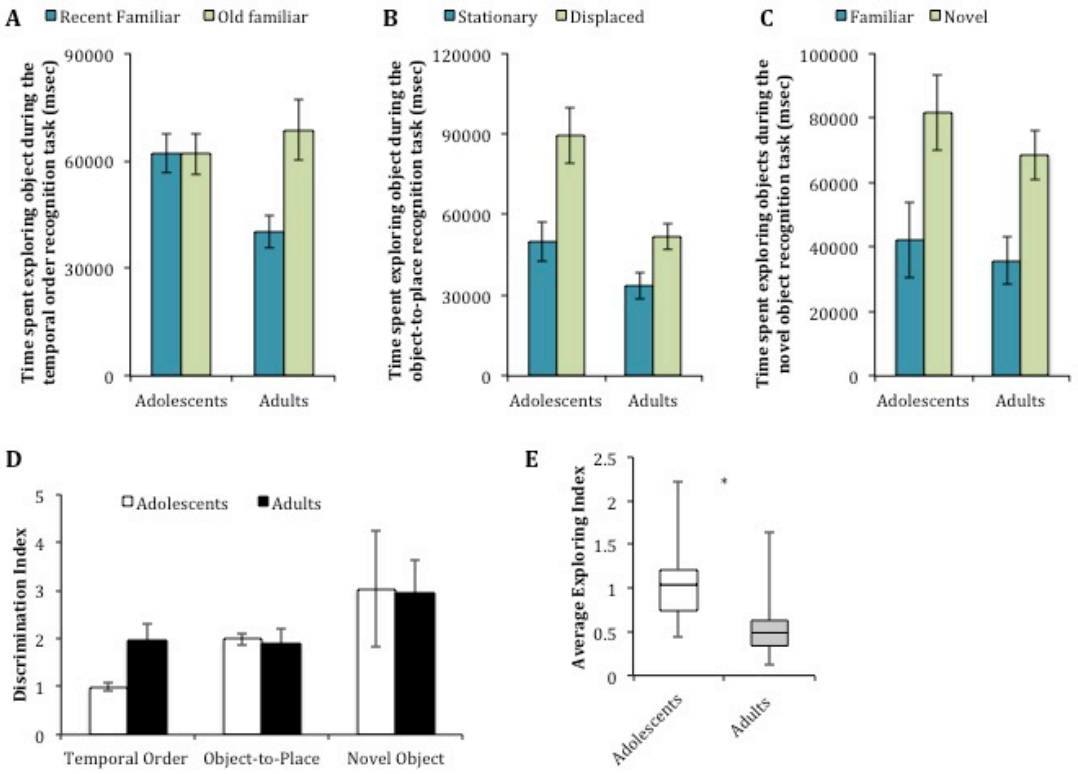


Figure 3.7. Decreased recency memory performance of adolescent mice in a object recognition task with temporal order.

A. *Plot showing the time adolescent and adult mice spent exploring the recent familiar and the old familiar object during the test trial of the object recognition task with temporal order. There is a significant effect of "object" and the interaction of "object" and "age" in the time exploring the two objects (2-way ANOVA, $p<0.05$). There is no significant difference in the time adolescents spent exploring the recent and the old familiar object (Tukey post hoc test, $p>0.1$), while adult mice spent significantly more time exploring the old familiar object compared to the recent familiar object (Tukey post hoc test, $p<0.05$).* ***B****. Plot showing the time adolescent and adult mice spent exploring the stationary and the displaced object during the test trial of the object-to-place recognition task. There is a significant effect of both "age" and "object" (2-way ANOVA, $p<0.05$). In particular, both age groups of mice spent significantly more time exploring the displaced object compared to the time spent exploring the stationary object (Tukey post-hoc test, $p<0.05$). Adolescents spent significantly more time exploring the displaced object compared to the adult mice (Tukey post-hoc, $p<0.05$)* ***C****. Plot showing the time adolescent and adult mice spent exploring the novel and the familiar object during the test trial of the novel recognition task. Thereis not a significant effect of factor "age" in the exploration of the objects (2-way ANOVA, $p>0.1$), but there is a significant effect of the factor "object" (2-way ANOVA, $p<0.05$), as both age groups of mice explore the novel object significantly more than the familiar one (2-way ANOVA, $p<0.05$).* ***D.*** *Plot showing the*

discrimination index of adolescent and adult mice during the three object recognition tasks. There is a significant improved discrimination ratio when performing the novel object recognition task compared to the temporal order recognition task (Tukey post hoc test, p<0.05). The discrimination ratio during the object-to-place recognition task is not significantly different with neither of the two other tests used (Tukey post hoc test, p>0.1). During the novel object recognition task adult and adolescents have similar discrimination indexes (Tukey post hoc test, p>0.1), as well as during the object-to-place recognition test (Tukey post hoc test, p>0.1). During the temporal order object recognition task the two age groups of mice have different discrimination indexes, although not significantly different (Tukey post hoc test, p=0.115). Adults seem to have similar discrimination indexes in all three tests used (Tukey post hoc test, p>0.1), while adolescent mice tend to have decreased discrimination index in the temporal order recognition task compared to the other two -not PFC-dependent- tests used (Tukey post hoc test, p<0.1) ***E.*** *Box-plot presenting the average exploring index. Adolescent mice explore the objects more compared to adult mice, as they have a significantly increased exploring index (one-way ANOVA, p<0.05).*

3.2.6. Decreased anxiety in adolescent mice

We used the open field test in order to identify age-dependent changes in trait anxiety levels, between adolescents and adults, but also detect possible changes in their ability to habituate in a novel environment, either within a session or between sessions. We find the levels of trait anxiety in the mice tested change as they grow. Mice were allowed to explore the open field for 20min, each day for 3 consecutive days. We measured their thigmotaxis index, which is an indication of the amount of time mice spend in the walls and corners of the open field relative to the amount of time mice explore the periphery and the center of the open-field. When analyzing the thigmotaxis index in adolescent and young adult mice, in 5min bins of the time they spent in the open-field, the factor “time” has a significant effect on thigmotaxis index (repeated measures, $p<0.05$), while there is not a significant interaction of “time” and “age” in thigmotaxis index during the test (repeated measures, $p>0.1$). The thigmotaxis index of two age groups tested (adolescents and adults) tend to differ during the test (repeated measures, $p<0.1$), while there is a significant difference on thigmotaxis index of the first and the third 5min of the test (repeated measures, $p<0.05$)

(Fig. 3.8A). Specifically, adolescent mice (PD 40) exhibit a significantly decreased thigmotaxis ratio compared to the young adult mice (PD 60) during the critical 1st 5min (One-way ANOVA, $p<0.05$) of the test (One-way ANOVA, $p<0.05$), suggesting that adolescent mice have a decreased amount of trait anxiety compared to adult mice, probably leading to the high-risk behavior that characterizes this time period of development (Jessor, 1991). Specifically, during this critical 1st 5min time bin in the open field "age" has a significant effect in the time spent in the center of the field (2-way ANOVA, $p<0.05$), while there is a tendency to effect the time spent in the area near the walls of the field (2-way ANOVA, $p<0.1$) (Fig. 3.8B).

We also measured the thigmotaxis during the 1st 5min period of the test for the three consecutive days that the mice were exposed to the open-field, in the two different age groups of mice. Our data show a significant difference between the two age groups of mice in their thigmotaxis index (repeated measures ANOVA, $p<0.05$). Adults exhibit significantly increased thigmotaxis index during the first (One-way ANOVA, $p<0.05$) and the second (One-way ANOVA, $p<0.05$) day of the test, while they tend to exhibit increased thigmotaxis during the third day of the test (One-way ANOVA, $p<0.1$) (Fig. 3.8C).

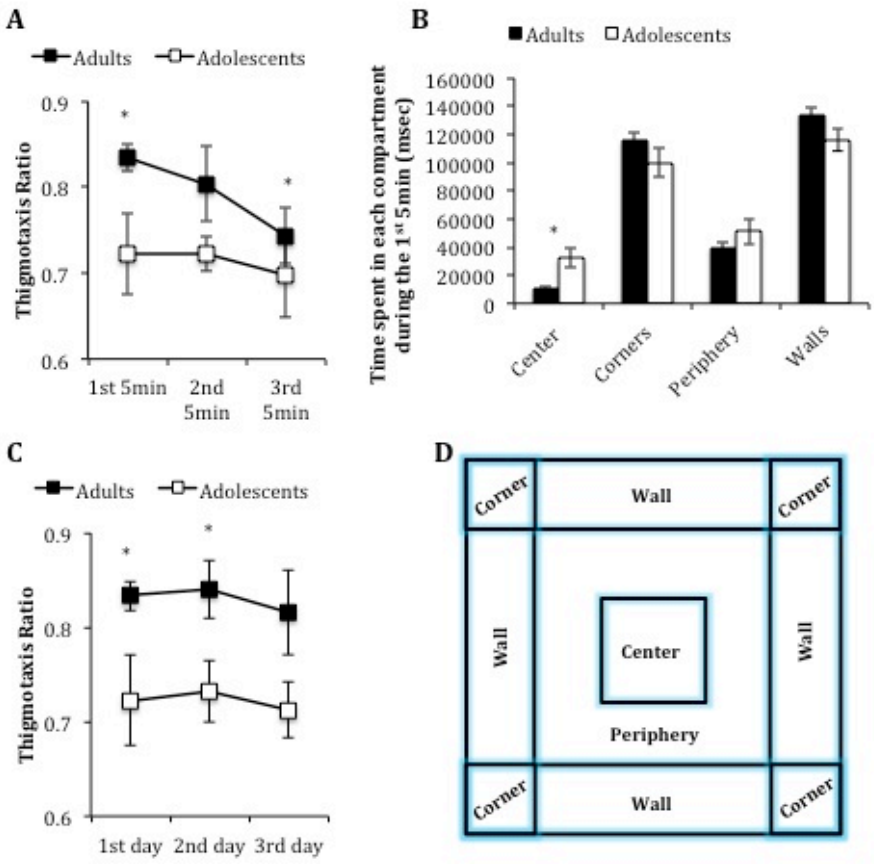


Figure 3.8. Decreased thigmotaxis in adolescent mice

A. *Graph showing the thigmotaxis ratio of adults and adolescents mice during the three 5min time bins in the open field. The thigmotaxis index of two age groups tested (adolescents and adults) tend to differ during the test (repeated measures ANOVA, p<0.1), while there is a significant difference on thigmotaxis index of the first and the third 5min of the test (repeated measures ANOVA, p<0.05). Adolescent mice (PD 40) exhibit a significantly decreased thigmotaxis ratio compared to the young adult mice (PD 60) during the 1st 5min of the test (one-way ANOVA, p<0.05).* ***B****. Graph showing the time spent in each compartment of the field by the adolescent and adult mice during the 1st 5min of the test. Adolescent mice spent significantly more time in the central area of the field (2-way ANOVA, p<0.05). Adolescents tend to spent less time towards the walls of the field (2-way ANOVA, p<0.1).* ***C.*** *Graph showing the thigmotaxis ratio of adults and adolescents mice during the 1st 5min for 3 consecutive days they took the test. There is a significant difference between the two age groups of mice in their thigmotaxis index (repeated measures ANOVA, p<0.05). Adults exhibit significantly increased thigmotaxis index during the first (one-way ANOVA, p<0.05) and the second (one-way ANOVA, p<0.05) day of the test, while they tend to exhibit increased thigmotaxis during the third day of the test (one-way ANOVA, p<0.1)*

3.2.7. Increased within-session habituation but decreased intra-session adaptation in adolescent mice

Habituation to a novel environment, measured as a change in exploratory activity over time, can be examined both within (intra-session) and between (intersession) sessions. It has been suggested that intra-session habituation measures adaptability, whereas inter-session habituation also reflects memory of the previous session (Bolivar, 2009). We measured the total number of entries in the different areas of the open field in order to assess the habituation percentage of the age groups of mice, both during a single session in the novel environment and between the three different sessions of the test. When analyzing the exploration within a session, the factor "time" has a significant linear effect in the total number of entries during the first day of the test (repeated measures, $p<0.05$). There is a significant difference in the total number of entries during the 1st 5min in the test compared to the 2nd and the 3rd 5min in the test (repeated measures, $p<0.05$). There is not a significant difference between the 2nd and the 3rd 5min (repeated measures, $p>0.1$), suggesting that both the adolescent and the adult mice exhibited equal habituation per time period. In addition, the total entries of the two different groups are not significantly different during the 1st, the 2nd or the 3rd 5min of the test (One-way ANOVA, $p>0.1$) (Fig. 3.9A). We also calculated the average percentage of intra-session habituation, taking into account the total time of entries during the 1st and the 3rd 5min time bin in the field for the two age groups of mice tested and found that adolescent mice seem to habituate more than adult mice, but this difference is not significant (One-way ANOVA, $p=0.132$) (Fig. 3.9B). Furthermore, we analyzed the total number of entries between different sessions during the three consecutive days that mice took the test. The interaction of the factors "age" and "time" does not have a significant linear effect in the total number of entries between the

three different sessions of the test (repeated measures ANOVA, $p<0.1$). There is not a significant difference in the number of entries between the two age groups of mice during the 1st and the 3rd days in the test (One-way ANOVA, $p<0.1$), while they differ significantly during the 2nd day in the test (One-way ANOVA, $p<0.05$) (Fig. 3.9C). We also calculated the average percentage of inter-session habituation, taking into account the total time of entries during the 1st and the 3rd day in the field for the two age groups of mice tested and found that adolescent mice seem to habituate less than adult mice, indicating decreased memory but this difference is not significant (One-way ANOVA, $p<0.1$) (Fig. 3.9D).

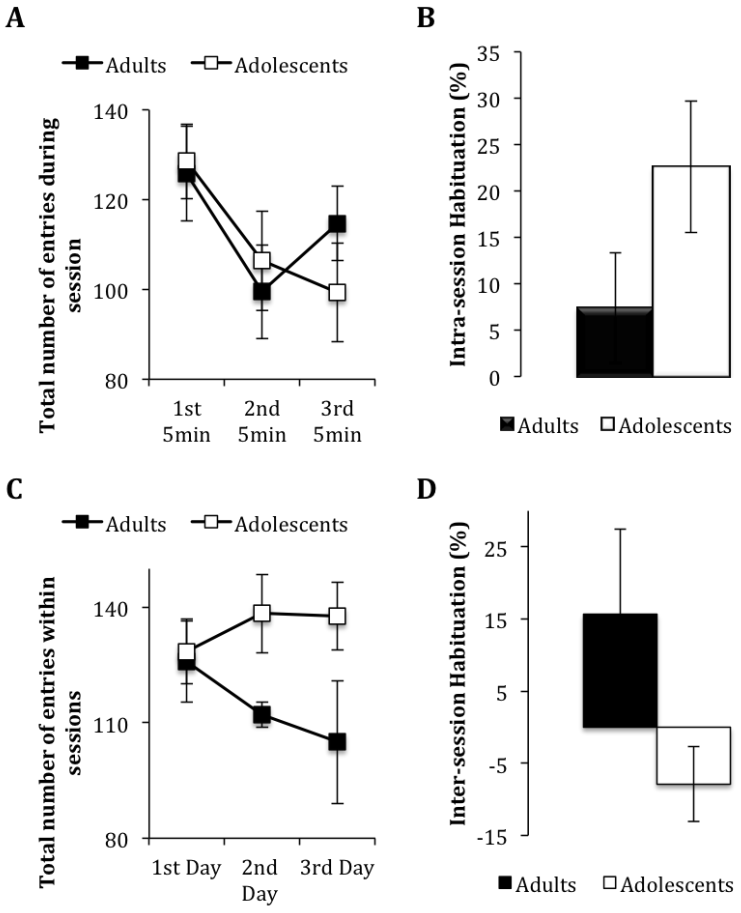


Figure 3.9. Increased adaptability but decreased memory for previous experiences in adolescent mice

A. *Graph showing the total number of entries in the different compartments of the field during the 3 5min time bins in the open field. There is a significant difference in the total number of*

entries during the 1st 5min in the test compared to the 2nd and the 3rd 5min in the test (repeated measures, p<0.05). There is not a significant difference between the 2nd and the 3rd 5min (repeated measures, p>0.1). The total entries of the two different groups are not significantly different during the 1st, the 2nd or the 3rd 5min of the test (One-way ANOVA, p>0.1). ***B.*** *Graph showing the average percentage of intra-session habituation. Adolescent mice seem to habituate more than adult mice, but this difference is not significant (One-way ANOVA, p=0.132)* ***C.*** *Graph showing the total number of entries in the different compartments of the field by the adolescent and adult mice during the 1st 5min for 3 consecutive days they took the test. There is not a significant difference in the number of entries between the two age groups of mice during the 1st and the 3rd days in the test (One-way ANOVA, p<0.1), while they differ significantly during the 2nd day in the test (One-way ANOVA, p<0.05)* ***D.*** *Graph showing the average percentage of inter-session habituation. Adolescent mice seem to habituate less than adult mice (One-way ANOVA, p<0.1).*

CHAPTER II

EFFECTS OF DEVELOPMENTALLY DECREASED INHIBITION IN BEHAVIORAL AND PHYSIOLOGICAL FUNCTIONS OF THE PFC

4.0. Chapter II: Effects of developmentally decreased inhibition in behavioral and physiological functions of the PFC

4.1. Introduction

Excitation-inhibition balance (E/I balance) refers to the relative degree of excitatory and inhibitory drive in a neural circuit, is established during development and is maintained in the mature nervous system. Excitation is provided by the pyramidal neuron function, while inhibition by the interneurons. Even small changes in the balance between excitation and inhibition can result in runaway excitability (Chagnac-Amitai & Connors, 1989) disruption of sensory responses (Nelson, 1991) and alteration of experience-dependent plasticity (T K Hensch et al., 1998). Severe behavioral deficits in psychiatric diseases such as epilepsy, anxiety, schizophrenia and autism have been hypothesized to arise from elevations in the cellular balance of excitation and inhibition (E/I balance) within neural microcircuitry. (Lewis, Hashimoto, & Volk, 2005; Marín, 2012; Yizhar et al., 2011). In this chapter, the focus will be on the role of GABA in epilepsy and anxiety.

4.1.1. Epilepsy

Epilepsy is a complex syndrome characterized by the emergence of epileptic seizures, caused by disturbances in normal neuronal function in the brain and particularly the normal balance between excitation and inhibition. During an epileptic seizure, groups of neurons exhibit massive, synchronous, abnormal firing behavior with very high frequencies of up to 500Hz. This leads to interruption of normal neuronal activity, causing strange behaviors and emotions, as well as muscle spasms and loss of consciousness. Epileptic seizures can be either focal or global, depending on the number of brain regions involved. Regarding focal seizures, synchronized neuronal activity initially appears in a specific

cortical area and then spreads to neighboring areas. At the level of individual cells, the seizure activity is characterized by high frequency cell firing, which may be caused by abnormalities in either passive or active properties of the cells, or alternatively, after the synaptic reorganization observed in cases of injury, leading the circuit to increased excitation capacity. In addition, defects in the GABAergic transmission have been linked to the emergence of epileptic seizures in patients (e.g., loss of a specific type of interneurons in patients with temporal lobe epilepsy) and in animal models of epilepsy (Jones-davis, Calcagnotto, & Sebe, n.d.). With regard to generalized seizures, the mechanisms are not yet so clear. The basic mechanism appears to be the defects in the function and communication of thalamocortical connections and involves interactions between GABAb receptors, calcium, and potassium, ion channels in thalamic neurons.

Temporal lobe epilepsy

The Temporal Lobe Epilepsy (TLE) is the most common form of focal seizures. Depending on their originating brain region, can be divided into subgroups: proximal and neocortical that originate in the hippocampus, the parahippocampal gyrus, amygdala and neocortex.

Proximal temporal lobe epilepsy is prevalent in patients with early onset of the disorder, unlike neocortical, which occurs frequently in patients who develop epilepsy at a later age (Villanueva & Serratosa, 2005). This could mean that the proximal structures, like the hippocampus, are more vulnerable to the early appearance of epilepsy. There are several animal models of temporal lobe epilepsy with a genetic, electrical or chemical basis for induction.

The pilocarpine model of temporal lobe epilepsy

The pilocarpine model of epilepsy is induced by an intraperitoneal administration of pilocarpine, which results in fast induction of acute seizures, while it also exhibits a latency to the onset of spontaneous recurrent seizures (chronic phase). The pilocarpine acts as an agonist of the M1 muscarinic acetylcholine receptor (mAChR) (Hamilton et al., 1997), and it induces epileptic seizures through activation of mAChR, but also through subsequent activation of NMDA receptors (Nagao, Alonso, & Avoli, 1996; Smolders, Khan, Manil, Ebinger, & Michotte, 1997). The pilocarpine animal model of epilepsy in rodents results in histopathological changes in areas such as the hippocampus, the neocortex, the olfactory cortex and the amygdala (Curia, Longo, Biagini, Jones, & Avoli, 2008), in reorganization of neural networks, probably resulting from the loss of cells, the creation of new dendritic segments (sprouting) induced by the seizure, in the occurrence of new ectopic cells, and in increased dendritic branching on their apical dendrites have been found (Silva, Sanabria, Cavalheiro, & Spreafico, 2002). Similar increases in dendritic spines have been observed in human patients as well.

4.1.2. Anxiety

Anxiety is a complex emotional state associated with elevated autonomic and behavioral arousal and a sustained increase in avoidance behavior in environmental situations characterized by a level of uncertainty, unpredictability, or uncontrollability, including situations where a conflict between approach and avoidance exists (Gray, 1987; Lowry et al., 2005). The physiological and behavioral arousal associated with anxiety states and

anxiety-related behaviors appears to be regulated by a distributed and interconnected system of forebrain and hindbrain structures including the septo-hippocampal system and entorhinal cortex (Gray, 1987), the PFC (Duncan et al., 1996), the amygdala (Campbell & Merchant, 2003; Spiga et al., 2006) and the raphe nuclei (N. Singewald & Sharp, 2000; Nicolas Singewald et al., 2003).

Anxiety is characterized by increased attentional capture by threat-related stimuli (MacLeod et al., 1986; Williams et al., 1996) resulting of a hyper-responsive pre-attentive threat-detection system centered on the amygdala (Mathews et al., 1997). PFC mechanisms influence the top-down control of selective attention to threat (Bishop, Duncan, & Lawrence, 2004; Öhman, 2005), therefore, it is suggested that increased trait anxiety is characterized by impaired recruitment of PFC mechanisms. It is proposed that this deficit does not arise as a result of current or state levels of anxiety, but instead reflects an underlying trait characteristic that influences attentional processing regardless of the presence or absence of threat-related stimuli (Bishop, 2009). Deficits in the GABAergic system have been suggested to contribute to an increased trait anxiety (Crestani et al., 1999).

Epilepsy and anxiety co-morbidity

In the recent years, it has become apparent that there is increased trait anxiety and/or emergence of anxiety disorders in epileptic patients (Beyenburg, Mitchell, Schmidt, Elger, & Reuber, 2005; Hamid, Ettinger, & Mula, 2011), and in animal models of epilepsy (Jones et al., 2008). Furthermore, the presence of an anxiety disorder in epileptic patients increases the probability for exhibiting resistance to anti-epileptic medication (Hamid et al., 2011). Therefore, it is necessary to identify mechanisms that contribute to the concurrent emergence of both conditions, in order to be able to test potential therapeutic interventions.

4.1.3. Transgenic animals of decreased number of interneurons

The new knowledge with regards to the transcription factors and intracellular mediators that regulate various aspects of interneuron development has generated several transgenic mice which show impaired proliferation and migration of cortical interneurons (Butt et al., 2008; Cobos et al., 2005; Neves et al., 2013; Vidaki et al., 2012). Few animal models have been found to replicate the above co-morbid phenotype. A genetic animal model in which the function of specific interneurons is impaired by removing the Nav1.1 ion channel from the interneurons has been shown to have both spontaneous epileptic seizures and increased anxiety as observed by the elevated plus maze (Han et al., 2012). Mice with a mutation in the urokinase plasminogen activator receptor have decreased numbers of interneurons in the anterior cingulate and behaviorally exhibit both increased anxiety and decreased threshold (increased susceptibility) for pentylenetretrazol-induced epilepsy (Powell et al., 2003). The WAG/Rij strain is also a well characterized animal model of absence epilepsy, in which several symptoms of depression have also been found, such as decreased consumption of sucrose, decreased active behaviors during the forced swim test and decreased exploration (rearings and grooming) (Sarkisova & van Luijtelaar, 2011). A working hypothesis, in agreement with the aforementioned data and our own, could be put forward postulating that the loss of interneuronal function could underlie co-morbidities that are associated with epilepsy. Therefore, these mice could be used as animal models for studying the emotional and cognitive behavior, as well as for testing pharmacological agents targeting specifically these co-morbidities, with the possibility of having better predictive values. These mice could be used to determine whether developmental defects in the functioning of interneurons would underlie the disease phenotype.

Rac1 Conditional KnockOut Mice

In our study here, we used a transgenic animal model that exhibits decreased number of interneurons, namely the Rac1 conditional knockout (Rac1 cKO) mouse, developed in the laboratory of Domna Karagogeos (Vidaki et al., 2012). In this transgenic mouse, the Rac1 protein was deleted from Nkx 2.1-expressing cells, using the LoxP/Cre technology. For the generation of the Rac1 cKO mice, mice expressing the Cre-recombinase system under the control of the promoter of Nkx2.1 (Fogarty et al., 2007) were mated to mice carrying the gene of Rac1 between 2 LoxP sequences recognized by the recombinase (Walmsley et al., 2003). The Nkx2.1 gene encodes a transcription factor expressed in the medial ganglionic eminence (MGE) and in the hypothalamic (preoptic) region of the globus pallidus (pallidal) of telencephalon. During fetal development, over 90% of the cells of the MGE (ventricular and subventricular zone) located in the S phase of the cell cycle; express this factor until they begin to migrate towards the cortex. Most MGE neurons expressing the Nkx2.1 factor are GABAergic interneuron progenitors, destined to population the cerebral cortex and to differentiate in PV/FS and Sst/RS interneurons (Wonders & Anderson, 2006). In the Rac1 cKO mice, the Cre-recombinase is expressed when the Nkx2.1 expression starts (i.e. embryonic day 10.5) and removes the Rac1 gene from MGE progenitor cells. The absence of Rac1, a rho-GTPase involved in regulating cell cycle, among other functions (Bongmba, 2012; Corbetta et al., 2009; de Curtis, 2014), from Nkx2.1 positive interneurons progenitor cells (derived from the MGE) caused abnormalities in the cell cycle, disrupting their progress of the G1 phase and leading to reduced migration of these cells in the layers of the cortex. Specifically, only 50% of GABAergic interneurons from the MGE, finally manage to migrate into the cortex with a delay. The vast majority of cells lacking Rac1, accumulate in the ventral MGE, in the area where they were born. As a result, the number of cortical interneurons in the Rac1 cKO mice is only half of that which is normal (Vidaki et al., 2012).

The ratio between different types of interneurons however is maintained. Thus, even in conditional knockout animals, 80% of defective cells are the PV and SST positive interneurons. Of course, interneurons immunopositive for proteins CR (Calretinin) and NPY (Neuropeptide Y) that originate from the region CGE of the telencephalon are not affected (Vidaki et al., 2012).

4.2. Results

4.2.1. Epileptic and anxiety behavior in Rac1 cKO mice

About 34.5% of Rac1 cKO mice that were born in standard cages died before reaching adulthood while only a 1.45% of breedings resulted in birth of a Rac1 cKO mouse. The 80% of them were observed having epileptic seizures when they were 15 to 35 days old and died shortly after. These seizures were characterized by long duration, with violent convulsions of the limbs (stage 5 of the scale Racine, (Racine, 1972)), which led to death immediately or after some time. Rac1 cKO mice that survived to adulthood did not exhibit spontaneous or mild-stress induced tonic-clonic seizures.

To determine whether adult Rac1 cKO mice had a lower threshold for induction of epileptic seizures, the pilocarpine model of epileptic seizures was used (Curia et al., 2008). Rac1 Het animals received three different doses of pilocarpine (100 mg/kg, 200 mg/kg or 300 mg/kg) or PBS 30 minutes following an injection of 1 mg/kg scopolamine in order to reduce discomfort due to peripheral effects of pilocarpine. Their behavior was monitored for 2 hours in order to record the stage, frequency, latency and duration of epileptic seizures.

As expected, epileptic seizures were of greater intensity and duration with increasing doses of pilocarpine. Specifically, mice that were treated with 100 mg/kg pilocarpine reached stage 3 on average, while all mice treated with 200 mg/kg or 300 mg/kg pilocarpine

reached stage 6. Rac1 Het mice that received 200 or 300 mg/kg pilocarpine reached significantly higher stages of epileptic seizures compared to Rac1 Het mice that received the lowest dose (1-way ANOVA, $p<0.001$) (Fig. 4.1*A*). In addition, mice treated with 300 mg/kg exhibited an increased number of stage 6 epileptic episodes (within 1hr) (Fig. 4.1*B*) and reached stage 6 faster compared to mice treated with 200 mg/kg (Tukey post-hoc test, $p<0.05$) (Fig. 4.1*C*). On the other hand, the average duration of each epileptic stage was the same in mice treated with either 200 mg/kg or 300 mg/kg pilocarpine (Fig. 4.1*D*). Rac1 KO mice received either PBS or 100 mg/kg pilocarpine. Higher doses of pilocarpine were lethal for the Rac1 cKO mice; therefore, doses higher than 100mg/kg in the Rac1 cKO could not be studied. No epileptic seizures were recorded in Rac1 cKO mice that received PBS, similar to Rac1 Het mice. Rac1 cKO mice that received 100 mg/kg pilocarpine exhibited significantly more intense seizures compared to Rac1 Het mice that received the same dose (Kruskal-Wallis test, $p<0.05$). Specifically, about 50% of Rac1 cKO mice reached stage 6 when administered with 100 mg/kg pilocarpine, while only 17% of Rac1 Het mice reached stage 6 with the same dose of pilocarpine. The average stage reached by Rac1 cKO mice was 5 while Rac1 Het mice that received a similar dose of pilocarpine only reached stage 3 on average (Fig. 4.1*A*). Furthermore, Rac1 cKO mice receiving 100 mg/kg pilocarpine have significantly decreased latency for the first stage 6 induction compared to Rac1 Het mice receiving 200mg/kg pilocarpine (1-way ANOVA, $p<0.05$, Tukey post-hoc test $p<0.05$) (Fig. 4.1B), while they have the same latency for first state 6 induction with the Rac1 Het mice receiving 300mg/kg pilocarpine (Tukey post-hoc test, $p>0.1$) (Fig. 4.1C). Finally, Rac1 cKO receiving 100mg/kg pilocarpine have the same average duration of epileptic seizures with the Rac1 cKO mice receiving 200 or 300mg/kg pilocarpine (1-way ANOVA, $p>0.1$) (Fig. 4.1D). Therefore, the epileptic seizures of Rac1 cKO mice that received 100 mg/kg

pilocarpine resembled closer the group of control mice that received 200 mg/kg or 300 mg/kg pilocarpine (Fig. 4.1*A*-*D*).

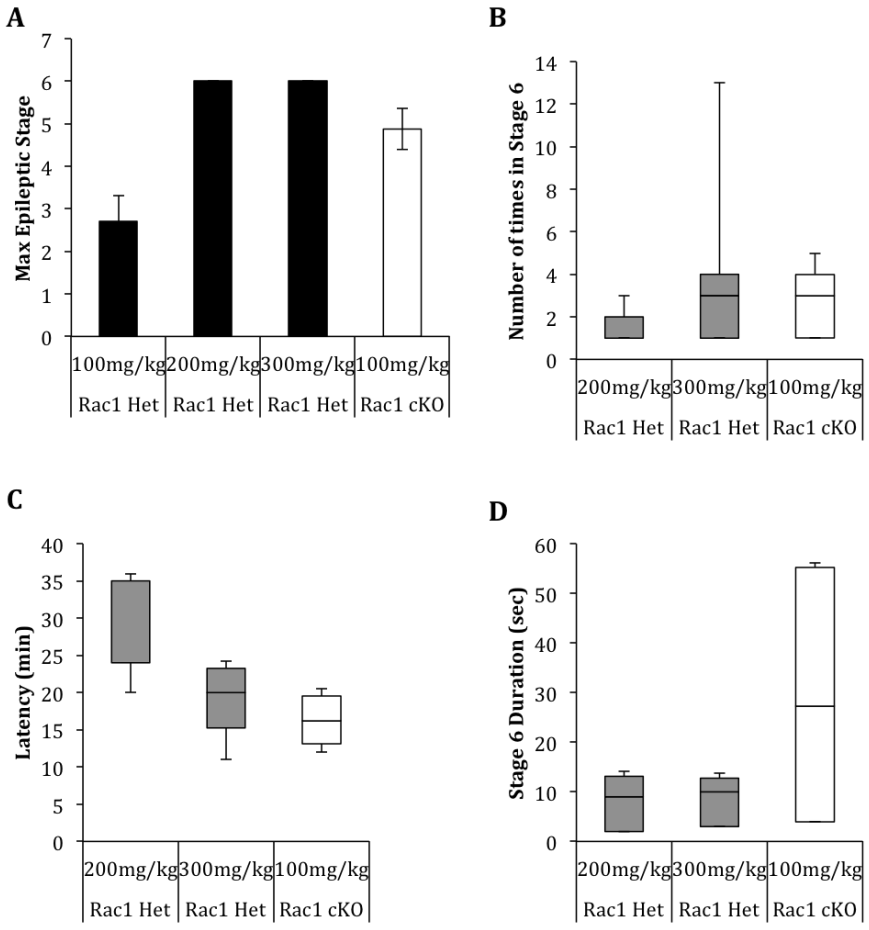


Figure 4.1. Rac1 cKO mice exhibit decreased threshold for pilocarpine-induced epileptic seizure*s*

A. *Graph showing the maximum epileptic stage reached in Rac1 Het mice (black bars) and Rac1 cKO mice (white bar). Rac1 cKO require a decreased pilocarpine dose in order to reach higher epileptic stages compared to Rac1 Het mice (Kruskal-Wallis test, p<0.05). Rac1 Het mice that received 200 or 300 mg/kg pilocarpine reached significantly higher stages of epileptic seizures compared to WT mice that received the lowest dose (1-way ANOVA, p<0.001).* ***B****. Graph showing the number of stage 6 occurrences in Rac1 Het (grey bars) and Rac1 cKO mice (white bar). Rac1 cKO mice receiving 100mg/kg pilocarpine have the same frequency of stage 6 occurrences with Rac1 Het mice receiving 200 or 300mg/kg pilocarpine (1-way ANOVA, p>0.1).* ***C.*** *Graph showing the latency for the first stage 6 induction in Rac1 Het (grey bars) and Rac1 cKO mice (white bar). Rac1 Het mice that received 200mg/kg pilocarpine had decreased latency for the first stage 6 epileptic episode compared to Rac1 Het mice that received 300mg/kg pilocarpine (Tukey post-hoc test, p<0.05).*

C. Rac1 cKO mice receiving 100mg/kg pilocarpine have significantly decreased latency for the first stage 6 induction compared to Rac1 Het mice receiving 200mg/kg pilocarpine (1-way

ANOVA, p<0.05, Tukey post-hoc test p<0.05). Rac1 cKO mice receiving 100mg/kg pilocarpine have the same latency for first state 6 induction with the Rac1 Het mice receiving 300mg/kg pilocarpine (Tukey post-hoc test, p>0.1). ***D.*** *Graph showing the average duration of stage 6 episodes in Rac1 Het (grey bars) and Rac1 cKO mice (white bar). Rac1 cKO receiving 100mg/kg pilocarpine have the same average duration of epileptic seizures with the WT mice receiving 200 or 300mg/kg pilocarpine (1-way ANOVA, p>0.1).*

Several reports from human studies suggest the presence of co-morbidity of epilepsy and anxiety (Hamid et al., 2011). However, the underlying mechanisms contributing to this co-morbidity are not known. Therefore, we investigated whether the Rac1 cKO mice have different anxiety levels compared to Rac1 Het mice, in addition to the decreased threshold for epileptic seizures. For this, both male and female mice were tested in the open-field test and the elevated plus maze to analyze their anxiety levels. When tested in the open-field arena, Rac1 cKO mice explored significantly more the peripheral zone (One-way ANOVA, $p<0.05$) and the area towards the walls of the field (One-way ANOVA, $p<0.05$) (Fig. 4.2A-C), a behavior which resulted in a significantly increased thigmotaxis index (Thigmotaxis Index: Time to Walls/Time Center + Time Periphery) not only during the critical 1st 5 min, but also during the 2nd and 3rd 5min in the test (repeated measures ANOVA, $p<0.05$) (Fig. 4.2B). Furthermore, Rac1 cKO mice travelled significantly shorter distance (repeated measures ANOVA, $p<0.05$) (Fig. 4.2D), with a significantly reduced speed during the critical 1st 5min in the field (repeated measures ANOVA, $p<0.05$) (Fig. 4.2E). However, in the 2nd and 3rd 5min period, they covered the same distance (repeated measures ANOVA, $p>0.1$) with the same mean speed as Rac1 Het mice (repeated measures ANOVA, $p>0.1$), indicating that they don't experience any locomotor disability (Fig. 2D-E). Finally, Rac1 cKO exhibit less horizontal and vertical movement during the 1st 5min in the open field. They stay immobile significantly more only during the 1st 5min in the field as they exhibit a significantly increased percentage of immobility time compared to Rac1 Het mice (repeated measures ANOVA, $p<0.05$) (Fig. 4.2F). As for their vertical movement Rac1 cKO mice

exhibited significantly less rears during the first 5 min of the test (repeated measures ANOVA, $p<0.05$) (Fig. 4.2G).

When tested in the elevated plus maze, Rac1 cKO mice enter both the closed and the open arms of the elevated plus maze significantly less than the Rac1 Het mice (ttest, $p<0.05$) (Fig. 4.3A). The Ratio of Entries in the Closed Arms to the Entries in the Open arms of the maze is significantly increased in Rac1 cKO mice, indicating their increased preference to the closed arms of the maze against the open arms (ttest, $p<0.05$) (Fig. 4.3B). Finally, Rac1 cKO mice show decreased total number of entries in all compartments of the maze (intersection, open arms, closed arms) indicating a decreased mobility that could be interpreted as increased anxiety caused by the device (ttest<0.05) (Fig. 4.3C). Collectively, the above results show that Rac1 cKO mice have an increased anxiety phenotype, which co-exists with an increased susceptibility to seizures.

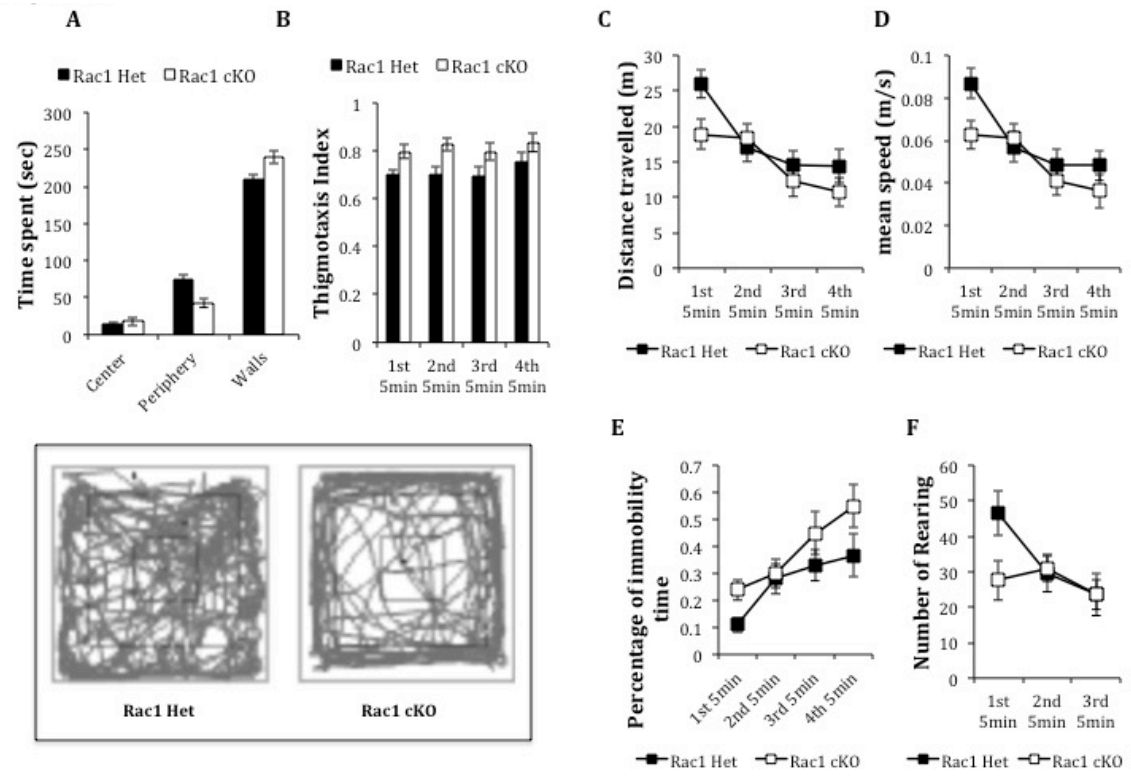


Figure 4.2. Rac1 cKO mice experience increased anxiety when placed in the open field.

A. *Graph showing the number of entries to the center, periphery and wall area in the open-field arena during the first 5min of the test. Rac1 cKO mice entered significantly less times the peripheral zones and significantly more times the wall area compared of the enclosure to the Rac1 Het mice (t-test, p<0.05).* ***B.*** *The thigmotaxis index in Rac1 cKO mice is significantly increased during the first 3 5min time bins in the field (t-test, p<0.05).* ***C.*** ***.****Representative figures showing the tracking of the movement trajectory of Rac1 Het and Rac1 cKO mice until the end of the 5th min in the test. It is very obvious that Rac1 Het mice have explored the whole open field area while Rac1 cKO mice prefered to travel towards the walls of the field, because of their increased thigmotaxis.* ***D.*** *Graph showing the distance travelled during the test. Rac1 cKO mice travelled significantly shorter distance during the critical first 5min in the field, this was not the case for the rest time of the test as they covered the same distance as Rac1 Het mice (repeated measures ANOVA, p<0.05).* ***E.*** *Graph showing the mean speed mice travelled in the field. Rac1 cKO mice travelled with a significantly reduced speed during the critical first 5min in the field, this was not the case for the rest time of the test as they covered the same distance with the same mean speed as Rac1 Het mice (repeated measures ANOVA, p<0.05).* ***F.*** *Graph showing the immobility percentage during the test. Rac1 cKO mice stay immobile significantly more only during the first 5min in the field as they exhibit a significantly increased percentage of immobility time compared to Rac1 Het mice (repeated measures ANOVA, p<0.05).* ***G.*** *Graph showing the number of rearing episodes during the test. Rac1 cKO mice rear significantly less during the first 5 min in the open-field test (repeated measures ANOVA, p<0.05).*

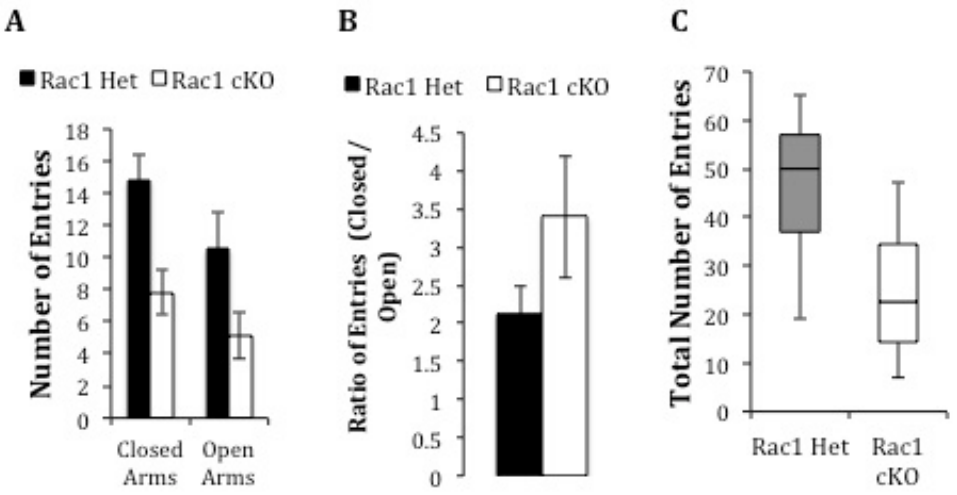


Figure 4.3. Rac1 cKO mice exhibit an increased anxiety phenotype in the elevated plus maze test.

A. *Rac1 cKO mice enter both closed and open arms significantly less times than Rac1 Het mice (t-test, p<0.05).*

B. *Rac1 cKO mice have increased ratio of entries in the closed arms of the maze compared to the Rac1 Het mice (ttest, p<0.05)*

C. *Rac1 cKO mice enter the different comparments of the mice significantly less (ttest, p<0.05)*

4.2.2. Epileptiform discharges in the Rac1 cKO mice brain slices

We next tested whether Rac1 cKO mice exhibit epileptiform discharges *in vitro* in two different cortical areas, namely the barrel cortex and PFC. We did not detect significant spontaneous epileptiform discharges in brain slices from Rac1 cKO mice, in either barrel cortex or PFC. We then tested for the emergence of spontaneous epileptiform discharges via making the brain slice more excitable, by removing the Mg^{++} ions from the aCSF solution. Therefore, spontaneous activity was measured in the control aCSF and in aCSF with no Mg^{++} ions. Spontaneous epileptiform activity emerged in brain slices from Rac1 cKO but not from Rac1 Het mice, showing a vulnerability of Rac1 cKO brain slices to induce epileptiform activity (Fig. 4.4A1,A2).

In addition, epileptiform discharges were also observed following electrical stimulation in the neocortex of the Rac1 cKO mice, which were not evident in brain slices from Rac1 Het mice (Fig. 4.4*B*). In order to quantify the stimulus-induced epileptiform activity in Rac1 Het and Rac1 cKO mice, we computed the histogram of the first derivative of the voltage response following stimulation, which shows that Rac1 cKO mice have increased values of the first derivative of the spontaneous voltage waveform, indicating increased activity (Fig. 4.4*C*). Thus, in addition to the increased susceptibility to behaviorally studied epileptic seizures, Rac1 cKO mice also have increased susceptibility for induction of epileptiform discharges in brain slices.

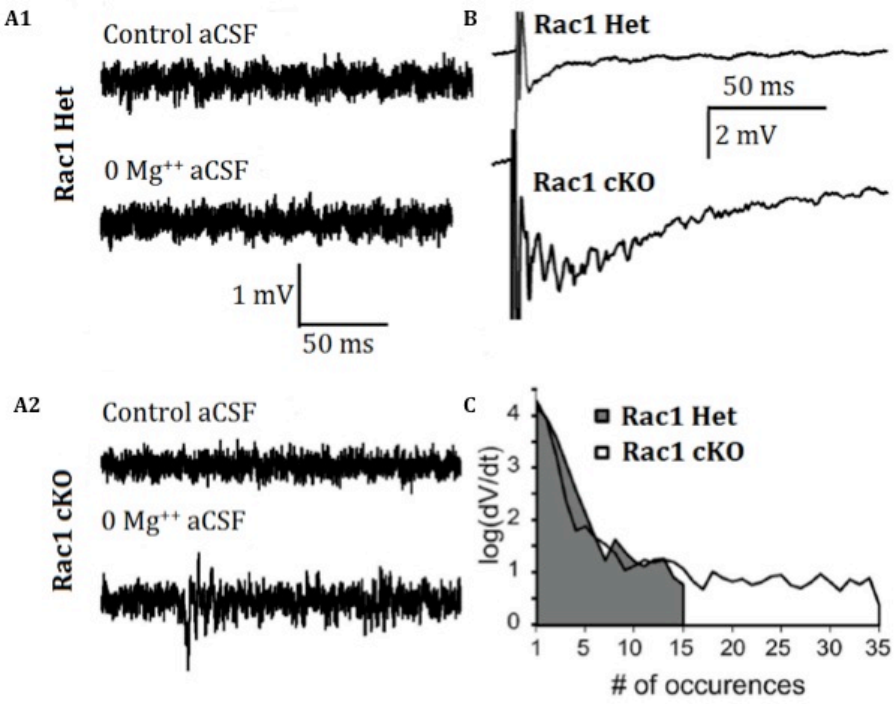


Figure 4.4. Rac1 cKO mice exhibit decreased threshold for spontaneous and stimulus induced epileptiform activity induction in brain slices
A1. *In brain slices from Rac1 Het animals, spontaneous epileptiform discharges did not emerge when the Mg^{++} ions were removed from the aCSF solution.* ***A2.*** *In brain slices from Rac1 cKO mice, spontaneous epileptiform activity emerged when the Mg^{++} ions were removed from the aCSF solution.* ***B.*** *Stimulus-induced epileptiform activity was evident when recording from Rac1 cKO neocortical slices, but not from Rac1 Het neocortical slices.* ***C.*** *The quantification of the stimulus-induced epileptiform activity noticed in Rac1 cKO slices, compared to that of Rac1 Het neocortical slices.*

4.2.3. Differential changes in basal synaptic transmission and short-term synaptic plasticity in the cortex

Next, we aimed to understand whether the synaptic properties of the Rac1 cKO cortex were altered in the presence of developmentally decreased inhibition. We studied basal synaptic transmission by delivering current pulses of increasing intensity and recording field excitatory potentials (fEPSPs) from neocortical layer II neurons. In the BC layer II, Rac1 cKO mice showed increased fEPSP responses in response to increasing stimulation currents, as it is expected, in the presence of decreased number of interneurons (repeated measures

ANOVA, $p<0.05$) (Fig. 4.5A). Furthermore, no difference in the paired-pulse ratio was observed between Rac1 Het and Rac1 cKO mice (repeated measures ANOVA, $p>0.1$) (Fig. 5B).

We also studied basal synaptic transmission in the PFC, because it is a brain area that mediates anxiety behaviors together with insular cortex and amygdala (Bishop, 2009). In contrast to the BC, the fEPSP responses to increasing amplitude of stimulation currents in the PFC were not different in Rac1 cKO mice compared to Rac1 Het mice (repeated measures ANOVA, $p>0.1$) (Fig. 4.5C). On the other hand, paired-pulse recordings showed an increased facilitation effect in Rac1 Het mice when the paired-pulse frequency was 20Hz, in agreement with other studies (Hernan, Holmes, Isaev, Scott, & Isaeva, 2013), which was impaired in the Rac1 KO mice (repeated measures ANOVA, $p<0.05$) (Fig. 4.5D). One reason behind this difference could be regional differences in the decrease in the number of interneurons. The addition of AP5 (NMDA antagonist) in the aCSF during the recordings of Rac1 Het slices had a similar impact both on basal synaptic transmission (Fig. 4.5E) and on paired-pulse ratio (Fig. 4.5F) leading to the hypothesis that there is a disruption in the function of the NMDA receptor of Rac1 cKO mice.

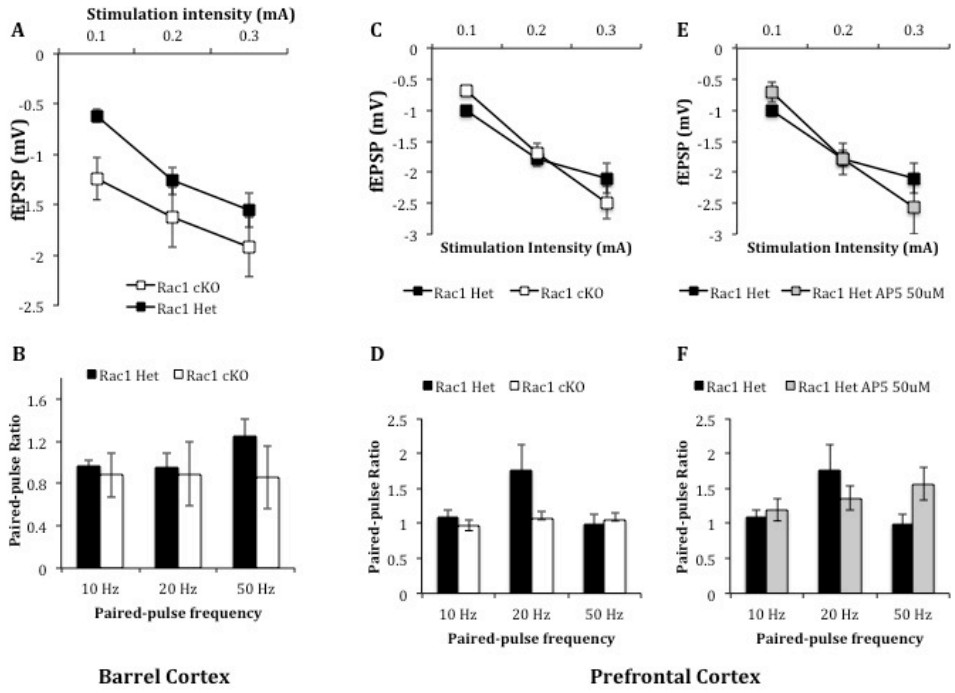


Figure 4.5. Differential changes in basal synaptic transmission and short-term synaptic plasticity in the cortex

A. *In the barrel cortex layer II, Rac1 cKO mice showed increased fEPSP responses in response to increasing stimulation currents, compared to Rac1 Het mice.* ***B.*** *No difference in the paired-pulse ratio when applying paired pulses of increasing frequency (10, 20 and 50 Hz) was observed between Rac1 Het and Rac1 cKO mice within layer II of barrel cortex.* ***C.*** *In the PFC, the fEPSP responses to increasing amplitude of stimulation currents were the same in Rac1 cKO mice and Rac1 Het mice (repeated measures ANOVA, p>0.1).* ***D.*** *In the PFC, paired-pulse recordings showed an increased facilitation effect in Rac1 Het mice when the paired-pulse frequency was 20Hz, which was impaired in the Rac1 cKO mice (repeated measures ANOVA, p<0.05).* ***E.*** *AP5 in the aCSF solution didn't change the fEPSP responses to increasing amplitude of stimulation currents in PFC.* ***F.*** *AP5 in the aCSF solution didn't change the paired-pulse ratio when applying paired pulses of increasing frequency (10, 20 and 50 Hz) in PFC.*

4.2.4. Long-term potentiation in the PFC

In light of short-term plasticity impairments in the PFC of Rac1 cKO mice, we also tested for the possible defects in long-term plasticity. For this, we studied LTP following a tetanic stimulation, which in Rac1 Het brain slices (n=17) resulted in ~50% increase of baseline fEPSP responses for at least 50 min. However, in PFC slices from Rac1 cKO mice (n=11), the same tetanic stimulation did not result in fEPSP potentiation (repeated measures ANOVA,

p<0.01) (Fig. 4.6*A*), suggesting that homeostatic changes in response to the significantly decreased inhibition result in impaired LTP in the PFC. LTP is known to require NMDA receptor activation in both the hippocampus (Bliss & Collingridge, 2013) and the PFC (Goto et al., 2010). Indeed, LTP in layer II PFC is also dependent on NMDA receptor activation, since no facilitation of the fEPSP is observed in the presence of AP5, ((2R)-amino-5-phosphonovaleric acid), a substance that blocks NMDA receptors (repeated measures ANOVA, p<0.05) (Fig. 6B).

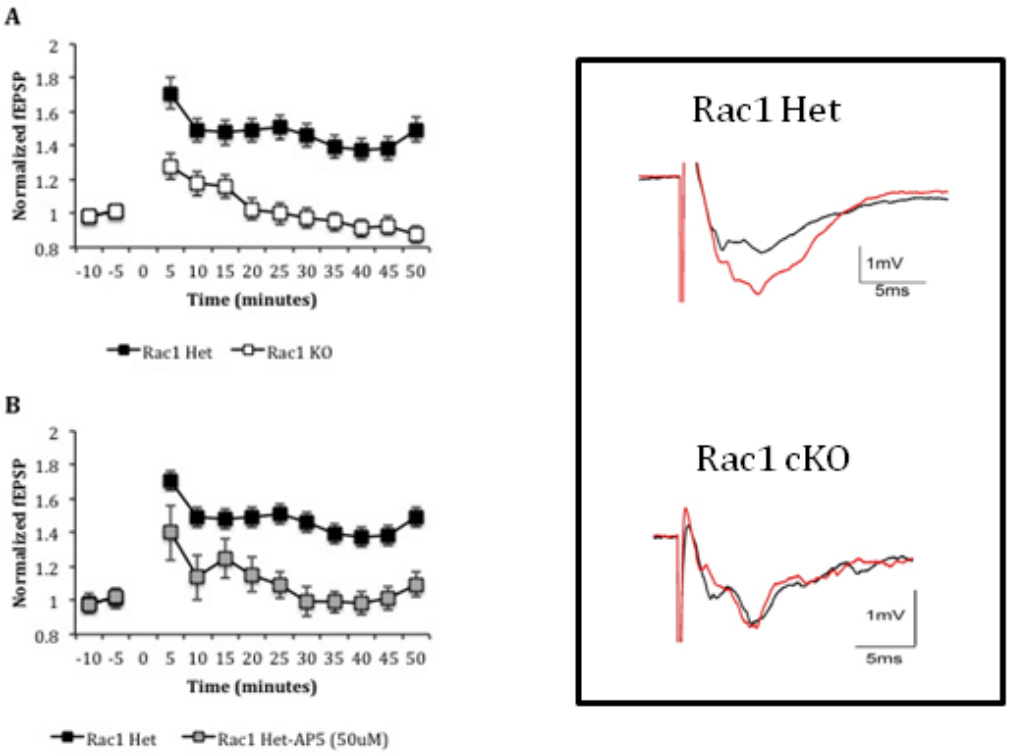


Figure 4.6. Long-term plasticity in the PFC
A. *In Rac1 Het mice, tetanic stimulation results in enhanced fEPSP for at least 45 minutes. In Rac1 cKO mice, the enhanced response is significantly smaller (inset: representative traces of baseline (black) and post-tetanic (red) response for Rac1 Het and Rac1 cKO mice) (repeated measures ANOVA, p<0.01).*
B. *The LTP observed in WT mice is NMDA-dependent, as there is no facilitation of the fEPSP in aCSF with 50uM AP5 (repeated measures ANOVA, p<0.05).*

It is also well-established that dendritic structure and particularly the number and shape of dendritic spines are positively correlated with the expression of LTP (Alvarez & Sabatini, 2007; De Roo et al., 2008; Matsuzaki, Honkura, Ellis-Davies, & Kasai, 2004; Zuo, Lin, Chang, & Gan, 2005). Therefore, we analyzed Golgi-Cox stained slides and examined the dendritic morphology of PFC pyramidal neurons in Rac1 Het and Rac1 cKO mice. Regarding the dendritic morphology of PFC pyramidal neurons, we find that Rac1 cKO mice have decreased total apical dendritic length (ttest, $p<0.1$) (Fig. 4.7A), with a significantly decreased apical dendritic diameter (ttest, $p<0.05$) (Fig. 4.7B), a decreased number of secondary apical dendritic segments (ttest, $p<0.1$) (Fig. 4.7C), with the same total secondary dendritic length (ttest, $p>0.1$) (Fig. 4.7D) and significantly decreased secondary dendritic diameter (ttest, $p<0.05$) (Fig. 4.7E). As for the number of spines measured, we find that Rac1 cKO mice have decreased total number of spines (ttest, $p<0.1$) (Fig. 4.7F) and decreased number of mushroom type spines but exhibit no significant change in the number of thin or stubby spines (Fig. 4.7G). Furthermore, the mushroom type spine density is significantly smaller (ttest, $p<0.05$) while the stubby (immature) type spine density tends to be increased in Rac1 cKO mice (ttest, $p>0.1$) (Fig. 4.7H). Proportionally there is a 1.43% and a 3.6% decrease in the percentage of thin and stubby spines for the Rac1 cKO mice respectively, while there is a 4.8% increase in the percentage of the immature stubby spines (Table 4.1). Mushroom type spines are mostly related to long-term potentiation while the thin spines are thought to mediate persistent activity in the PFC (Kasai et al., 2003). Therefore, our results show that alterations in the dendritic structure of PFC neurons could mediate the reduction in LTP in the PFC.

Table 4.1

	% thin spines	% stubby spines	% mushroom spine
Rac1 Het	34.1	44.5	21.7
Rac1 cKO	32.6	49.3	18.1
% change	-1.43	+4.8	-3.6

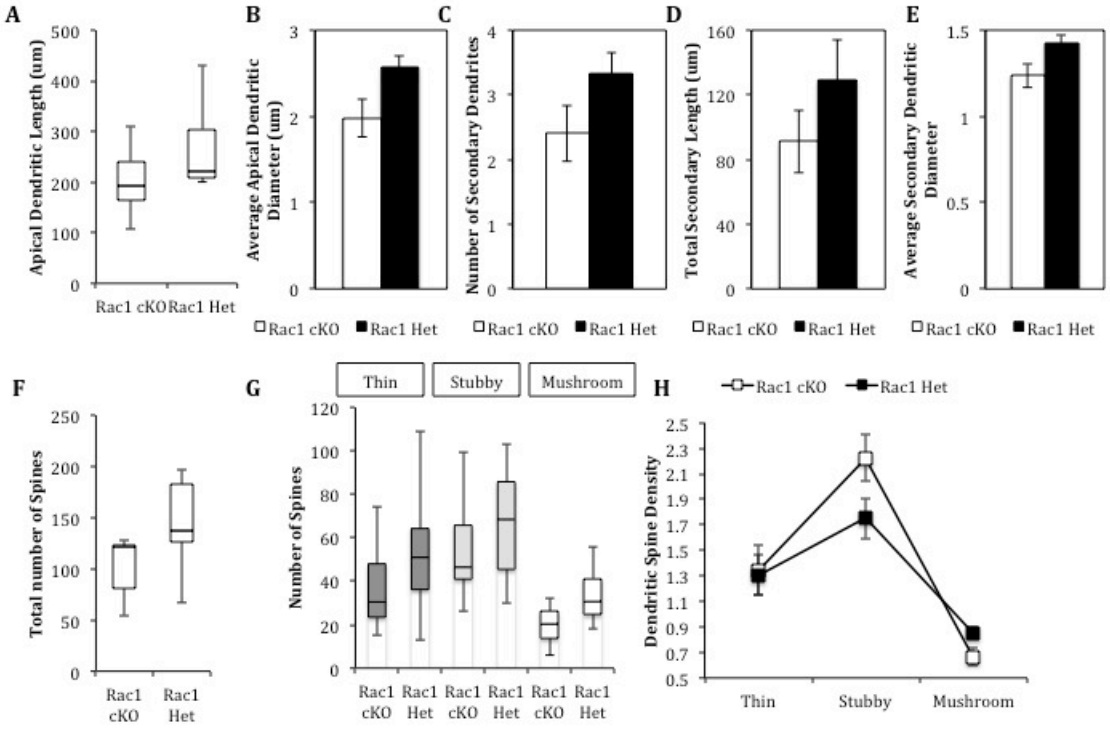


Figure 4.7. Dendritic structure in Rac1 Het and Rac1 cKO mice.
A. *Rac1 cKO mice have decreased total apical dendritic length (ttest, p<0.1)* ***B.*** *Rac1 cKOhave a significantly decreased apical dendritic diameter (ttest, p<0.05)* ***C.*** *Rac cKO have a decreased number of secondary apical dendritic segments (ttest, p<0.1)* ***D****. Rac1 cKO have the same total secondary dendritic length as Rac1 Het mice (ttest, p>0.1)* ***E.*** *Rac1 cKO mice have significantly decreased secondary dendritic diameter (ttest, p<0.05)* ***F.*** *Rac1 cKO mice have decreased total number of spines (ttest, p<0.1)* ***G.*** *Rac1 cKO mice have decreased number of mushroom type spines but exhibit no significant change in the number of thin or stubby spines* ***H.*** *In Rac1 cKO slices the mushroom type spine density is significantly smaller (ttest, p<0.05) while the stubby (immature) type spine density is tends to be in Rac1 cKO mice (ttest, p>0.1)* ***I.*** *Proportionally there is a 1.43% and a 3.6% decrease in the percentage of thin and stubby spines for the Rac1 cKO mice, while there is a 4.8% increase in the percentage of the immature stubby spines.*

4.2.5. Effect of GABAa enhancement/blocking on LTP of Rac1 cKO and Rac1 Het mice

The above findings led us to further hypothesize that the PFC network of Rac1 cKO mice exhibit an imbalance of excitation and inhibition, that it is caused both by deregulation of the glutamatergic in the presence of decreased number of interneurons in the cortex. The question is whether this reduced inhibition caused homeostatic changes to the glutamatergic system developmentally in order to moderate the increased excitability making it impossible to induce LTP in Rac1 cKO mice or some manipulation of the GABA system could reverse the abnormal state of LTP. To test this, we examined the impact of a GABA enhancer, diazepam (2uM), and a competitive GABAa receptor antagonist, bicuculline (5uM) on synaptic properties of Rac1 Het and Rac1 cKO mice. Blockade of GABAaRs with bicuculline abolished the emergence of LTP in Rac1 Het slices (repeated measures ANOVA, $p<0.1$) (Fig. 4.8A), while it didn't change the absence of LTP in Rac1 cKO slices (repeated measures ANOVA, $p>0.1$) (Fig. 4.8B). Diazepam in the aCSF while performing the LTP induction protocol in Rac1 Het mice brain slices induced an enhancement of post-tetanic augmentation followed by LTP that is maintained for at least 50 minutes which is not significant (repeated measures ANOVA, $p>0.1$) (Fig. 4.8C), while in Rac1 cKO mice is able to rescue the reduced LTP effect causing a significant LTP that maintains at least 50 minutes following the tetanic stimulation (repeated measures ANOVA, $p<0.05$) (Fig. 4.8D). These results indicate that an upregulation of GABA receptor-mediated neurotransmission is sufficient to normalize the reduced LTP induction within layer II of PFC caused by developmentally decreased inhibition.

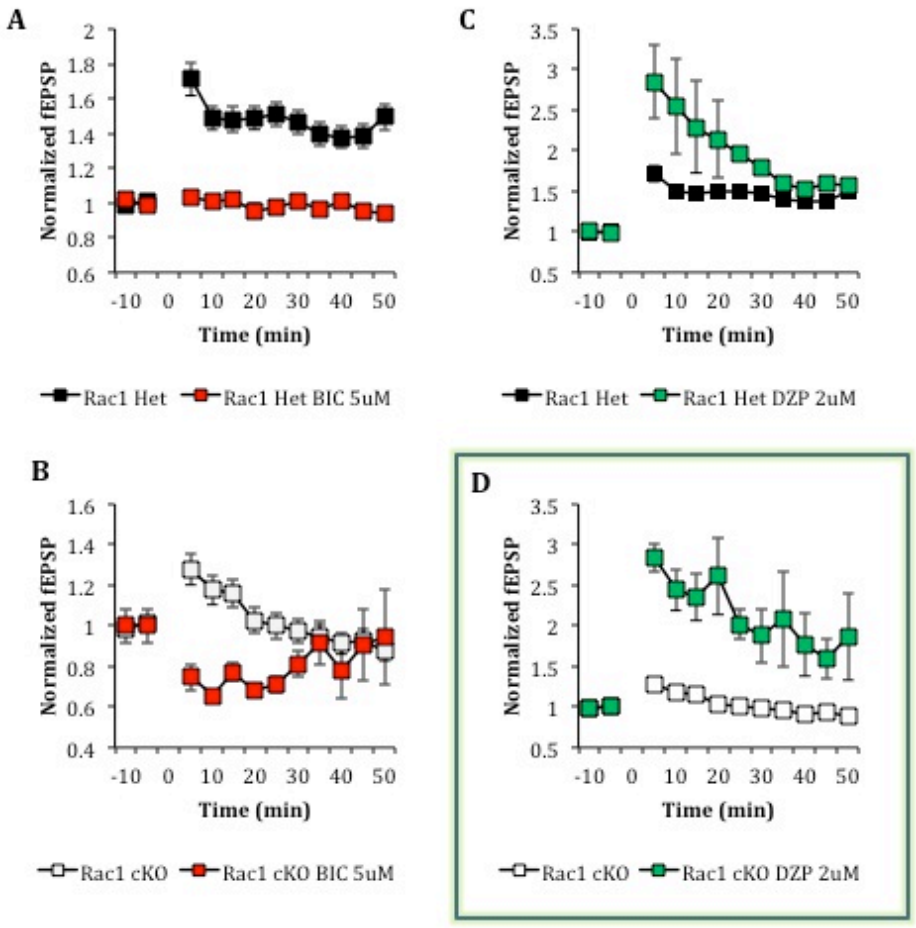


Figure 4.8. Effect of GABAa enhancement/blocking on LTP in Rac1 cKO and Rac1 Het mice

8A. *Blockade of GABAaRs with bicuculline (5uM) in the aCSF solution change the fEPSPs following tetanic stimulation, as long term synaptic depression is induced in Rac1 Het slices (repeated measures ANOVA, p<0.1)*

8B. *Blockade of GABAaRs with bicuculline (5uM) in the aCSF solution doesn't cause a change in post-tetanic synaptic activation in Rac1 cKO slices (repeated measures ANOVA, p>0.1)*

8C. *Diazepam in the aCSF while performing the LTP induction protocol to Rac1 Het causes an enhance of post-tetanic augmentation followed by LTP that is maintained for at least 50 minutes in which is not significant (repeated measures ANOVA, p>0.1)*

8D. *Diazepam in the aCSF in Rac1 cKO mice is able to rescue the reduced LTP effect causing a significant LTP that maintains at least 50 minutes following the tetanic stimulation (repeated measures ANOVA, p<0.05)*

4.2.5. Increased locomotor activity and decreased anxiety in Juvenile Rac1 cKO mice

We investigated whether the juvenile Rac1 cKO mice (PD<30) have different anxiety levels compared to juvenile Rac1 Het mice. For this, both male and female mice were tested in the open-field test and the elevated plus maze to analyze their anxiety levels. When tested in the open-field arena, juvenile Rac1 cKO mice didn't exhibit in significant differences in the number of entries in the distinct zones of the open field arena (ttest, $p>0.1$) (Fig. 4.9A), resulting in no changes in the thigmotaxis index during the critical first five minutes in the test compared to juvenile Rac1 Het mice (Thigmotaxis Index: Time to Walls/Time Center + Time Periphery) (ttest, $p>0.1$) (Fig. 4.9B). Juvenile Rac1 cKO mice exhibit increased mobility in the OFT arena in all three 5min intervals analyzed (repeated measures ANOVA, $p<0.05$) (Fig. 4.9C), indicating less anxiety caused by the open novel environment that they were exposed.

When tested in the elevated plus maze, juvenile Rac1 cKO mice enter the open arms of the elevated plus maze significantly more than the juvenile Rac1 Het mice (ttest, $p<0.05$) (Fig. 4.9D). The Ratio of Entries in the Open arms of the maze is significantly increased in Rac1 cKO mice (ttest, $p<0.05$), indicating their decreased anxiety caused by the open elevated arms of the maze (Fig. 4.9E). Collectively, the above results show that juvenile Rac1 cKO mice have a decreased anxiety phenotype that is apparent in both tasks used.

4.2.6. Altered physiological properties within layer II of PFC in juvenile Rac1 cKO mice

Next, we aimed to understand whether the synaptic properties of the juvenile Rac1 cKO cortex were altered in the presence of developmentally decreased inhibition. We studied

basal synaptic transmission by delivering current pulses of increasing intensity and recording field excitatory potentials (fEPSPs) from neocortical layer II neurons of PFC.

The fEPSP responses to increasing amplitude of stimulation currents in the PFC were decreased in juvenile Rac1 cKO mice compared to Rac1 Het mice (repeated measures ANOVA, $p<0.1$) (Fig. 4.10A). On the other hand, paired-pulse recordings showed an increased facilitation effect in Rac1 cKO mice mice when the paired-pulse frequency was 50Hz (Fig. 4.10B).

Long-term potentiation in the PFC of juvenile Rac1 cKO mice

In light of this short-term facilitation effect in the PFC of juvenile Rac1 cKO mice, we also tested for the possible alterations in LTP. For this, we employed a tetanic stimulation, which in juvenile Rac1 Het brain slices resulted in ~20% decrease of baseline fEPSP responses that maintained for at least 50 min. However, in PFC slices from juvenile Rac1 cKO mice, the same tetanic stimulation did not result in fEPSP depression but instead to induction of LTP, while fEPSP responses following titanic stimulation increased ~25% and this potentiation maintained for at least 50 min (repeated measured ANOVA, $p=0.197$) (Fig. 4.10C).

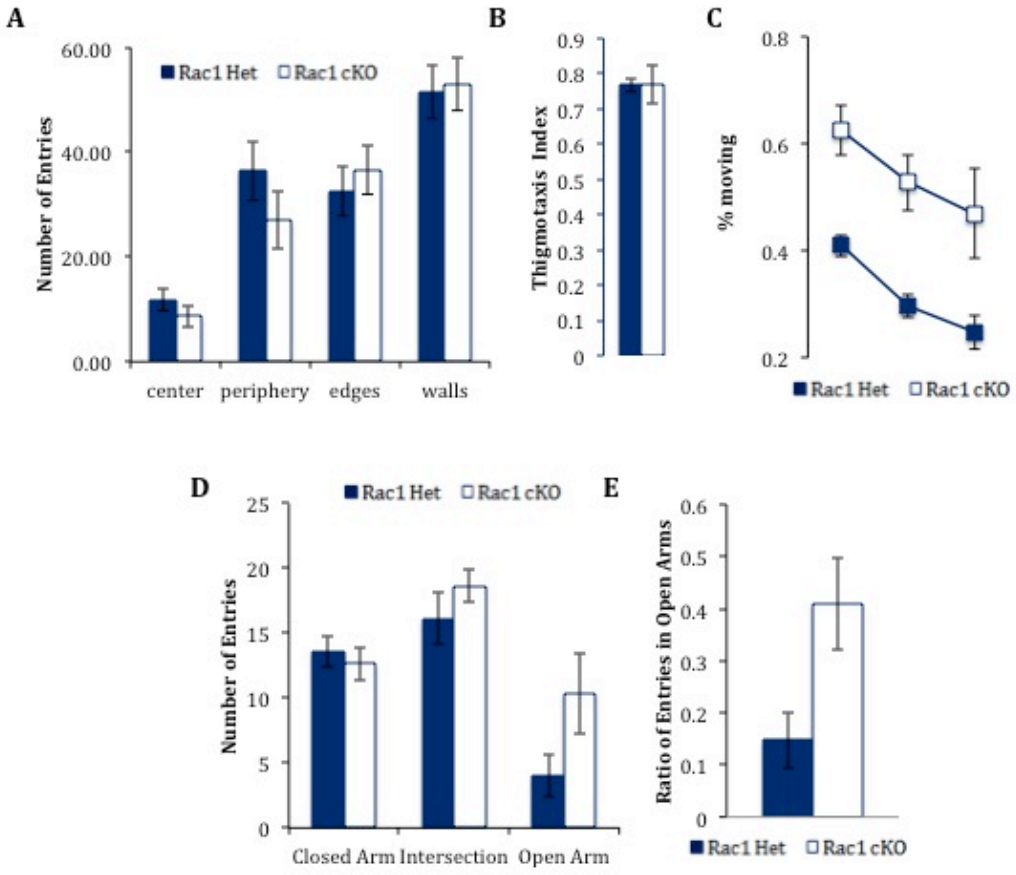


Figure 4.9. Decreased anxiety of juvenile Rac1 cKO compared to Rac1 Het mice.
1A. *Juvenile Rac1 cKO mice didn't exhibit in significant differences in the number of entries in the distinct zones of the open field arena (ttest, p>0.1)*
1B. *No changes in the thigmotaxis index during the critical first five minutes in the test compared to juvenile Rac1 Het mice (ttest, p>0.1)*
1C. *Juvenile Rac1 cKO mice exhibit increased mobility in the OFT arena in all three 5min intervals analyzed (repeated measures ANOVA, p<0.05)*
1D. *In the elevated plus maze, juvenile Rac1 cKO mice enter the open arms of the elevated plus maze significantly more than the juvenile Rac1 Het mice (ttest, p<0.05)*
1E. *The Ratio of Entries in the Open arms of the maze is significantly increased in Rac1 cKO mice (ttest, p<0.05)*

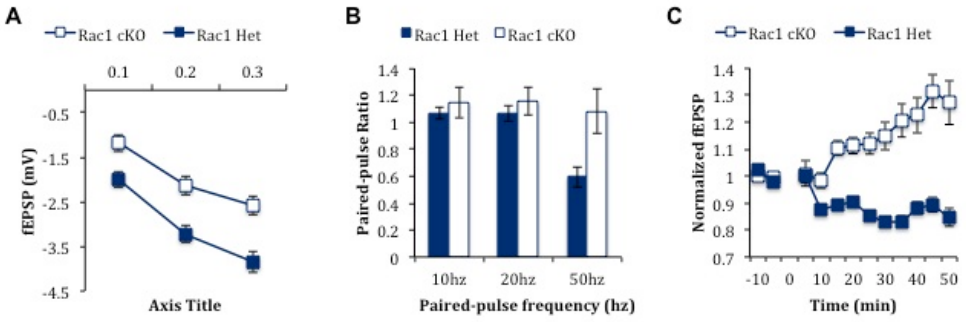


Figure 4.10. Altered physiological properties within layer II of PFC in juvenile Rac1 cKO mice

A. *fEPSP responses to increasing amplitude of stimulation currents in the PFC were decreased in juvenile Rac1 cKO mice compared to Rac1 Het mice (repeated measures ANOVA, p<0.1)* ***B.*** *Paired-pulse recordings showed an increased facilitation effect in Rac1 cKO mice mice when the paired-pulse frequency was 50Hz* ***C.*** *Tetanic stimulation, in juvenile Rac1 Het brain slices resulted in ~20% decrease of baseline fEPSP responses that maintained for at least 50 min. In PFC slices from juvenile Rac1 cKO mice, the same tetanic stimulation resulted in increased fEPSP responses by ~25% and this potentiation maintained for at least 50 min (repeated measured ANOVA, p=0.197).*

Juvenile Rac1 cKO Dendritic Morphology and Spine Density

Dendritic structure and particularly the number and shape of dendritic spines are positively correlated with the expression of LTP (Alvarez & Sabatini, 2007; De Roo et al., 2008; Matsuzaki et al., 2004; Zuo et al., 2005). Therefore, we analyzed Golgi-Cox stained slides and we quantified the dendrtitic spines and examined the dendritic morphology of PFC pyramidal neurons in juvenile Rac1 Het and Rac1 cKO mice. We find that juvenile Rac1 cKO mice have increased total apical dendritic length (ttest, $p<0.1$) (Fig. 4.11A), with an increased number of secondary apical dendritic segments (ttest, $p<0.1$) (Fig. 4.11B). As for the number of spines measured, we find that Juvenile Rac1 cKO mice have significantly increased density of stubby spines on their apical dendrite (ttest, $p<0.05$) (Fig. 4.11C), while the number of all types of spines (thin, stubby and mushroom) is significantly increased on

the apical dendrite (ttest<0.05) (Fig. 4.11D). Finally, we measured increased number of spines on the secondary dendrites of Juvenile Rac1 cKO mice (ttest<0.05) (Fig. 4.11E).

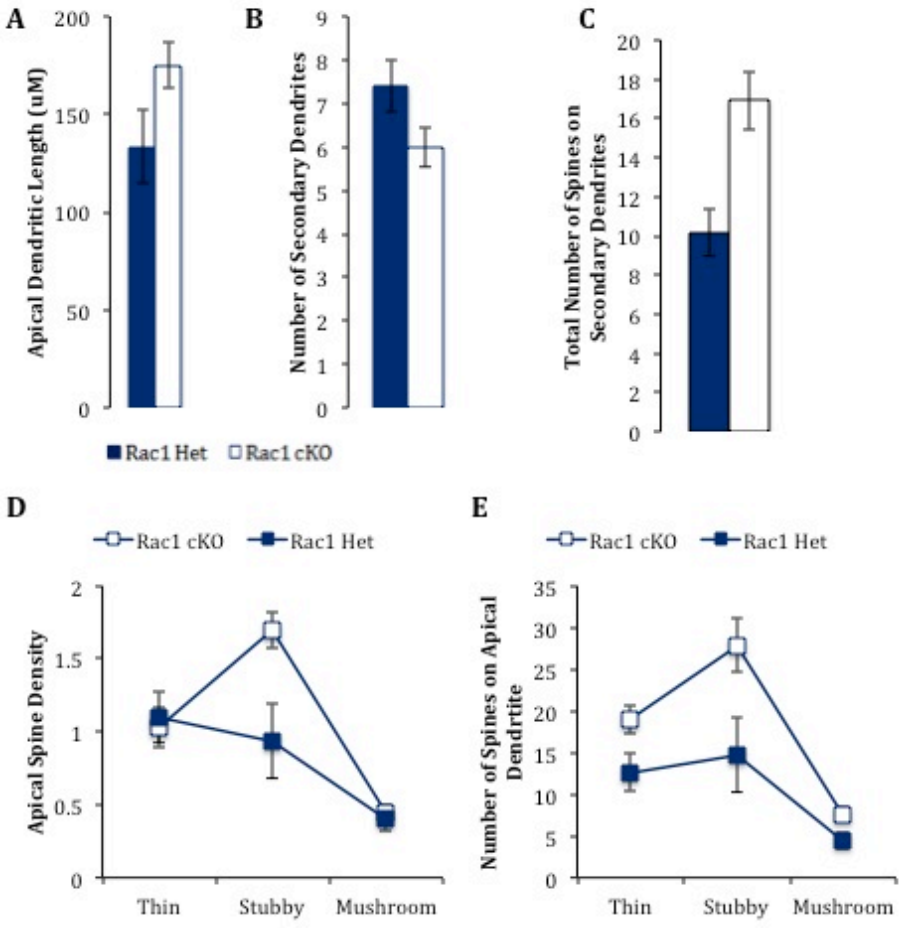


Figure 4.11. Juvenile Rac1 cKO Dendritic Morphology and Spine Density
A. *Juvenile Rac1 cKO mice have increased total apical dendritic length (ttest, p<0.1)* ***B.*** *Juvenile Rac1 cKO mice have an increased number of secondary apical dendritic segments (ttest, p<0.1)* ***C.*** *Juvenile Rac1 cKO mice have significantly increased density of stubby spines on their apical dendrite (ttest, p<0.05)* ***D.*** *In Juvenile Rac1 cKO mice the number of all types of spines (thin, stubby and mushroom) is significantly increased on the apical dendrite (ttest<0.05)* ***E.*** *Increased number of spines on the secondary dendrites of Juvenile Rac1 cKO mice (ttest<0.05)*

4.2.7. Developmental Effect of Decreased Inhibition

It seems that during development of Rac1 cKO mice the necessary changes to obtain proper excitation/inhibition balance do not occur. Basal synaptic transmission in the Rac1 cKO

mice does not differ between the juvenile and adult age groups (repeated measures ANOVA, $p>0.1$) (Fig. 4A). Specifically, juvenile Rac1 cKO mice synaptic responses resemble closer to the adult control group (repeated measures ANOVA, $p>0.1$) (Fig. 4.12A-B). The basal synaptic responses between juvenile and adult Rac1 Het mice differ (repeated measures ANOVA, $p<0.1$). In particular, basal synaptic transmission is significantly increased in Rac1 Het juvenile mice indicating reduced inhibition in this age group. As a result, although in Rac1 Het mice we see a gradual decrease of basal synaptic transmission as mice reach adulthood this effect doesn't exist in Rac1 cKO mice (Fig. 4.12B). Moreover, in Rac1 cKO mice there are no differences in short-term plasticity in response to paired-pulse stimulation between juvenile and adult Rac1 cKO mice, but it is stable (Fig 4.12C). In Rac1 Het mice short-term depression, turns to short-term facilitation in response to 20 Hz paired-pulse stimulation (Fig. 4.12D).

In Rac1 cKO mice, juvenile mice show moderate LTP following tetanic stimulation, while adult Rac1 cKO mice show a small LTD (repeated measures ANOVA, $p>0.1$) (Fig. 4E). This is the inverse relationship compared to Rac1 Het juvenile and adult mice that show a gradual increase of LTP following tetanic stimulation (repeated measures ANOVA, $p<0.05$). Response following tetanic stimulation increases as a function of age, as Rac1 Het juvenile mice exhibit long term depression of their synapses following tetanic stimulation, while Rac1 Het adult mice express LTP of about 50% (Fig. 4.12F). Together, these results indicate that the presence of decreased inhibition during the juvenile period is critical for the proper development of synaptic properties and plasticity within layer II of PFC.

Behaviorally, as indicated from the thigmotaxis index in the OFT, the Rac1 cKO adult mice have similar anxiety levels compared to their juvenile counterparts (ttest, $p>0.1$), but significantly increased thigmotaxis index compared to Rac1 cKO adult mice (ttest, $p<0.05$) (Fig. 4.13A). During the elevated plus maze test Rac1 cKO adult mice spent significantly

more time to the closed arms of the maze compared to the Rac1 Het mice (ttest, $p<0.05$), while the performance of Rac1 cKO mice in this test remain stable during development (ttest, $p>0.1$) (fig. 4.13B-C).

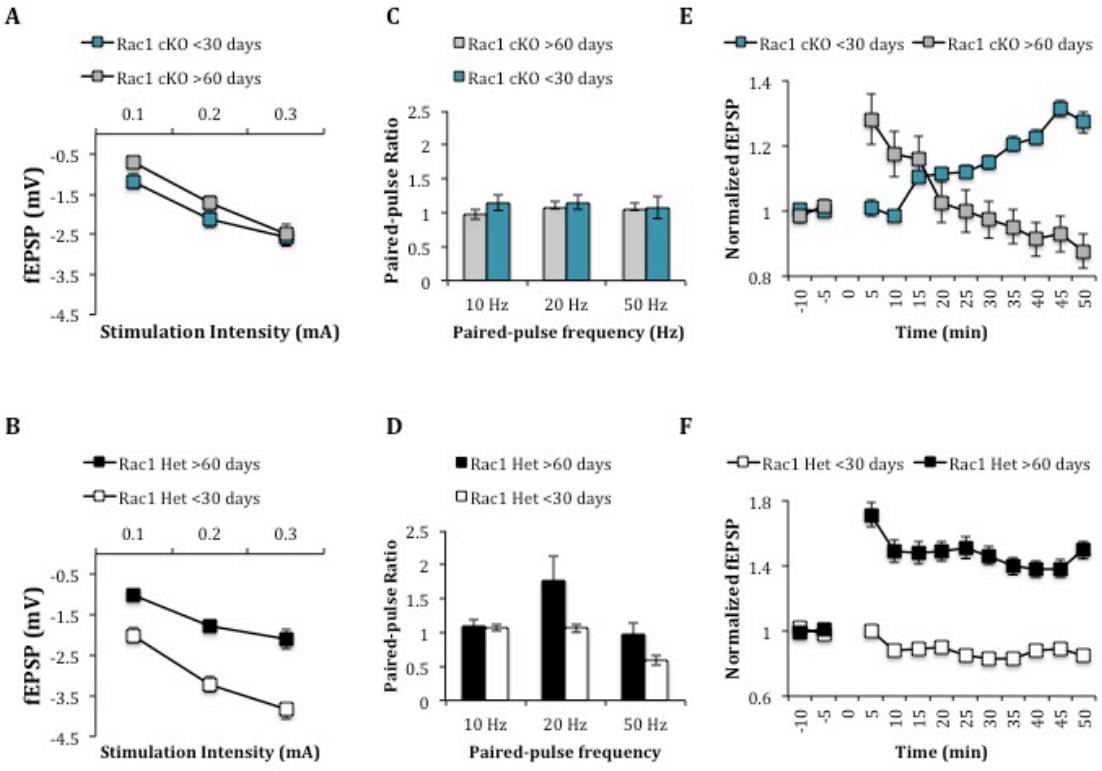


Figure 4.12. Developmental effect of decreased inhibition

A. *Basal synaptic transmission in the Rac1 cKO mice does not differ between the juvenile and adult age groups (repeated measures ANOVA, $p>0.1$)* ***B.*** *Basal synaptic transmission is significantly increased in Rac1 Het juvenile mice (repeated measures ANOVA, $p<0.1$).* ***C.*** *There are no differences in short-term plasticity in response to paired-pulse stimulation between juvenile and adult Rac1 cKO mice* ***D.*** *In Rac1 Het mice short-term depression, turns to short-term facilitation in response to 20 Hz paired-pulse stimulation* ***E.*** *In Rac1 cKO mice, juvenile mice show moderate LTP following tetanic stimulation, while adult Rac1 cKO mice show a small LTD (repeated measures ANOVA, $p>0.1$)* ***F.*** *Rac1 Het juvenile and adult mice show a gradual increase of long term potentiation following tetanic stimulation (repeated measures ANOVA, $p<0.05$). Response following tetanic stimulation increases as a function of age, as Rac1 Het juvenile mice exhibit long term depression of their synapses following tetanic stimulation, while Rac1 Het adult mice express LTP of about 50%*

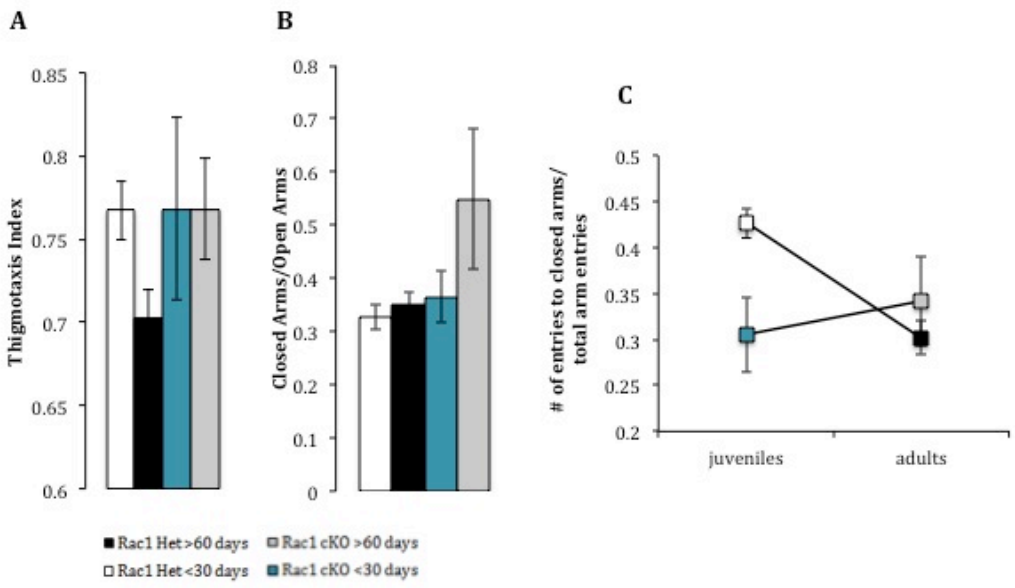


Figure 4.13. Developmental effect of decreased inhibition in mice behavior
A. *As indicated from the thigmotaxis index in the OFT, the Rac1 KO adult mice have similar anxiety levels compared to their juvenile counterparts, but significantly increased thigmotaxis index compared to Rac1 Het adult mice.* ***B-C.*** *During the elevated plus maze test Rac1 KO adult mice spent significantly more time to the closed arms of the maze compared to the Rac1 Het mice, while the performance of Rac1 KO mice in this test remain stable during development.*

4.2.8. Effect of acute disruption of NMDA or GABAa receptors on PFC physiological properties in different age groups of mice

Effect of low AP5 concentration

AP5 is a selective NMDA receptor antagonist that competitively inhibits the glutamate binding site of NMDARs, but it can also block the conversion of a silent synapse to an active one. In order to study the effect of a low concentration of AP5 in basal synaptic transmission with layer II of the PFC, we recorded field potentials from PFC layer II neurons while stimulating in layer II, about 200um away from the recording site. The application

AP5 (10uM) - in contrast to AP5 (50uM) - seems to disinhibit the cortical network, by blocking only the glutamate input towards the interneurons in adult Rac1 Het mice. There is not a significant effect of AP5 (10uM) in the basal synaptic transmission within layer II of PFC in adult Rac1 Het mice (repeated measures ANOVA, $p>0.1$) (Fig. 4.14A).

In juvenile Rac1 Het mice in slices treated with AP5 (10uM), the fEPSP caused by stimulation of low intensities is decreased. Assuming that there is decreased GABA neurotransmission in juvenile Rac1 Het mice, as revealed by their basal synaptic responses, AP5 blocks both NMDA on interneurons and on pyramidal neurons, resulting to this decreased basal synaptic response (Fig. 4.14B). Paired-pulse ratio of 20 Hz frequency loses its facilitation effect, which is apparent in adult Rac1 Het mice, in the presence of AP5 (10uM) in the aCSF solution (repeated measures ANOVA, $p>0.1$) (Fig. 4.14C). On the contrary, in juvenile Rac1 Het mice we see a facilitation affect when applying paired-pulses of 50Hz frequency (repeated measures ANOVA, $p>0.1$) (Fig. 4.14D). The short-term plasticity modulation caused by AP5 (10uM) led as to test for possible alterations in long-term plasticity. For this, we employed a tetanic stimulation, which in adult Rac1 Het mice caused a post-tetanic augmentation followed by LTP maintenance for at least 50 minutes (repeated measures ANOVA, $p>0.1$) (Fig. 4.14E). In the case of Juvenile Rac1 Het slices, AP5 (10uM) caused a greater effect, when applying the LTP induction protocol, of inducing LTP instead of LTD (repeated measures ANOVA, $p>0.1$) (Fig. 4.14F).

Effect of bicuculline

Administration of bicuculline (5uM) in the aCSF solution during recordings, in adult Rac1 Het mice causes increased fEPSP responses when applying stimulation of lower intensities within layer II of PFC (repeated measures ANOVA, $p>0.1$) (Fig. 4.15A). In juvenile Rac1 Het bicuculline (5uM) seems to decrease the basal synaptic responses (repeated measures

ANOVA, $p<0.1$). This could be considered as an indication of excitatory GABA responses, following the hypothesis that GABAa receptors in this age group in this particular area of PFC are still depolarizing (Fig. 4.15B).

Paired-pulse responses showed a significant short-term depression effect in adult Rac1 Het mice, in all stimulation frequencies (repeated measures ANOVA, $p<0.05$) (Fig. 4.15C). In juvenile Rac1 Het slices, this short-term depression effect, was also apparent, but in a milder level, when applying paired-pulses of 10 or 20 Hz frequency (repeated measures ANOVA, $p<0.1$) (Fig. 4.15D). The differential contribution of bicuculline (5uM) on PFC physiology was also apparent when measuring responses following tetanic stimulation, whereas in adult Rac1 Het mice it causes ablation of LTP (repeated measures ANOVA, $p<0.05$) (Fig. 4.15E), while it causes a mild enhancement of fEPSP responses in juvenile Rac1 Het mice (repeated measures ANOVA, $p>0.1$) (Fig. 4.15 F).

The acute pharmacological manipulations that cause excitation/inhibition balance disruption, using classical receptor antagonists, seem to contribute differentially in the two age-groups of Rac1 Het mice, revealing the developmental alterations of GABAa and NMDA receptors that occur in PFC.

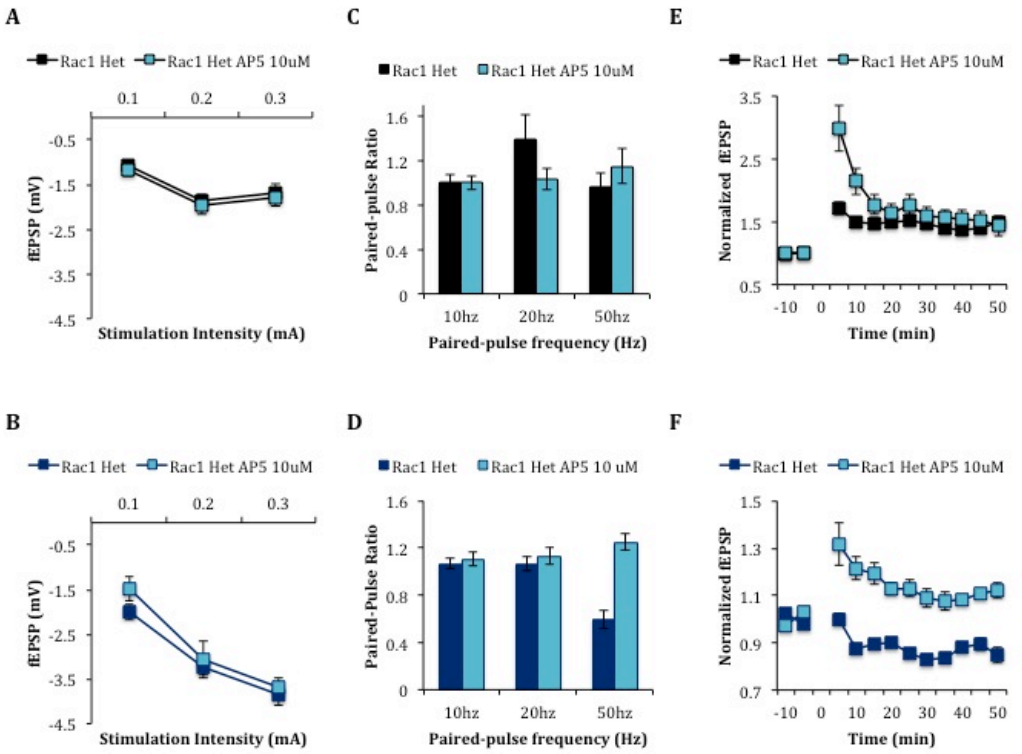


Figure 4.14. Effect of low concentration of AP5 in PFC physiological properties in different age groups of mice

A. There is not a significant effect of AP5 (10uM) in the basal synaptic transmission within layer II of PFC in adult Rac1 Het mice (repeated measures ANOVA, p>0.1) **B.** In juvenile Rac1 Het mice in slices treated with AP5 (10uM), the fEPSP caused by stimulation of low intensities is decreased. **C**. Paired-pulse ratio of 20 Hz frequency loses its facilitation effect, which is apparent in adult Rac1 Het mice, in the presence of AP5 (10uM) in the aCSF solution (repeated measures ANOVA, p>0.1) **D.** in juvenile Rac1 Het mice we see a facilitation affect when applying paired-pulses of 50Hz frequency (repeated measures ANOVA, p>0.1) **E.** Tetanic stimulation in adult Rac1 Het mice caused a post-tetanic augmentation followed by LTP maintenance for at least 50 minutes (repeated measures ANOVA, p>0.1) **F.** In the case of Juvenile Rac1 Het slices, AP5 (10uM) caused a greater effect, when applying the LTP induction protocol, of inducing LTP instead of LTD (repeated measures ANOVA, p>0.1)

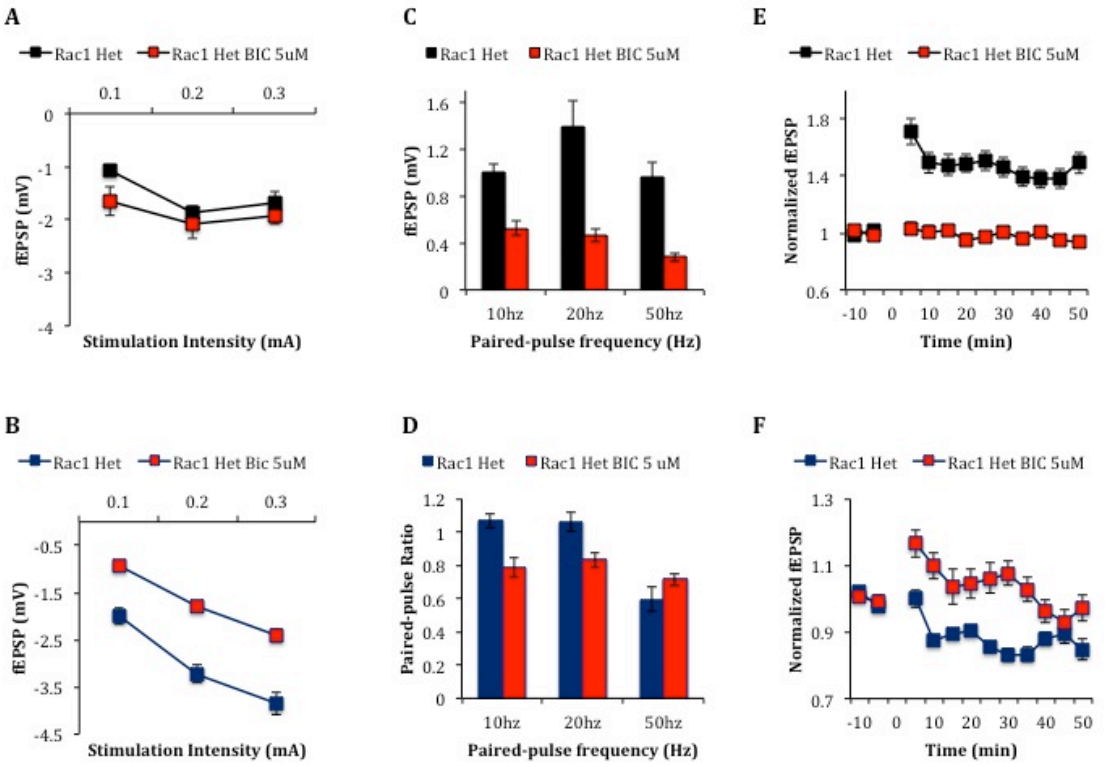


Figure 4.15. Effect of bicuculline (5uM) on PFC physiological properties in different age groups of mice

A. *Administration of bicuculline (5uM) in the aCSF solution during recordings, in adult Rac1 Het mice causes increased fEPSP responses when applying stimulation of lower intensities within layer II of PFC (repeated measures ANOVA, p>0.1)* ***B.*** *In juvenile Rac1 Het bicuculline (5uM) seems to decrease the basal synaptic responses (repeated measures ANOVA, p<0.1).* ***C.*** *Paired-pulse responses showed a significant short-term depression effect in adult Rac1 Het mice, in all stimulation frequencies (repeated measures ANOVA, p<0.05)* ***D.*** *In juvenile Rac1 Het slices, short-term depression effect apparent in a milder level (than in Fig. 2C), when applying paired-pulses of 10 or 20 Hz frequency (repeated measures ANOVA, p<0.1)* ***E.*** *In adult Rac1 Het mice bicuculline (5uM) causes ablation of LTP (repeated measures ANOVA, p<0.05)* ***F.*** *Bicuculline (5uM) in the aCSF solution causes a mild enhancement of fEPSP responses in juvenile Rac1 Het mice (repeated measures ANOVA, p>0.1)*

4.2.9. Effect of picrotoxin treatment during the juvenile period on adult mice

Finally, we used a pharmacological approach to study the developmentally decreased inhibition in behavior and PFC physiology. Picrotoxin acts as a noncompetitive antagonist for the GABAa receptor chloride channels. It is therefore a channel blocker rather than a

receptor antagonist. Juvenile Rac1 Het mice (pd20) were given daily injection of picrotoxin (1 mg/ml – non epileptic dose) or PBS intraperitoneally in their home cages for 10 days. When they reached adulthood (>2 months old) they were tested in the open field and the elevated plus maze. Subsequently female mice were handled for object recognition memory tests (preliminary data presented). While brains were taken from male picrotoxin treated mice in order to measure local field potentials, within layer 2 of PFC (Fig. 4.16A).

Picrotoxin-treated animals exhibit greater anxiety in the OFT as they seem to move less in the periphery and the center of the open field (Fig. 4.16B), resulting to a significantly increased thigmotaxis ratio as compared to Rac1 Het mice (ttest, $p<0.05$) (Fig. 4.16C). Picrotoxin-treated mice move significantly less during the first five minutes in the open field (ttest, $p<0.05$) (Fig. 4.16D). We do not see any effect of picrotoxin when performing the elevated plus maze test (ttest, $p>0.1$) (Fig. 4.16E).

In order to study possible cognitive deficits of the picrotoxin-treated mice, we performed a set of object recognition experiments. Although, these are extremely preliminary results, it seems that picrotoxin-treated mice have reduced performance in the object recognition for temporal order, indicating a low performancy in recency memory, PFC-dependent cognitive function (Fig. 4.17) It doesn't seem that they exhibit deficits in the other two recognition tasks they performed; the object-to-place (spatial memory test – hippocampus dependent) and the novel object recognition test (object recognition test – perirhinal cortex dependent).

Electrophysiological recordings within layer II of PFC revealed that stimulation intensity affected significantly the fEPSP responses (repeated measures ANOVA, $p>0.1$) while the interaction of the stimulation with the treatment was not significant (repeated measures ANOVA, $p>0.1$) (Fig. 4.18A). The application of paired-pulses of increasing frequencies (10 Hz, 20 Hz, 50 Hz) induced paired-pulse ratios with no significant difference between the two groups of mice, although we observe short term depression induced by paired pulses of

20Hz frequency (repeated measures ANOVA, p>0.1) (Fig. 4.18B). Furthermore, LTD was induced, following tetanic stimulation in slices taken from Picrotoxin-treated Rac1 Het mice (repeated measures, p<0.05). All these results resemble the results obtained by Rac1 conditional KO mice (Fig. 4.18C).

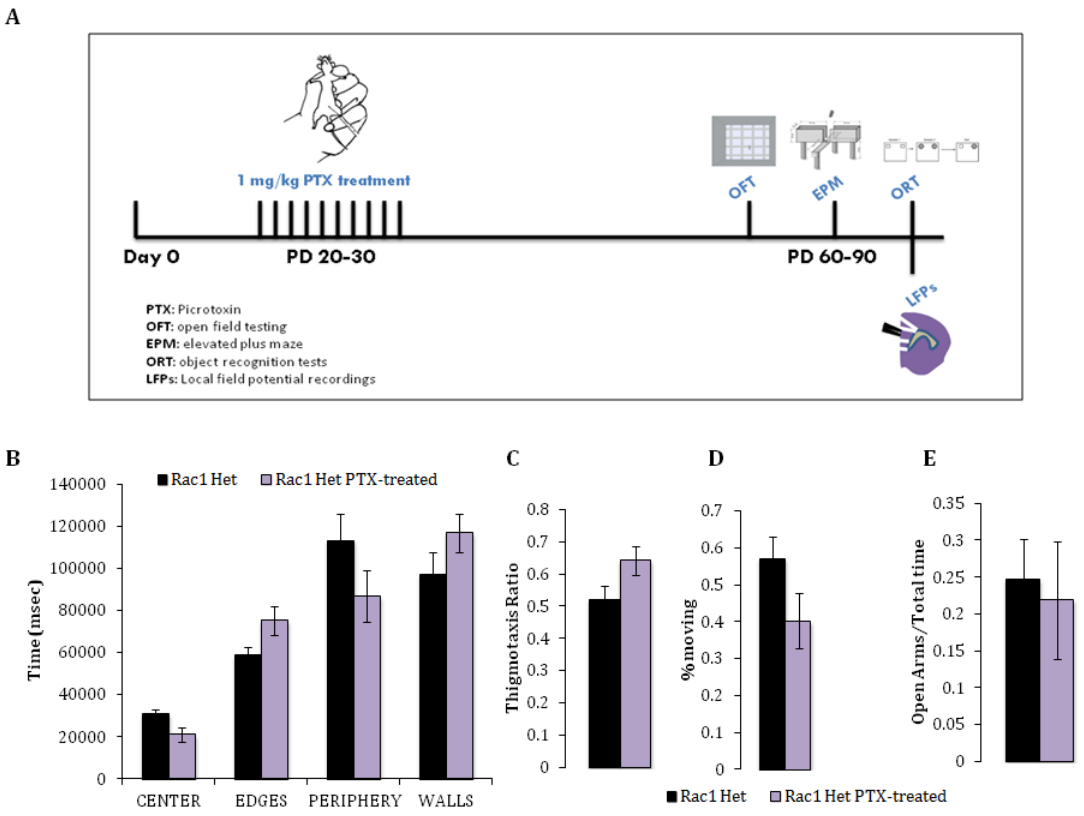


Fig. 4.16. Pharmacological induction of developmentally decreased inhibition: Picrotoxin-treated mice

A. *Juvenile Rac1 Het mice (pd20) were given daily injection of picrotoxin (1 mg/ml – non epileptic dose) or PBS intraperitoneally in their home cages for 10 days. When they reached adulthood (>2 months old) they were tested in the open field and the elevated plus maze. Subsequently female mice were handled for object recognition memory tests, while brains were taken from male picrotoxin treated mice in order to measure local field potentials, within layer 2 of PFC* ***B.*** *Picrotoxin-treated animals move less in the periphery and the center of the open field* ***C.*** *Picrotoxin-treated mice exhibit a significantly increased thigmotaxis ratio as compared to Rac1 Het mice (ttest, p<0.05)* ***D.*** *Picrotoxin-treated mice move significantly less during the first five minutes in the open field (ttest, p<0.05)* ***E.*** *We do not see any effect of picrotoxin when performing the elevated plus maze test (ttest, p>0.1)*

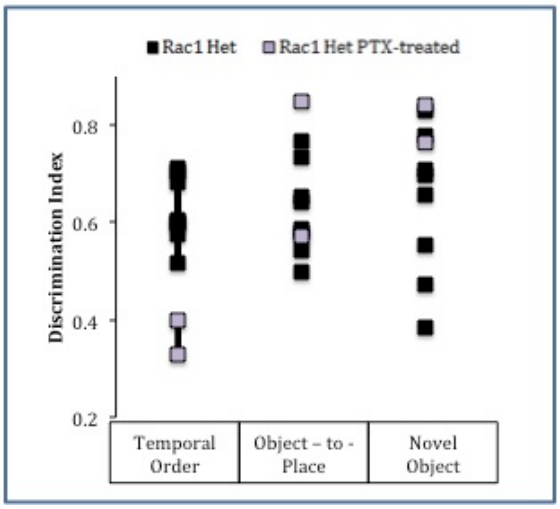


Fig. 4.17. Cognitive deficits in mice with developmentally decreased inhibition
Discrimination index of Rac1Het Picrotoxin –treated mice in the temporal order object recognition test is reduced compared to Rac1 Het non-treated mice. Discrimination indexes in the other two object recongnition tasks doesn't seem to change (preliminary results)

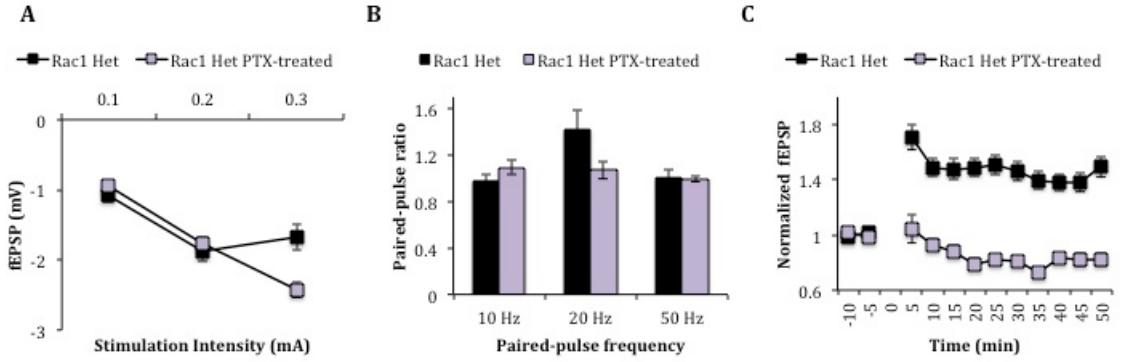


Fig. 4.18. Physiological alterations in within PFC of Picrotoxin-treated mice
A. *Stimulation intensity affected significantly the fEPSP responses (repeated measures ANOVA, p>0.1) while the interaction of the stimulation with the treatment was not significant (repeated measures ANOVA, p>0.1)* ***B.*** *The application of paired-pulses of increasing frequencies (10 Hz, 20 Hz, 50 Hz) induced paired-pulse ratios with no significant difference between the two groups of mice, although we observe short-term depression induced by paired pulses of 20Hz frequency (repeated measures ANOVA, p>0.1)* ***C.*** *LTD was induced, following tetanic stimulation in slices taken from Picrotoxin-treated Rac1 Het mice (repeated measures, p<0.05)*

CHAPTER III

MODULATORY EFFECTS OF INHIBITION

5.0. Chapter III: Modulatory effects of inhibition

5.1. Model construction

5.1.1. Microcircuit model

All models were implemented in the Neuron simulation environment (Hines & Carnevale, 2001; Papoutsi, Sidiropoulou, Cutsuridis, & Poirazi, 2013) and simulations were executed on a Xeon cluster (8 core Xeon processors). We constructed a microcircuit of 20 neuron models: 16 pyramidal models, based on (Papoutsi et al., 2013), 2 parvalbumin/fast-spiking (FS) interneuron models, 1 calbindin/regular spiking (RS) interneuron model and 1 calretinin/irregular spiking (IS) interneuron model, so that the relative number of interneurons to pyramidal model neurons was 20% (Dombrowski, Hilgetag, & Barbas, 2001) and the relative inhibitory input coming from FS interneurons was 50% (Fig. 5.1). The four different compartmental model cells were built, based on known electrophysiological data: one pyramidal neuron and three different interneurons, an FS model, an RS model and an IS model. They were connected in a network, which comprised 16 pyramidal models and 4 interneuron models (2 FS models, 1 RS and 1 IS model). Connectivity properties including the location and number of synaptic contacts, the latencies between pairs of neurons, as well as the electrophysiological properties of their synaptic connections, were based on anatomical and electrophysiological data, similar to the values reported in (Papoutsi et al., 2013). Specifically, pyramidal neuron models were fully connected recurrently (Y. Wang et al., 2006) at their basal dendrites with latencies drawn from a Gaussian distribution with $\mu = 1.7$ ms and $\sigma = 0.9$ (Alex M Thomson & Lamy, 2007). Autaptic contacts were also included and were adapted to 1/3 of excitatory connections (Lübke, Markram, Frotscher, & Sakmann, 1996).

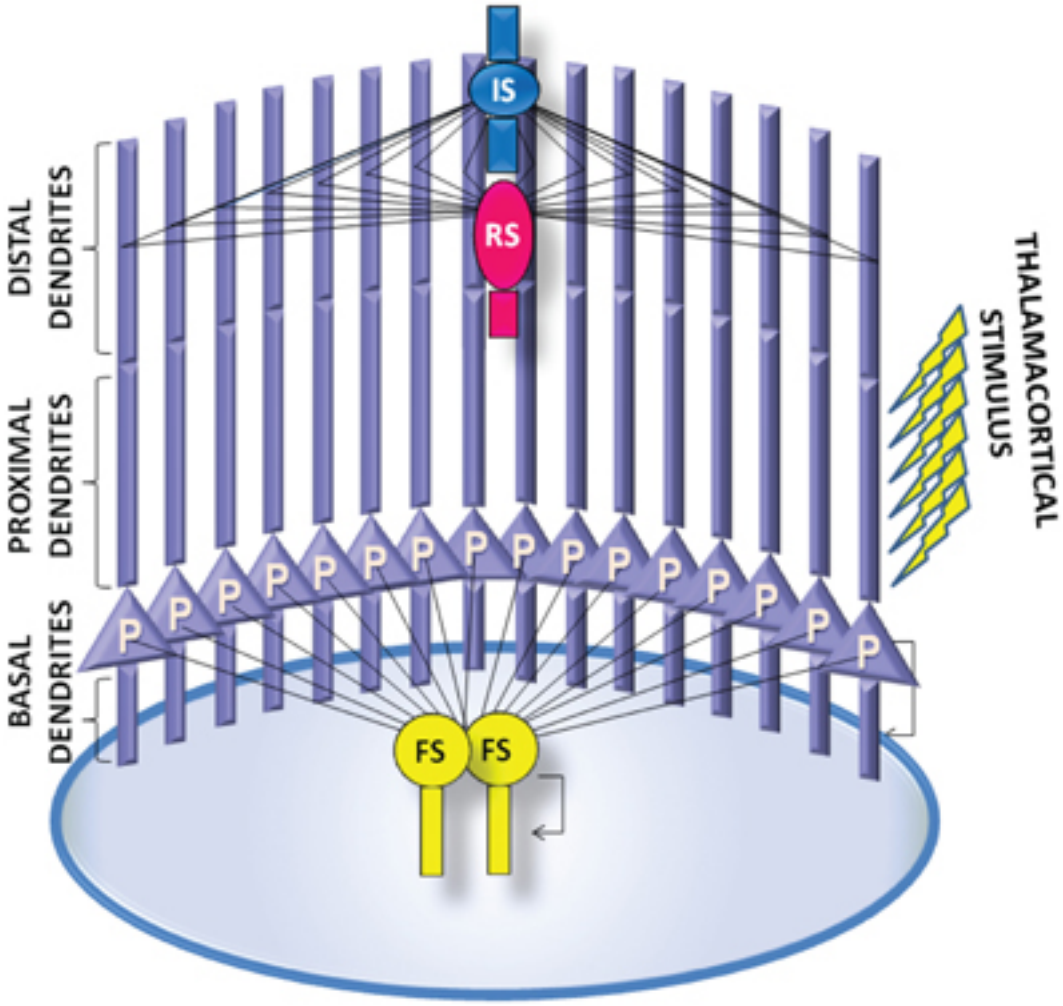


Fig. 5.1. A schematic of the PFC microcircuit. *The PFC microcircuit consists of 16 pyramidal neuron models and 4 interneuron models; 2 FS interneuron models, 1 RS and 1 IS. All neurons are fully connected through recurrent connections. The axon of each pyramidal neuron model projects to the basal dendrite of the other pyramidal neuron models. The axon of the FS interneuron model projects to the soma of all pyramidal neuron models. The axons of the RS and the IS interneuron models project to distal apical dendrite of all pyramidal neuron models. The axon of the IS interneuron model projects to the dendrite of the RS interneuron model. The pyramidal neuron and the FS interneuron models also form autaptic synapses. Providing external synaptic simulation to all 16 pyramidal neurons in their proximal apical dendrites induces persistent activity in the microcircuit.*

5.1.2. Construction of the different neuronal models

Pyramidal neuron model

The pyramidal neuron model used was based on the one published in (Dombrowski et al., 2001; Papoutsi et al., 2013) and consists of a soma, a basal, a proximal and a distal dendritic compartment. It includes modeling equations for 14 types of ionic mechanisms, known to be present in these neurons, as well as modeling equations for the regulation of intracellular calcium (same equation as in(Papoutsi et al., 2013)). The passive and active properties of the pyramidal neuron model was validated according to experimental results of (Nasif, Sidiropoulou, Hu, & White, 2005; Papoutsi et al., 2013) (Figure 5.2 and appendix, table A6). The dimensions of the somatic, axonic, and dendritic compartments of the pyramidal model cell, as well as the passive and active parameters of the model neuron are listed in Appendix, tables A1 and A2.

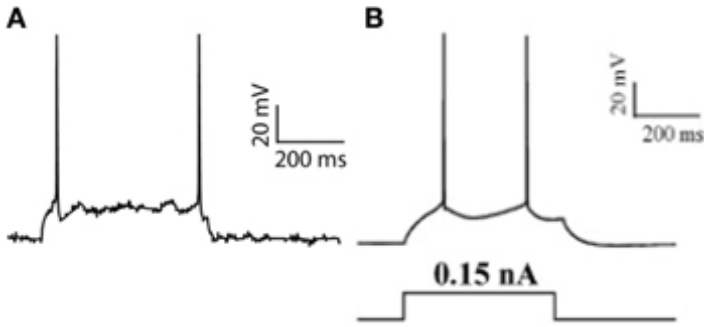


Figure 5.2. Pyramidal neuron model validation
A. Model response to a current-step pulse at the soma (0.17nA). B. Experimental response of a PFC layer V pyramidal neuron to a current step-pulse (adapted from (Nasif et al., 2005)*).*

Interneuron models

All three interneuron models included ionic mechanisms for the fast Na^+, A-type K^+ and delayed-rectifier K^+ currents, as well as modeling equations for the regulation of intracellular calcium buffering mechanism (same equations as in (Papoutsi et al., 2013)). In addition, each different interneuron model subtype included additional ionic mechanisms known to be present in each type (Toledo-Rodriguez et al., 2004; Y. Wang et al., 2006), as detailed in the following paragraphs.

FS interneuron model

The FS interneuron model consisted of three compartments: a somatic, a dendritic and an axonic compartment (Table A1, *APPENDIX*). The somatic compartment included mechanisms for the slow K^+ current (I_{Kslow}), the N-type high-threshold activated Ca^{++} current (N-type) and the hyperpolarization-activated cation current (Ih) (Table 2, *APPENDIX*), in addition to the ones mentioned above. The membrane capacitance was set to 1.2μF/cm^2 and axial resistance to 150ohm/cm (Table A3, *APPENDIX*). The resting membrane potential was adjusted to -73mV and its resulting input resistance was 250 MΩ (Y Kawaguchi & Kubota, 1993; Alex M Thomson & Lamy, 2007) (Table A6, *APPENDIX*). The action potentials of this FS model neuron had short duration and large afterhyperpolarization. It responded to a depolarizing current pulse (0.05 nA, 500 ms) with six spikes, as shown in Figure 5.3A (top), with an action potential threshold of -53mV. A depolarizing current of 0.2 nA, 500 ms resulted in a (10 spikes 100 msec) 100 Hz response (Figure 5.2A, bottom).

RS interneuron model

The RS interneuron model consisted of three compartments: a somatic, a dendritic and an axonic compartment (Table A1, *APPENDIX*) and included mechanisms for the low-threshold Ca^{++} current (T-type) and the Ih (Table A, *APPENDIX*). The membrane potential was adjusted to -64 mV (Y Kawaguchi & Kubota, 1993; Lübke et al., 1996). The membrane capacitance was set to 1.2μF/cm^2 and the axial resistance to 150ohm/cm (Table A4, *APPENDIX*). The resulting input resistance is 487 MΩ (Table A6, *APPENDIX*). The model neuron responded to a depolarizing current pulse (0.05, 500ms) with 15 spikes, with an action potential threshold of -51 mV (Figure 5.3B, bottom). A depolarizing current of 0.2 nA, 500ms resulted in a 60 Hz response (Figure 5.3B, top).

IS interneuron model

The IS interneuron model consisted of four compartments: a somatic, two dendritic and an axonal compartment, simulating a bipolar cell (Table A1, *APPENDIX*), and included mechanisms for slow K+ current, fast Ca^{++}-activated K+ current and N-type Ca^{++} current (Table A5, *APPENDIX*). The membrane potential was adjusted to -70 mV (Y Kawaguchi & Kubota, 1993)(Y Kawaguchi & Kubota, 1993), the membrane capacitance to 1.2μF/cm^2, and axial resistance to 150ohm/cm (Table A5, *APPENDIX*). Its input resistance (~545 MΩ) as indicated by electrophysiological data (Zaitsev et al., 2005) (Table A6, *APPENDIX*). The typical discharge of this cell in response to depolarizing current pulses consisted of the emission of an initial cluster of two to six action potentials, depending on the level of depolarization, followed by action potentials emitted at an irregular frequency (Cauli et al., 1997). The discharge frequency increases as a function of the stimulation intensity according to electrophysiological results of (Cauli et al., 1997) (Figure 5.3C).

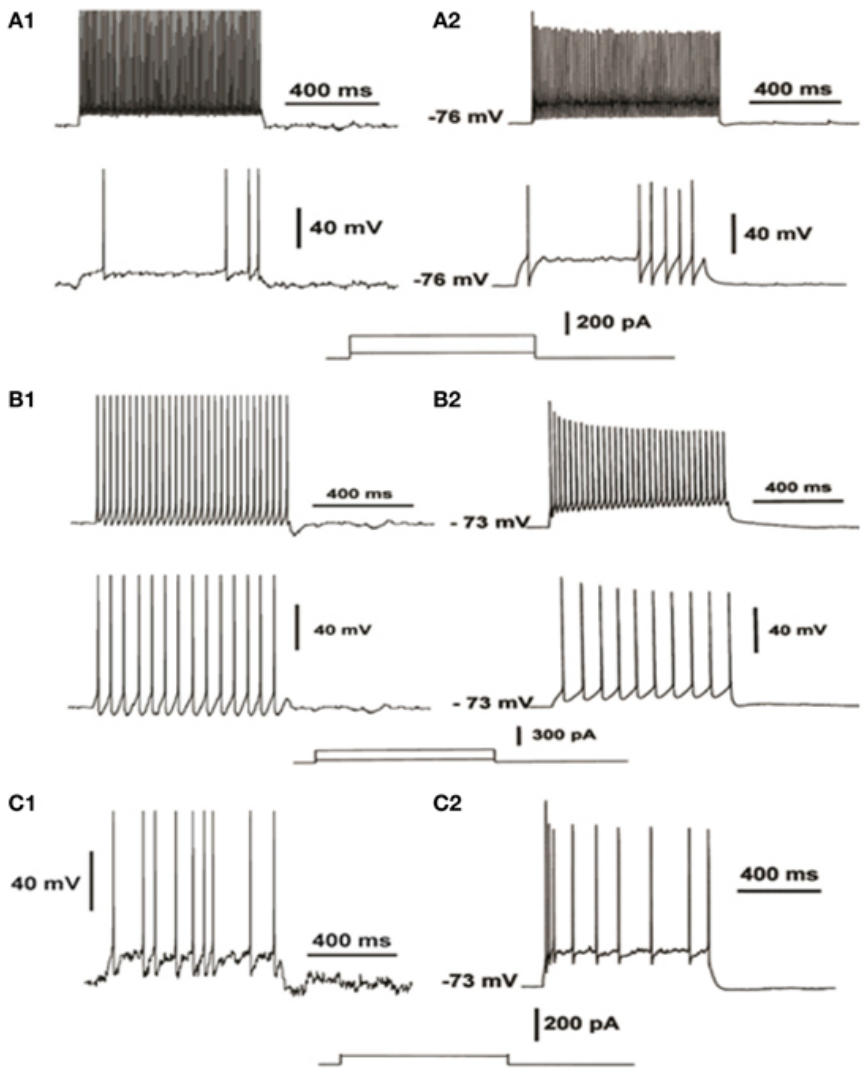


Figure 5.3. Interneuron model validation.
Spike trains in response to depolarizing current injections in the FS (A1), RS (B1), and IS (C1) interneurons models. The biophysical mechanisms were adjusted so that the model neurons exhibit similar spike trains with the experimental data (A2, B2 and C2). A2. Current-clamp recording during injection of depolarizing current pulses of 50 and 200 pA (top trace) in a layer V FS cell. B2. Current-clamp recording obtained in response to application of current pulses of 50 and 200 pA in layer II-III RS interneuron. C2. Current-cliamp recording obtained in response to depolarizing current pulse of 50 pA in a layer V IS cell. (Experimental data taken from Cauli et al.,1997)

5.1.3. Microcircuit Connectivity and properties

The axon of each pyramidal neuron model projects to the basal dendrite of other pyramidal neuron models. Pyramidal neuron models also projected to the dendrites of FS models, IS model and RS model. However, specificity of synaptic innervations in the neocortex implies that the recurrent network is not randomly arranged (Yoshimura & Callaway, 2005). The axons of the FS interneuron models project to the soma of all pyramidal neuron models. The axon of the RS interneuron model projects to the distal apical dendrite of all pyramidal neuron models (Murayama et al., 2009). The axon of the IS interneuron model projects to the soma of the RS interneuron model, providing disinhibitory input to the micorcircuit, as well as the distal apical dendrite of all pyramidal neuron models. Furthermore, inhibitory autapses are present in the FS interneuron models (Bacci, Huguenard, & Prince, 2003). A summary of synaptic connections present in the microcircuit is described in Table A7 *(APPENDIX).*

Number of synapses

The total number of excitatory synapses to the three types of interneuron models and of inhibitory synapses on the pyramidal neuron model was based on the anatomical data (Markram et al., 2004; Tamás, Buhl, & Somogyi, 1997). The total number of inhibitory synapses onto each pyramidal model neuron was 13% of the total excitatory synapses (Peters, Sethares, & Luebke, 2008). A summary of the number of synapses introduced between each type of connection is described in Table A8 *(APPENDIX)*.

Validation of the synaptic mechanisms

The conductances of excitatory and inhibitory synaptic mechanisms were adjusted according to electrophysiological recordings (Angulo, Rossier, & Audinat, 1999; Bacci, Huguenard, et al., 2003; A M Thomson & Deuchars, 1997; A. M. Thomson & Destexhe, 1999; H. Wang et al., 2008; H.-X. Wang & Gao, 2009; Woo et al., 2007; Xiang, Huguenard, & Prince, 2002). The conductance of a single AMPA-R synapse onto the pyramidal neuron model was adjusted so that it generated a voltage response of 0.1mV at the soma (Nevian, Larkum, Polsky, & Schiller, 2007). The NMDA current was validated with a simulated voltage clamp protocol to replicate the results of (H. Wang et al., 2008) (Figure 5.4A). AMPA- and NMDA-mediated currents were recorded at -70mV and +60mV, respectively, in FS and RS neuron models, according to (H.-X. Wang & Gao, 2009). Our results correspond to the experimental data, as shown in Fig. 5.4B and 5.4C. The relative proportion of NMDA and AMPA receptor mediated synaptic components of the FS models is standardized at 0.5 (H.-X. Wang & Gao, 2009). The relative proportion of NMDA and AMPA receptor mediated synaptic components of RS models is standardized at 0.8 (H.-X. Wang & Gao, 2009). In lack of experimental data for the IS neuron model, its AMPA- and NMDA- mediated currents were also simulated to match those of the FS and RS neuron models, whereas the NMDA-to-AMPA ratio was adapted so that the IS interneuron model could fire action potentials during the stimulus.

Furthermore, $GABA_A$ receptor mediated currents (IPSCs), between the FS interneuron and the pyramidal neuron were validated, based on (Woo et al., 2007) and the $GABA_B$ receptor mediated IPSC was validated against experimental data from (A M Thomson, Deuchars, & West, 1996) as in (Papoutsi et al., 2013). According to (Xiang et al., 2002), the amplitude of IPSCs for FS-Pyramidal pairs had a mean value significantly larger than RS-Pyramidal pairs. In particular, the $GABA_A$ mediated current between the RS-Pyramidal neuron pair should be

1/10 of the $GABA_A$ mediated current between FS-Pyramidal cell pair (Xiang et al., 2002). Due to lack of experimental data for the IPSCs of the IS-Pyramidal neuron pair, $GABA_A$ mediated current of this pair was estimated to be 1/10 of the $GABA_A$ of RS-Pyramidal pair. The autoinhibition of PV interneurons is much stronger than the inhibition between interneurons of different types, such as IS-RS pairs (Bacci, Rudolph, Huguenard, & Prince, 2003). Autaptic inhibitory currents in FS interneurons evoked a relatively large transient current of 0.35mA amplitude (Bacci, Huguenard, et al., 2003). The aforementioned current was simulated as described in (Papoutsi et al., 2013). Across different experiments, the NMDA-to-AMPA ratio of 1.25 and the GABAb-to-GABAa ratio of 0.2 were taken as control states.

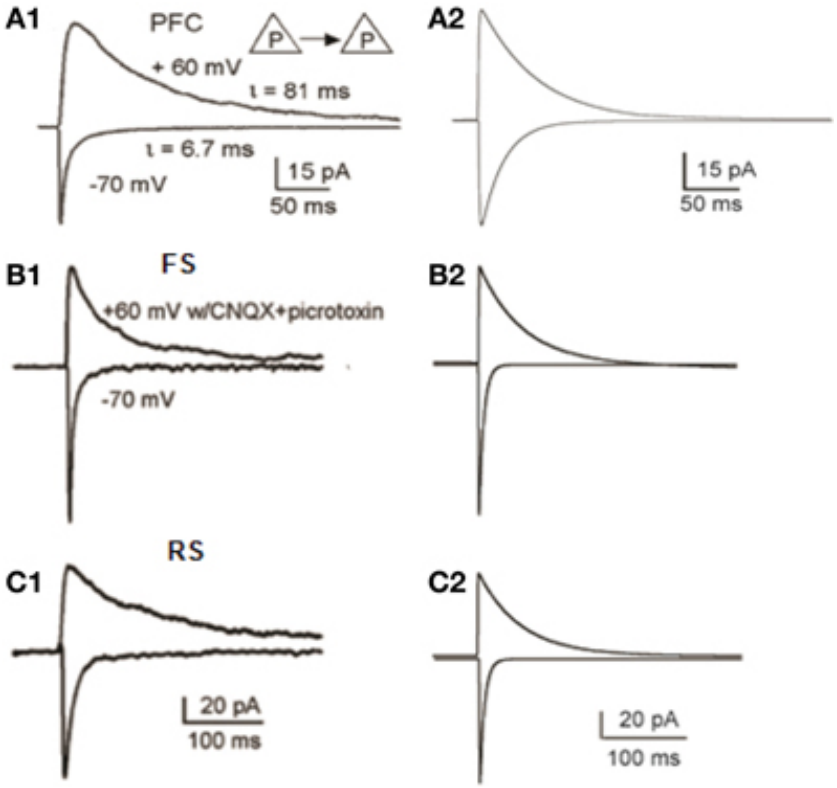


Figure 5.4. Validation of Synaptic Properties within the microcircuit
A1. Examples of single-pulse recordings of layer 5 P-P pairs from PFC. When membrane potentials were held at -70 mV, the currents are predominantly mediated by AMPA receptors; whereas at +60 mV with CNQX and picrotoxin in the bath solution blocking the AMPA and GABAa channels, respectively, the currents were largely mediated by NMDAR channels. (Wang et al., 2008) A2. Modeling validation of AMPA and NMDA receptors' properties of the

pyramidal neurons in the PFC microcircuit. B1-B2. Properties of AMPAR-mediated currents in FS interneurons. B1. Samples of AMPAR- and NMDAR- mediated currents recorded at -70 and +60 mV, respectively FS interneurons (Wang & Gao, 2009). B2. Modeling validation of AMPA and NMDA receptors' properties of FS interneurons in the PFC microcircuit. C1-C2. Properties of AMPAR-mediated currents in RS interneurons. B1. Samples of AMPAR- and NMDAR-mediated currents recorded at -70 and +60 mV, respectively RS interneurons (Wang & Gao, 2009). B2. Modeling validation of AMPA and NMDA receptors' properties of RS interneurons in the PFC microcircuit.

Background noise

In addition, for best simulation of membrane potential fluctuations as observed *in vitro* due to the stochastic ion channel noise (Linaro, Storace, & Giugliano, 2011), an artificial current with Poisson characteristics (mean rate 0.02Hz) was injected in the soma of all neuron models. Specifically, for the IS neuron model, the amplitude of this mechanism was larger (mean rate 0.035Hz) (Golomb et al., 2007).

5.1.4. Stimulation protocol

The proximal apical dendrites of the pyramidal neuron models were stimulated with 120 excitatory synapses (containing both AMPA and NMDA receptors), which were activated 10 times at 20Hz (yellow arrows in Figure 5.1) (Kuroda, Yokofujita, & Murakami, 1998). Since neurons within a microcircuit share similar stimulus properties (Petreanu, Mao, Sternson, & Svoboda, 2009; Yoshimura & Callaway, 2005), the same initial stimulus was delivered to all pyramidal neurons.

5.1.5. Analysis

Data analysis was performed in Matlab (Mathworks, Inc). Inter-Spike-Intervals (ISIs) were calculated for the neuronal response of each neuron model of the microcircuit during the stimulus and during persistent activity. An average of the ISIs of each neuron of the network, as well as coefficient of variations, in 500ms time bins was measured for each experimental state.

The Synchronization or de-synchronization of the neurons was measured using the SPIKE-distance measurement, which is sensitive to spike coincidences (Kreuz, Chicharro, Greschner, & Andrzejak, 2011). For this measurement we obtained the spike trains simultaneously from the neuronal population of the microcircuit and then we calculated the time intervals between successive spikes occurring in any of the participating neurons. If there are no phase lags between the spike trains (neurons fire synchronously) the synchronization index will have values of zero. In general, small values of synchronization index indicate synchronicity, whereas large values indicate asynchronous spiking activity (as in (Papoutsi et al., 2013)).

As an additional estimation of the synchronization or de-synchronization among spiking neurons in the microcircuit during each different condition, we measured the total number of spikes recorded in 1 millisecond time bins, and constructed plot with the discrete -time firing rate.

Power spectra were generated on the summed synaptic currents (AMPA, NMDA and $GABA_A$) generated by the pyramidal neurons in the network, averaged for 10 trials, over a one-second period of steady-state persistent activity, 3 seconds after the end of the stimulus. The averaged synaptic currents were first decimated and then, the mean square power spectrum was calculated using the periodogram method.

5.2. Results

We used a 20-neuron PFC microcircuit model that included 16 biophysically-detailed pyramidal cell models and 4 interneuron models: 2 FS, 1RS and 1IS interneuron model, in order to study the role of these interneuron cell-types in persistent activity emergence and maintenance properties. All modeled neurons were validated against experimental data from intracellular recordings in brain slices (Figure 5.3 - see methods for details). In addition, the synaptic mechanisms were validated against experimental data (AMPA current, NMDA-to-AMPA ratio, GABA currents) (Figure 5.4 – see methods for details).

Persistent activity in the network was induced by an external excitatory stimulus to the apical dendrite (Figure 5.5). Similar to a smaller version of the microcircuit model (which included 7 pyramidal model neurons and 2 FS interneurons (Papoutsi et al., 2013), persistent activity induction was dependent on the $GABA_B$-to-$GABA_A$ and NMDA-to-AMPA ratio on the pyramidal neuron models (Figure 5.5A). Each neuron model had a different firing pattern during persistent activity, depending on its own electrophysiological characteristics (Figure 5.5B). The interspike intervals (ISIs) of the pyramidal neuron model during persistent activity were between 60-120ms, i.e. firing frequency of 8-17Hz (Figure 5.5C). The coefficient of variation (CV) of the ISIs, although not very high as observed in vivo (Compte, 2006), was greater during persistent activity compared to the CV during the stimulus (Fig. 5D). Furthermore, we find that spiking activity of neurons in the network was synchronized both during the stimulus response and the persistent activity, although synchronicity during the stimulus was greater compared to that during persistent activity (Figure 5.5E). These properties are similar to the corresponding properties observed in persistent activity during working memory tasks (Constantinidis & Procyk, 2004).

Increasing the NMDA-to-AMPA ratio onto pyramidal neuron models decreased the ISIs, especially during the initial phases of persistent activity (Figure 5.5F), suggesting an

increase in the firing frequency. On the other hand, modulating the NMDA-to-AMPA ratio onto FS models does modulate the % probability for induction of persistent activity (Figure 5.5G) but not the ISIs of the pyramidal neuron model (Figure 5.5H). This is in accordance with the notion that regulation of NMDA receptors in FS interneurons modulates prefrontal cortical function (Homayoun & Moghaddam, 2007), although the slow kinetics of the NMDA receptors may not allow for immediate change in network firing (Rotaru, Yoshino, Lewis, Ermentrout, & Gonzalez-Burgos, 2011). For the rest of the study we used the following conditions: $GABA_B$-to-$GABA_A$ ratio=0.2, NMDA-to-AMPA=1.25 (pyramidal neuron model), and NMDA-to-AMPA=0.5 (FS interneuron model).

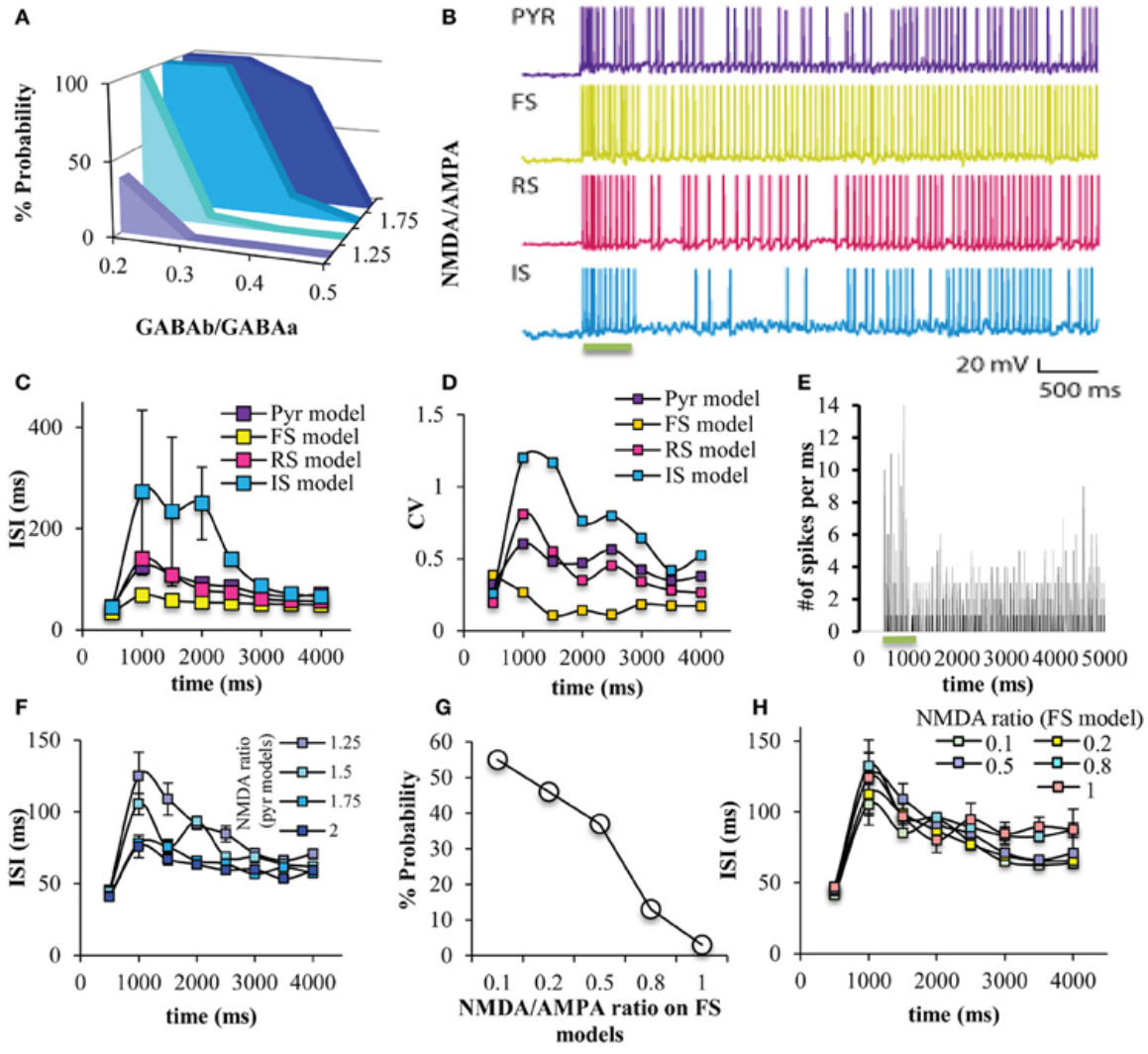


Figure 5.5. Persistent activity in the network was induced by an external excitatory stimulus to the apical dendrite
A. The probability of persistent activity induction (measured out of 100 trials) was dependent on the GABAB-to-GABAA and NMDA-to-AMPA ratio on the pyramidal model neurons, as previously seen (Papoutsi et al., 2013). B. Representative traces of all neuron models during the stimulus and during persistent activity. C. Graph showing the ISIs during the stimulus and persistent activity in 500ms bins, for all neuron models. D. Graph showing the coefficient of variation during the stimulus and persistent activity in 500ms bins, for all neuron models. The ISI is increased during the initial phase of persistent activity for all model neurons compared to the stimulus. This is not the case for the FS interneuron model. E. Discrete-time firing rate plot showing the number of neurons that fire synchronously during the stimulus and during persistent activity. F. Changing NMDA-to-AMPA ratio on pyramidal neuron models modulated the ISIs, especially during the initial phases of persistent activity. G. Changing NMDA-to-AMPA ratio on FS interneuron models modulated the probability for induction of persistent activity. H. Changing NMDA-to-AMPA ratio on FS interneuron models did not modulated the ISIs.

In order to study the role of the different interneuron cell types in persistent activity, we next simulated 'knock-out' networks for each interneuron subtype, Thus, we generated a PFC microcircuit without the FS models ('FS KO') (Figure 5.6A1), a microcircuit without a RS model ('RS KO' network) (Figure 5.6A2), and a microcircuit without an IS model ('IS KO' network) (Figure 5.6A3). We find that the probability for persistent activity induction is always 1, across all $GABA_B$-to-$GABA_A$ and NMDA-to-AMPA ratios in the 'FS KO' network (Figure 5.6B1), while it is not significantly altered in the 'RS KO' and 'IS KO' network models (Figure 5.6B2-3). Therefore, the sensitivity to biophysical modulation is completely lost in the 'FS KO' network. In addition, the ISIs during the stimulus and during persistent activity are significantly decreased in the 'FS KO' network to 15ms (i.e. close to 80Hz frequency), but not significantly altered in the 'RS KO' and 'IR KO' networks (Figure 5.6C). As well, the CV of the ISIs of pyramidal neurons during the stimulus and during persistent activity is significantly decreased in the 'FS KO' network (Figure 5.6D). This indicates that the firing rate and its variability of pyramidal neuron models is tightly controlled by the activity of FS interneuron models, but not the RS and IR interneurons. Finally, neuronal synchronicity during persistent activity is also significantly decreased in the 'FS KO' network, as evident

by the desynchronization index measure and the discrete-time firing rate plot (Figure 5.6E-F). This is again in accordance with other studies suggesting a contribution of FS interneuron spiking on neuronal synchronization and oscillations (Sohal, Zhang, Yizhar, & Deisseroth, 2009).

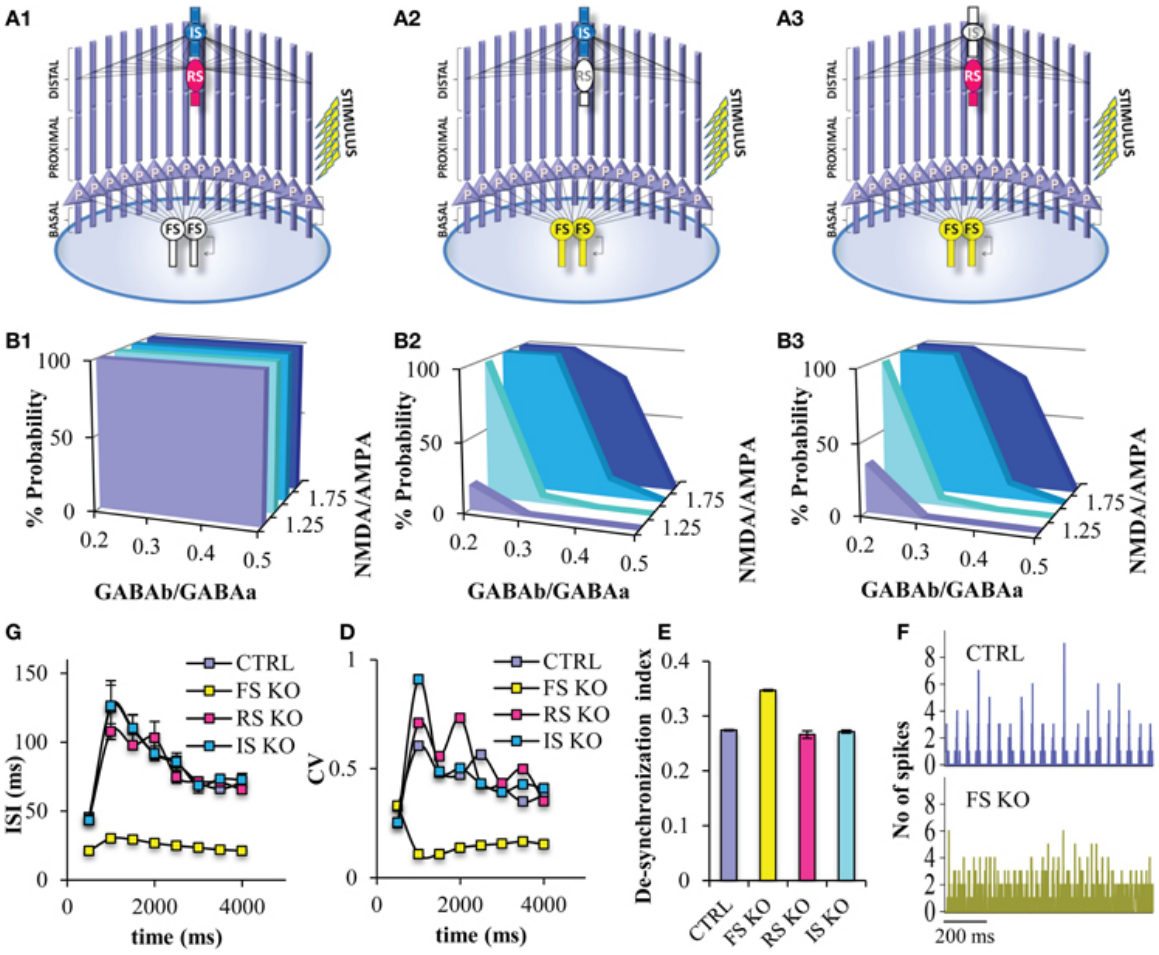


Figure 5.6. Persistent activity properties at different simulated 'interneuron KO' network models

A. All representations of the FS interneuron KO network model (A1), the RS interneuron KO network model (A2), the IS interneuron KO network model (A3). B. Graphs showing persistent activity induction across different NMDA-to-AMPA and $GABA_B$-to-$GABA_A$ ratios in the FS interneuron KO network model (B1), the RS interneuron KO network model (B2), the IS interneuron KO network model (B3). C. Graph showing the ISIs before and during persistent in 500ms bins for the control and the different 'KO' network models. D. Graph showing the CVs of ISIs of the pyramidal neuron model before and during persistent in 500ms bins for the control and the different 'KO' network models. E. Graph showing the de-synchronicity index in the

control and different 'KO' network models. F. Discrete-time firing rate plot showing the synchronization among all neuron models during persistent activity in the 'control' (top) and 'FS KO' network models (bottom)

Since the 'FS KO' net was the only one showing significant differences with regards to persistent activity properties, we wanted to further study the role of the FS interneuron model. Thus, we gradually decreased the number of GABAergic synapses (both $GABA_A$ and $GABA_B$) from the FS interneuron model onto the pyramidal neuron model in order to simulate a less severe, and possibly more realistic, disruption in the FS neuronal functioning. We find that decreasing the FS model inputs onto the pyramidal neuron model increases the probability for persistent activity induction across the different $GABA_B$-to-$GABA_A$ (NMDA-to-AMPA=1.25), while when 40% or less of FS inputs remain, persistent activity is induced across all $GABA_B$-to-$GABA_A$ ratios tested (Figure 5.7A). This suggests that once more than 50% of PV inputs are lost, then the PFC microcircuit behaves as if no FS model is present, with regards to induction of persistent activity. This large increase in persistent activity induction renders the microcircuit insensitive to modulation of $GABA_B$. Furthermore, as FS model inputs decrease, the ISIs of the pyramidal neuron model during persistent activity gradually decrease, hence the firing frequency gradually increases (Figure 5.7B). The variability of ISIs is also decreased when FS inputs decrease to 60% or more, making this index the most sensitive to FS inputs (Figure 5.7C). Finally, the desyncrhonization index among neuron models in the microcircuit gradually decreases while decreasing the number of FS inputs, but then increases when no FS inputs are present (i.e. 'FS KO' net) (Figure 5.7D). This suggests that synchronicity actually increases when a percentage of FS inputs to the pyramidal neuron models are blocked but then decreases when no inputs are present.

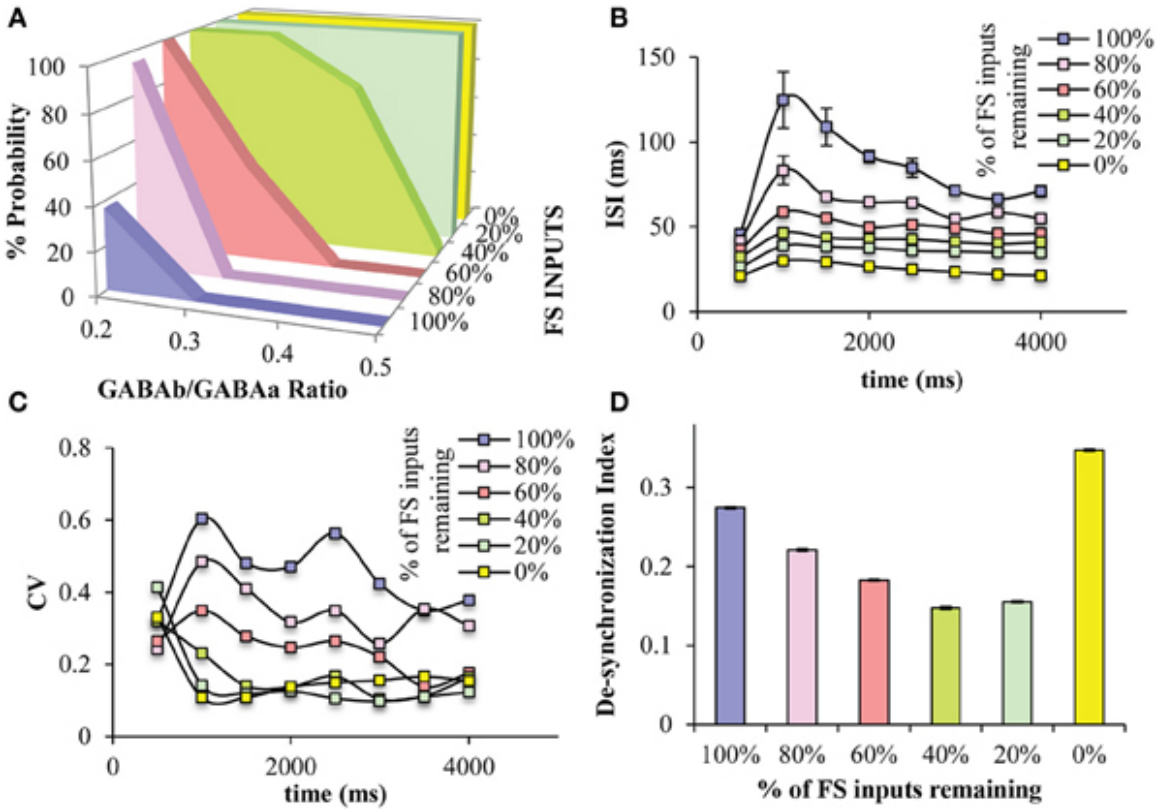


Figure 5.7. Effects of decreased number of GABAergic synaptic inputs from the FS models to the pyramidal neuron models.
A. Decreasing the number of synaptic inputs from the FS model neurons to the pyramidal model neurons increases the range of $GABA_B$-to-$GABA_A$ ratios, in which persistent activity is induced. When less than 40% of FS inputs are present in the microcircuit, persistent activity is induced 100% across all $GABA_B$-to-$GABA_A$ ratios. B. As the number of the FS inputs decreases, the ISIs of the pyramidal neuron model decreases. C. As the number of the FS inputs decreases, the CV of the ISIs of the pyramidal neuron model decreases. D. The synchronicity among all neuron models during persistent activity, is significantly reduced when no PV inputs are present in the microcircuit, while it increases by decreasing the number of synaptic inputs from the FS model neurons to the pyramidal model neurons

Many of the roles of FS neurons on cortical network functions have been attributed to its specific connectivity, specifically the projection of FS neurons to the soma of the pyramidal neurons (Lovett-Barron et al., 2012; Royer et al., 2012). However, by design experimental manipulations cannot differentiate between the target location of an interneuron and its physiological characteristics. So, the next step was to study in detail the role of this specific

connectivity by changing the projection site of the FS neuron model to different dendritic locations of the pyramidal neurons other than the soma; on the basal dendrites (D0 net) (Figure 5.8A1), on the proximal dendrites (D1 net) (Figure 5.8A2), on the distal dendrites (D2 net) (Figure 5.8A3). When the FS input is located anywhere else but the soma, then, the probability for induction of persistent activity increases to 100% (Figure 5.8D), while the ISIs and ISI variability during persistent activity significantly decrease (Figure 5.8B-C). Furthermore, when the FS model input is located to dendritic locations and not the soma the desynchronization index decreases, suggesting an increase in synchronicity (Figure 5.8E). Therefore, if FS neuron models do not project to the soma, network activity during persistent activity resembles the state where 50% of less FS inputs to the soma are active (Figure 5.7).

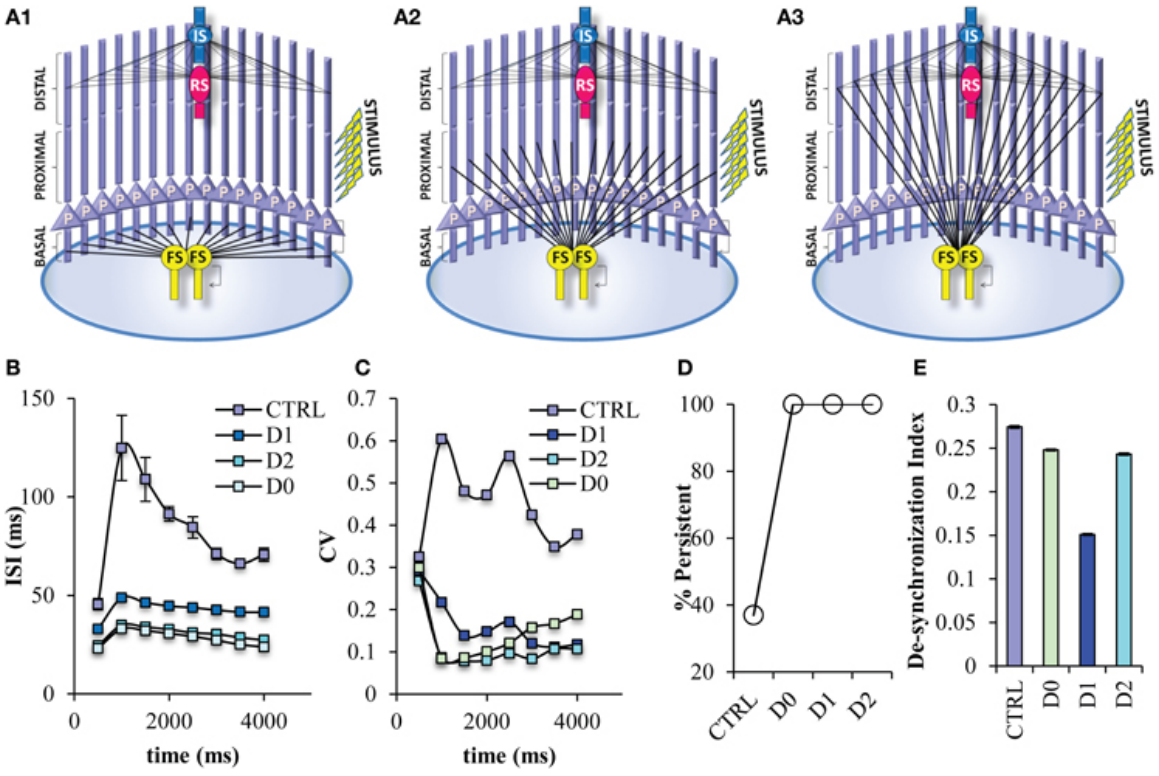


Figure 5.8. Persistent activity properties when the FS interneuron model projects to different dendritic compartments.

Different variations of the PFC microcircuit were constructed in order to study the effect of the projection site of FS model neurons onto the pyramidal neurons. A. Graphical representations of the FS interneuron model projecting to the basal (D0) (A1), the proximal (D1) (A2), and distal (D2) (A3) dendritic compartment of the pyramidal neuron model. B. Graph showing ISIs before and during persistent in 500ms bins for the control and the different projecting site networks. The ISIs of pyramidal neuron models are decreased in all microcircuits in which the FS model neurons project to the dendritic compartments of the pyramidal neuron models. C. Graph showing CVs of ISIs before and during persistent in 500ms bins for the control and the different projecting sites. The CVs of the ISIs of pyramidal neuron models are decreased in all microcircuits in which the FS model neurons project to the dendritic compartments of the pyramidal neuron models. D. Graph showing persistent activity induction in the control and the different FS projecting site networks. E. Graph showing the synchronicity index in the control and different projecting site networks.

Somatic inhibition provided by the FS interneuron seems to be necessary for the induction of proper firing frequencies during persistent activity. In order to eliminate the possibility that the same perisomatic inhibitory effect could be achieved by the other types of interneurons, we modified the network by reversing the projection and number of FS interneuron with RS interneuron (Reverse RS net) (Figure 5.9A1) and with IS interneuron (Reverse IS net) (Figure 5.9A2). When one FS interneuron is projecting in distal dendrites of pyramidal neurons, but two RS or two IS interneurons provide somatic inhibition on pyramidal neurons, the probability for induction of persistent activity increases (Figure 5.9E), while the ISIs and ISI variability during persistent activity significantly decrease (Figure 5.9B-C). Finally, the desynchronization index decreases in both of the reverse networks (Figure 5.9D). These results suggest that somatic inhibition provided specifically by the FS neuron is necessary for the firing frequency during persistent activity and allowing for modulation of persistent activity induction. On the other hand, changing either the projection of the FS model or changing the physiological profile of the interneuron models projecting to the soma could increase synchronicity of the PFC microcircuit.

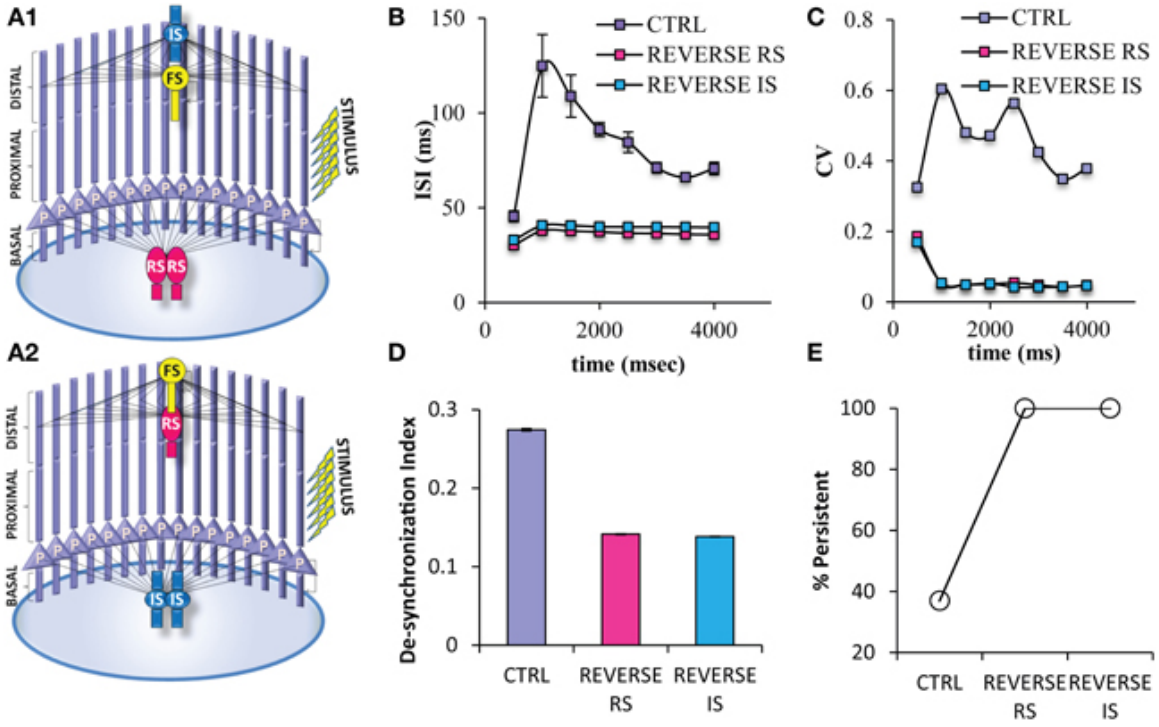


Figure 5.9. Different variations of the PFC microcircuit were constructed in order to study the effect of the spiking profile of the neuron model that provides somatic inhibition to the pyramidal neuron.

A. Graphical representations of the microcircuit in which 2 RS interneuron models are projecting to the soma, while 1 FS interneuron model projects to the distal dendritic compartment of pyramidal neuron models (Reverse RS) (A1), and of another microcircuit in which 2 IS interneuron models are projecting to the soma, while 1 FS interneuron model projects to the distal dendritic compartment of pyramidal neuron models (Reverse IS). B. Graph showing ISIs before and during persistent in 500ms bins for the control and the two "reverse" states of the network. The ISIs of pyramidal neuron models are decreased in all microcircuits in which the FS model neuron project to the distal dendritic compartments of the pyramidal neuron models while either of the other two neuron models are projecting to the soma of pyramidal neurons. C. Graph showing CVs of ISIs before and during persistent in 500ms bins for the control and the two "reverse" states of the network. The CVs of ISIs of pyramidal neuron models are decreased in all microcircuits in which the FS model neuron project to the distal dendritic compartments of the pyramidal neuron models while either of the other two neuron models are projecting to the soma of pyramidal neurons. D. Graph showing the synchronicity index in the control and the two "reverse" states of the network. E. Graph showing persistent activity induction in the control and the the two "reverse" states of the network.

In an effort to compare our results to the available literature with regards to changes in network oscillations in the presence of defects in inhibition, we analyzed the power spectra

of the summed synaptic currents in the different model networks reported above. In our control network, we observe the presence of a peak in the power spectrum at 20Hz and a smaller peak at 40Hz (Figure 5.10A, dark blue trace). Both peaks are absent in the FS KO network, suggesting a significant role of the FS model neuron in maintaining these oscillations. In addition, only the 40Hz peak is decreased in the RS KO network, while the power spectrum of the IS KO network is the same as the control (Figure 5.10A). Both peaks are absent when 50% or less of the FS input to the pyramidal model neuron remain, however, the peak at 40Hz is already decreased when 80% of the FS inputs remain (Figure 5.10B). Finally, when the somatic inhibition is provided by the RS or IS neuron, the peaks at 20Hz and 40Hz are even larger (Figure 5.10C). Therefore, our results suggest that the FS input is critical for maintaining network oscillations, and also reveals a novel role of the RS neuron model in maintaining primarily the 40Hz oscillation.

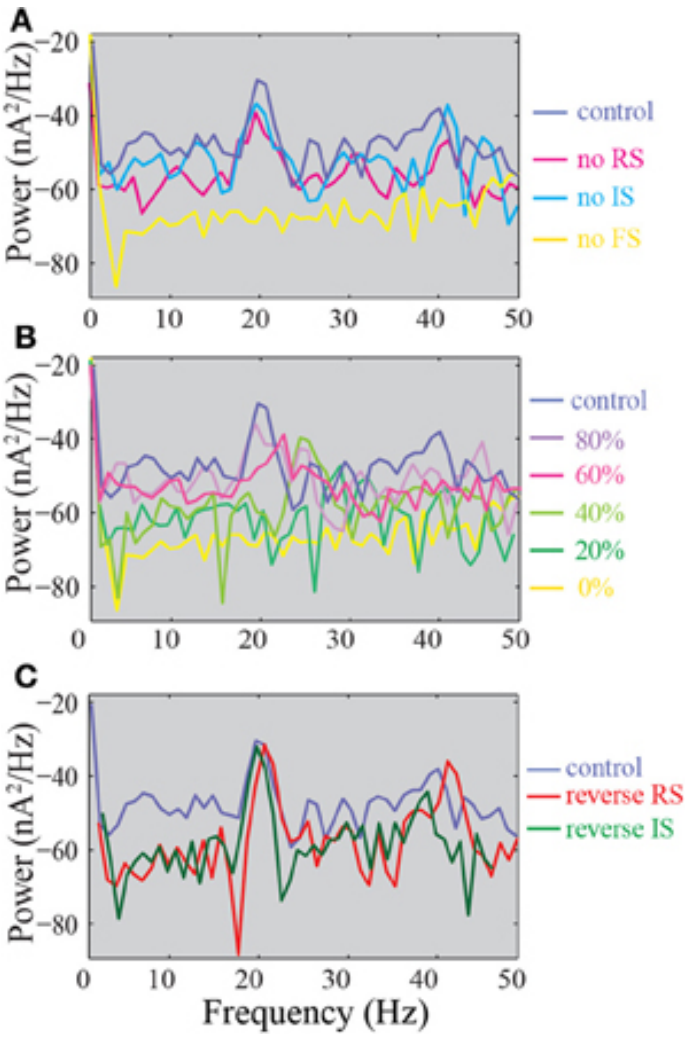


Figure 5.10. Power spectra of the summed synaptic activity in the model network.
A. Power spectra of the control, FS KO, RS KO and IS KO networks.
B. Power spectra of networks with different % of remaining FS inputs on pyramidal models.
C. Power spectra of the control compared to reverse RS and reverse IS networks.

GENERAL DISCUSSION

6.0. Discussion

In this dissertation, we studied the age-dependent effects of PFC function as well as the role of GABAergic inhibition in shaping PFC function. We found that:

a) The age-dependent development of dendritic spines and long-term plasticity of the PFC differs from other primary sensory cortices, and specifically during the period of adolescence and early adulthood. Correlated with the cellular changes in these time periods, behavioral functions that depend on the PFC are also modulated.

b) Developmentally decreased inhibition impairs the emergence of LTP in the PFC as well as it modulates dendritic spine formation. In addition, developmentally decreased inhibition results in pathological phenotype of increased susceptibility to epilepsy and increased anxiety.

c) Decreased inhibition, particularly from the PV interneurons, modulates the properties of persistent activity in a PFC microcircuit model, such as the probability for induction, the firing rate, neuronal synchronicity and network oscillations.

6.1. Age-dependent development of dendritic spines in the PFC

It has been a long-standing hypothesis that the last part of the brain to develop is the region humans prize for its ability to regulate thoughts and to control behavior, namely the PFC. So, PFC is the cause why cortical development is considered heterochronous, as its age-dependent synaptic density curve does not “catch up” with the auditory or other primary cortices. One of the aims of our study was to detect temporal windows of PFC development that coincide with transition periods of dendritic morphology, physiological phenomena, behavioral and cognitive capabilities. To achieve this goal, we used a multidisciplinary approach including cellular, electrophysiological and behavioral techniques in different age groups of mice.

Golgi-cox staining was used to study the dendritic morphology of pyramidal neurons of the PFC as it is a primary technique that it is used to visualize neural cell somata, dendrites and dendritic spines or even axons with clarity in their entirety under light microscopy. We performed an extensive study to uncover changes in the dendritic arborization and spines of pyramidal neurons in the PFC in 6 different age groups of postnatal development starting from the juvenile period until adulthood. We found a differential expression of distinct types of dendritic spines in pyramidal neurons of PFC among different age groups of mice. In particular, "adolescent" pyramidal neurons exhibit the lowest spine density measured, with an increased percentage of stubby spines, reflecting the reduced ability to induce LTP in "adolescent" brain slices of PFC that was also found in electrophysiology experiments.

More specifically, we detected a growth of spine density in juveniles (PD35), followed by a decreased spine density during adolescence (PD 40-45). These results are consistent with previous findings investigating dendritic changes in pyramidal neurons of the BLN and Layer V of the PFC at PD20, PD35 and PD90 in rats, using the Golgi-cox technique, that showed high levels of dendritic spine density between PD20 and PD35, followed by a decrease between PD35 and PD90 (W. A. Koss, 2013). Similar findings reporting the growth of dendritic spine density early in development came from studies on Layer 3 pyramidal neurons of PFC (Markham, Mullins, & Koenig, 2013) and in a preliminary study of Layer V apical dendrites in the mPFC (Heng, Markham, Hu, & Tseng, 2011).

In our study, we also examined an intermediate group at PD60 (early adults) and found a second peak of increased dendritic spine density at that time point, followed by a decrease again at PD90. Our result doesn't contradict the other studies mentioned, as they do not take into account this age stage of development (PD60). This result could suggest that there could be multiple periods of heightened plasticity in the PFC, the second occurs during early adulthood.

Changes in spine density indicate remodeling of synapses throughout adolescent development, especially in excitatory synapses. The phenomenon of synaptic loss has been identified during adolescence in previous studies of the primate PFC (Bourgeois & Rakic, 1993; P. R. Huttenlocher & Dabholkar, 1997; P. Huttenlocher, 1979). The functional consequences of these modifications involve changes in many neurotransmitter systems across the juvenile and adolescent period. In the rat PFC, both NMDA receptors and the GluA2 AMPA receptor subunit peak around PD28-PD30 and then decline by PD60 (Murphy, Tcharnaia, Beshara, & Jones, 2012). Kalsbeek et al. (1988)(Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988) found that dopaminergic fibers increased in the mPFC between PD20 and PD60 which coincides with peak D1 and D2 receptor density at PD40-PD60 (S L Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000; Brenhouse, Sonntag, & Andersen, 2008). As far as the GABA system is concerned, GABAa receptors decrease in sensitivity in the adolescent rat cerebral cortex (Kellogg, Taylor, Rodriguez-Zafra, & Pleger, 1993). All these neural changes together with the alterations of spine density may be responsible for changes in PFC cognitive abilities reported in humans and in rats (Anderson, Anderson, Northam, Jacobs, & Catroppa, 2001; Casey, Giedd, & Thomas, 2000; W. a Koss, Franklin, & Juraska, 2011; L. Spear, 2000).

6.2. Studying LTP with local field potentials

In order to extend our study of the PFC postnatal development, we studied the properties of synaptic plasticity and synaptic properties by performing recordings of local field potentials (LFPs) in layer II of PFC brain slices. For the LFP recordings, an extracellular electrode records electric fields generated by the potential difference (Ve) between two points in a given volume of brain tissue, with a time resolution in the scale of subdivisions of a millisecond. The main advantage of this method compared with other techniques

investigating the activity of neural circuits, is that the principles of biophysics associated with these measurements is largely understood. This has allowed the development of reliable quantifying mathematical models, to clarify the way in which the transmembrane currents collectively contribute to the local field potentials recorded. In addition, it provides indications regarding the type of neural circuits under study (e.g. open or closed loop) (Freeman, 1972) and the study of their subthreshold activity, which is required to investigate the changes in synaptic strength (Buzsáki, Anastassiou, & Koch, 2012), which is the aim of the present study.

6.3. Age-dependent changes in LTP in the PFC

The developmental trajectory of spine density that we found with our Golgi-Cox analysis coincides with the developmental trajectory of LTP induction. Specifically, we found two main peaks for LTP induction, one at PD35 and one at PD60. In other words, the adolescent PFC brain slices responded less to the tetanic stimulation, exhibiting no synaptic potentiation. However, in all other age groups tetanic stimulation resulted in synaptic potentiation, with the level of potentiation being the greatest at the juvenile and early adult period. The paired-pulse ratio in response to a 20 Hz frequency paired pulse stimulation also follows a similar developmental trajectory, with the increased paired-pulse ratio occurring at PD35 and PD60 and the smallest paired-pulse ratio occurring at PD40. On the other hand, the average fEPSP response to current stimulation follows an inverse developmental curve; we find the largest fEPSP response during adolescence and the smallest fEPSP response at PD35 and PD60.

The same “LTP induction” protocol applied within layer II of barrel cortex induced LTP of at least 50% in all age groups tested, indicating, firstly, the different developmental trajectory followed by these two brain regions and, secondly, the possibility that PD40-PD50

developmental time bin is a sensitive period for the development of PFC, while this period precedes the PD35 for the primary cortices, as barrel cortex. The theoretical framework that describes the developmental changes that take place in the brain as a function of age provides ample evidence to justify this finding.

Firstly it is now known that the degree of synchronization of activity within neural circuits is closely linked to synaptic plasticity, which has two directions: the well-synchronized arrival of afferent input can induce the expression of LTP and lead to maturation and stabilization of synapses, while the non-synchronized activity may reduce synaptic strength through LTD and increase the chances for elimination of synapses (Selemon, 2013). Regarding the mechanism involved, it appears that an active (strongly depolarized) neuron can recognize coincident pre- and post-synaptic activity, within a local flow of Ca^{2+} through NMDA receptors of the postsynaptic membrane, resulting in the strengthening of the synaptic transmission (Hebbian LTP) (Bear, 2003). Since GABAergic interneurons, including the PV-positive (PV+ or fast-spiking (FS)) interneurons, which are important for the creation of high-frequency gamma oscillations, take longer to complete their maturation, the expected result was the observation of greater synaptic strength in the group of adults (PD60) compared with that of adolescents (PD40), which was confirmed experimentally.

A further element that enhances the reliability of the difference in long-term synaptic plasticity between different ages is that AMPA receptors are beginning to be integrated at a higher rate in the postsynaptic membrane during adolescence, so at the earliest stages of development their concentration is less than during adulthood (Bossong & Niesink, 2010). The increased presence of AMPARs enhances expression of LTP, as it enhances the responsiveness of the cell to the postsynaptic glutamate acid, whereas their absence has been associated with the appearance of LTD (Dineley et al., 2001). Indeed, during the LTP

the CaMKII kinase phosphorylates cytoplasmic stocks of AMPA receptors leading to their integration in the cell membrane, which highlights the importance of AMPA-dependent ion flux to achieve LTP. In short, the greater presence of AMPA receptors in the postsynaptic membrane in adulthood puts the cell into a "LTP-receptive" state.

Finally, the baseline concentrations of DA and the density of dopaminergic afferent neurons in the PFC peak during peri-adolescence, and then reduced (Bossong & Niesink, 2010). Previous studies have shown that stimulation of the D1 receptor *in vivo* activates a pathway that promotes long-term potentiation. However, overstimulation of this receptor has an inhibitory effect on the expression of LTP (Otani et al., 1998). Also, in brain slices in which dopaminergic inputs in the PFC have been cut and the local DA concentration is lower than the stable tonal levels retained in the intact brain, the high frequency tetanic stimulation, induces phasic release of dopamine contained in DA neurons remaining edges, which promotes expression of LTD (Kolomiets, Marzo, Caboche, Vanhoutte, & Otani, 2009). So this promotion signal for synaptic depression is expected to be more pronounced in brain sections from younger animals, since their neurons express greater amounts of dopamine and D1 receptors.

Comparison with LTP in barrel cortex

The results related to the induction or not of LTP after tetanic stimulation in barrel cortex showed that neurons within layer II of barrel cortex tend to the induction of at least 50% LTP in all age groups tested. Our hypothesis that a sensitive period of decreased plasticity precedes the PD35, that is the youngest age group that we tested, is in accordance with studies showing that LTP in barrel is strongest in PD3-PD5 rats, weaker in PD0-PD1 and PD6 and PD7 rats and disappears at PD8-PD14 (An, Yang, Sun, Kilb, & Luhmann, 2012).

This provides us an indication that the age of decreased plasticity in a primary cortical area as barrel cortex occurs, indeed, during earliest stages of postnatal development, locating it at PD8-PD14.

6.3. Age-dependent effects on basal synaptic transmission and short-term plasticity

Basal synaptic transmission was found decreased in the adolescent group. Similar electrophysiological studies in rodents of different age groups revealed changes in the sensitivity of layer V mPFC pyramidal neurons in the stimulation of dopamine receptors (Tseng & O 'Donnell, 2007), but also a reduction in the postsynaptic response induced in pyramidal neurons of mPFC during adolescence (Heng et al., 2011). This reduction was associated, within the latter study, with the expression levels of the endocannabinoid receptor CB1, as its excitatory action mediates the moderation of GABAergic inhibition. Throughout the cortex, but mainly in structures of the limbic system and the mPFC, the expression levels of CB1 peak during early childhood and then begin to decrease during adolescence. So the CB1-mediated withdrawal of inhibition is more widespread in the early postnatal developmental stages, participating to the induction of increased excitatory postsynaptic potentials (EPSPs).

Finally the results of the paired-pulse recordings within PFC revealed a tendency to switch to short-term synaptic inhibition (PPD) during PD40 and then switched back to short-term facilitation (PPF) and increases with age. Our result is in agreement with other studies (Betz, 1970; Metherate & Ashe, 1995; Zucker & Regehr, 2002). During the last years, models have been structured in order to explain the mechanism of the phenomenon of short-term depression. One of the dominant models is the "depletion model of depression", according to which the first pulse in turn causes the release of a large proportion (say F) of synaptic vesicles of the readily releasable pool (RRP), i.e. all synaptic vesicles that are

available immediately after the arrival of the signal at the presynaptic terminal, resulting in a reduction of available stocks of neurotransmitter. The inhibition observed in response to the second pulse is due to this reduction, since fewer amounts of neurotransmitter secretion results in stimulation of fewer receptors on the postsynaptic membrane. The extent of inhibition depends firstly on the interval between the two pulses: the shorter it is, the smaller percentage of the releasing quantity F anticipates restoring in the stocks of RRP until the arrival of the second pulse. Secondly, it depends on the value of the quantity of F, i.e. the larger neurotransmitter stock consumed during the synaptic response to the first pulse, the less amount remains in the RRP for response to the second pulse, hence, the stronger the observed inhibition (Regehr, 2012). An analysis of our basal synaptic transmission results showed that neurons of the adolescent brain have increased synaptic responses after the same stimulation, compared to the adult neurons, which could be an indication that the value of the quantity F is greater in the case of immature neurons. Based on the "depletion model of depression", the observation that brain sections of adolescent animals exhibit a stronger tendency to short-term synaptic inhibition can therefore be justified.

6.4. Age-dependent effects of PFC-dependent and PFC-independent behaviors

We finally wanted to determine whether the developmental changes observed in dendritic spine density and in synaptic properties and plasticity result in developmental changes in behaviors mediated by the PFC. For this reason, we studied different behavioral tasks in two age-groups that showed the greatest changes in morphology and plasticity: the adolescent age group (PD40-50) and the early adulthood age group (PD60-80). The adolescent mice exhibited all the characteristic behavioral features of their age; increased exploration, decreased anxiety suggesting increased risk taking behavior, enhanced

habituation, but they lack in their performance in PFC-dependent cognitive functions, such as working memory, recency memory, memory of previous exposure to a novel environment, without exhibiting differences in non-PFC-dependent tasks compared to adult mice, such as the novel object and the object-to-place recognition memory.

The delayed alternation test using the T-maze belongs to a broader group of delayed response tasks (DR), whereby an information-element is presented initially to the animal/person, then removed from the perceptual field for a particular time (delay) and finally the same element is presented again in conjunction with a new element. The animal is asked to remember which the item that was presented before the delay was. Such assays are used routinely for the study of the working memory and the importance of the PFC to the DR performance tests has been revealed using electrophysiological techniques and neuroimaging studies and with targeted lesions within the PFC in primates (P S Goldman-Rakic, 1990).

The protocol used for our study specifically requires the function of spatial working memory, and provides information on other aspects of the functioning of the PFC, such as changes in behavioral strategy depending on the circumstances. The use of reward, against the simplest spontaneous alternation task was preferred, which is based entirely on the natural tendency of rodents to alternate arm choice, because the latter is considered to have given inconsistent and variable results (Deacon & Rawlins, 2006). Indeed, during the first days of the experiment, it was apparent that mice select the same arm in a row, perhaps because they were not completely free of stress and prefer the more familiar side that had been visited previously. For these reasons, the experimental procedure of delayed alternation with reward was the most appropriate tool for the study of the development of the PFC at the functional level during adolescence, compared with the adult brain.

As it is known from the literature, and indicated by our cellular and physiological studies, the developmental changes of PFC are not yet completed before the end of adolescence and the various functionalities of this region, such as working memory, vary dramatically depending on the age until full maturity (Kolb et al., 2012). Our working hypothesis, therefore, was that the performance of the group of adolescent mice would be lower than that of adults. Indeed, adolescent mice required more efforts (i.e. sessions) to successfully complete the test. Furthermore, their overall performance in the task was lower compared to adults. There are very few studies that have investigated the function of the PFC, and more specifically the mPFC, at the behavioral level during adolescence, particularly in rodents. The only published study that directly compares the performance of adolescent and adult rodents of both sexes to test delayed alternation agrees with the findings of our study, as the adolescent rats scored progressively lower rates of correct choices as the duration of the delay increased (W. A. Koss, 2013), On the other hand, the overall disability of adolescents in cognitive functions such as decision making, the focus of attention and inhibitory control during response, in relation to the optimum level reached in adulthood has been identified by many studies in humans, primates and mammals (Anderson et al., 2001; W. A. Koss, 2013; Schenk, 1985). For example, rodents that are near adolescence lack in performance in a popular test of working memory using Morris water maze, compared to adults (Schenk, 1985). Moreover, human's performance on tests that examine working memory and control of attention, such as remembering a range of digits (digit span) and the contingency naming task, was found to improve throughout the course of adolescence (Anderson et al., 2001).

Another behavioral index revealed by the delayed alternation task in the T-maze is the perseveration index, which is quantified as the maximum number of consecutive wrong choices done by the mice of both age groups. Clinical trials in people who have suffered

injuries to the anterior orbital cortex, which is part of the PFC, as well as experiments in monkeys have shown that this type of damage can cause symptoms of increased impulsivity and rigidity in behavior (Rolls, Hornak, Wade, & McGrath, 1994). Behavioral inhibition, therefore, has been associated with control of mindless behavior, impulses, instinctive behaviors and emotional maintenance. Based on the above data, the adolescent age group of mice was expected to show greater persistence in wrong choices than adults. The findings of our experiments confirmed the expected result, based on the hypothesis that adolescents have impaired PFC function. The findings of a corresponding study of delayed alternation were similar; adolescent rats made significantly larger number of consecutive errors compared with adults (W. A. Koss, 2013). This probably reflects the immaturity of the mPFC, as supported by our results, although other associated areas, such as the amygdala, a brain area involved in the processing of fear, could also be involved. This nucleus exhibits, as the PFC, prolonged postnatal maturation and even a reduction in the number of neurons during adolescence (Rubinow & Juraska, 2009), while it forms a circuit with dense innervation with the mPFC, which is involved in learning processes (Uylings et al., 2003). Furthermore, in general, the interaction of the PFC with the amygdala plays a role in the regulation of emotions and fear responses. Therefore, a factor that possibly contributed to the increased number of repeated mistakes made by the adolescent mice is the lack of moderation of fear responses by the immature amygdala, which pushed them to continuously visit the more familiar arm of the maze.

The recency memory is the memory associated with free recall of a list of items, in which people tend to recall better the items found at end of the list. One explanation is related to the temporal context. Temporal context can serve as a retrieval cue, so that more recent items have a higher recall probability. Neuroimaging studies of normal young adults have

consistently found PFC activity during the performance of recency memory tasks serving general strategic organizational or monitoring processes (Rajah & McIntosh, 2006).

We studied recency memory in mice in the context of a set of recognition memory tests for objects, for place and for temporal order. Recognition memory in general requires judgments of previous occurrence of stimuli based on the relative familiarity of individual objects, location of objects, or by using recency information. It is the ability to discriminate novel from familiar stimuli. In the case of object recognition memory, the familiar stimulus is an object and under normal conditions the animal will investigate the novel object more (familiarity discrimination) (dependent on the presence of a functioning perirhinal cortex) (Barker, Bird, Alexander, & Warburton, 2007), while in object-to-place recognition memory (spatial recognition) the familiar stimulus is the previous location of the object. Under normal conditions, the animal will explore the object that has been displaced more than the one that is stationary. This is known to be dependent on the hippocampus. Recency memory is the discrimination of familiar stimuli by their relative distance in time, called temporal order memory. Recency memory and recognition memory depend partly on different neural pathways. Studies have shown that mPFC and perirhinal cortex are crucial for recency discriminations, whereas the perirhinal cortex but not the mPFC is important for the discrimination of novel and familiar individual objects (Barker & Warburton, 2011; Brown & Aggleton, 2001; Abdelkader Ennaceur, Neave, & Aggleton, 1997; Mumby & Pinel, 1994).

In our experiments, adolescent mice have the same performance with adult mice in the two recognition memory tests; the novel object recognition and the object-to-place (spatial recognition) memory task, while the exhibited significantly lower performance in the recency memory test (temporal order recognition memory task). As, recency memory is a PFC-dependent cognitive ability, this result gives us one more indication that PFC is not yet

fully matured during the adolescent period of development, in contrast to perirhinal cortex and hippocampus, as in the other two object recognition tests adolescent mice had the proper performance.

Adolescence is associated with increased sensation-seeking, exploratory activity reckless and risk-taking behaviors, low levels of harm avoidance and anxiety (Arnett, 1992; T A Wills, Vaccaro, & McNamara, 1994). Our results show indeed that PD40-50 mice have all these characteristic behaviors of adolescents, as studied by measuring their exploratory activity, their trait anxiety levels, and their habituation to a novel environment. Firstly, exploratory activity of adolescent was measured during the object recognition tests, by calculating the time they spent exploring the objects, compared to the adults. Adolescent mice had increased exploratory activity, in accordance with findings from previous studies (Macrì, Adriani, Chiarotti, & Laviola, 2002). Secondly, the use of the open field test enabled us to study their risk-taking and/or their exploratory and/or their adaptability in a novel environment, by measuring their thigmotaxis index and their intra-session habituation in the open field (Walsh & Cummins, 1976). The duration of time spent in the central square is a measure of exploratory behavior and anxiety. Increased number of entries and increased duration of stay in the central square indicate high exploratory behavior and low levels of anxiety. Furthermore repeated exposure to a novel environment results in time dependent changes in behavior (Choleris, Thomas, Kavaliers, & Prato, 2001). At first, more fear-related behaviors are displayed, because of the novelty of the environment and then, with time or with repeated trials, more exploratory and locomotor activity is observed. Of course, strain differences exist; some strains show increased activity, others show habituation and decreased activity levels and others show no change (Bolivar, 2009). Our results showed that adolescent mice have decreased levels of trait anxiety, increased exploratory behavior, or even increased risk-taking behavior compared to the adults, as they spent significantly

more time in the central area of the field during the first minutes of the test. Our results are in agreement with other studies, which also report elevated levels of novelty seeking and reduced behavioral and physiological responses to stressful situations in mice and rats around this age (Laviola et al., 2003; Philpot & Wecker, 2008; Stansfield & Kirstein, 2006; Sturman, Mandell, & Moghaddam, 2011).

Habituation is a form of non-associative learning and it is defined as the decrease in responsiveness upon repeated exposure to a stimulus. According to the 'cognitive map theory' (O'Keefe & Nadel, 1979), an internal representation of the spatial properties of an environment is constructed in the hippocampus whenever novelty is detected (during the early stages of exploration in a new environment). When enough information has been gathered for this cognitive map, exploration decreases and habituation occurs. Habituation to an open field, measured as a change in exploratory activity over time, can be examined both within (intrasession) and between (intersession) measurement sessions. It has been suggested that intra-session habituation measures adaptability, whereas inter-session habituation also reflects memory of the previous novel environment exposure (Bolivar, 2009), a function that depends on the PFC.

Our results showed that while adolescent mice show improved adaptability, they exhibit decreased memory to the previous exposures (inter-session habituation), further provided evidence for impaired PFC function during this developmental period. Collectively, our results show that the adolescent period is a significant period for the development of PFC when changes occur at the cellular, the physiological and the behavioral level.

6.5. The role of decreased inhibition in epilepsy and anxiety

Defects in GABAergic transmission are known to exist in the epileptic brain, both in animal models (Treiman, 2001) and in humans (Scheffer & Berkovic, 2003), however, it is not clear

whether the decreased GABAergic function precedes or results from epileptic seizures. The recent advantage in the knowledge of interneuron development and the generation of several transgenic mice resulting in impairments of the interneuron population has led to a possibly new etiology for epileptic phenotypes, that of 'interneuropathies' (Sebe & Baraban, 2011), in which interneuron deficits definitely precede the emergence of the epileptic phenotype.

Early removal (E9.5 or 10.5, but not at E12.5), of Nkx2.1, a transcription factor involved in development of MGE-derived interneurons, resulted in several behavioral abnormalities, such as prolonged periods of immobility, with several episodes of violent tremors and absence or reduction of voluntary movement. EEG recordings revealed the emergence of prolonged abnormal bursting activity in the cortex (Butt et al., 2008). Mice lacking Dlx1, a transcription factor also involved in interneuron development, showed a decrease in calretinin and somatostatin interneurons in the somatosensory cortex (about 20-30% decrease), and exhibited seizures upon exposure to mild stressor such as noise or handling (Cobos et al., 2005). Specific deletion of the Na^+ channel subunit, Nav1.1, from interneurons resulted in spontaneous seizures and sporadic deaths, hyperactivity, increased grooming and increased circling behavior. These mice also showed increased anxiety in the elevated plus maze (less entries in the open arms) (Han et al., 2012). Mice deficient in CNTNAP2, a protein shown to be involved in autism spectrum disorders, have reduced number of interneurons and epileptic seizures, along with other behavioral changes (Peñagarikano et al., 2011). Our results reinforce the above findings that a defect in the development of interneurons results in spontaneous epileptic seizures or to a decreased threshold for induction of seizures.

Epilepsy is known to co-exist with other psychiatric conditions, one of which is anxiety disorder and/or depression (Beyenburg et al., 2005; Tellez-Zenteno, Patten, Jetté, Williams,

& Wiebe, 2007), however, the course and treatment of epilepsy in the presence of anxiety is not well studied. In general, the presence of anxiety-epilepsy co-morbidity has been shown to decrease the therapeutic potential of several anti-epileptic drugs (Hamid et al., 2011; Reilly, Agnew, & Neville, 2011). Few animal models have been found to replicate the above co-morbid phenotype. A genetic animal model mentioned above in which the function of specific interneurons is impaired by removing the Nav1.1 ion channel from the interneurons has been shown to have both spontaneous epileptic seizures and increased anxiety as observed by the elevated plus maze (Han et al., 2012). Mice with a mutation in the urokinase plasminogen activator receptor have decreased numbers of interneurons in the anterior cingulate and behaviorally exhibit both increased anxiety and decreased threshold (increased susceptibility) for pentylenetretrazol-induced epilepsy (Powell et al., 2003). The WAG/Rij strain is also a well characterized animal model of absence epilepsy, in which several symptoms of depression have also been found, such as decreased consumption of sucrose, decreased active behaviors during the forced swim test and decreased exploration (rearings and grooming) (Sarkisova & van Luijtelaar, 2011). A working hypothesis, in agreement with the aforementioned data and our own, could be put forward postulating that the loss of interneuronal function could underlie co-morbidities that are associated with epilepsy. Therefore, these mice could be used as animal models for studying the emotional and cognitive behavior, as well as for testing pharmacological agents targeting specifically these co-morbidities, with the possibility of having better predictive values.

6.6. The role of PFC long-term potentiation in anxiety

The PFC is known to regulate the emotion of anxiety (Etkin, 2010). Inactivation of the PFC with GABA agonists increases anxiety in the elevated plus maze (de Visser, Baars, van 't Klooster, & van den Bos, 2011). Specifically, activation of PFC neurons seems to represent the safer parts of the environment, such as the closed arms of the elevated plus maze. In the absence of such activation, however, mice become more anxious (Adhikari, Topiwala, & Gordon, 2011). Specifically, activation of the PFC is probably important for decreasing the amygdala output that would normally generate an anxious behavior. In agreement with the above studies, our results show that mice with decreased inhibition (the Rac1 cKO) are more anxious and have impaired LTP in layer II of their PFC. The LTP impairment could render the PFC not capable in adjusting to novel environments and discriminating between safe and unsafe regions of the environment, hence, allowing the increased activity in the amygdala to generate increased anxiety.

Although the function and mechanisms underlying LTP have been studied extensively in the hippocampus, they have been relatively overlooked in the PFC, primarily due to the contribution of the PFC in short-term and working memory processes. However, studies suggest that LTP has an important role in PFC function and PFC mediated cognitive tasks. LTP in the PFC has been studied either in the connections between layer II and layer V or in the ventral hippocampal input to layer V PFC neurons (Goto et al., 2010). In addition, spike timing dependent plasticity has also been observed in layer V of the PFC (Couey et al., 2007). The LTP identified in the local in-between layers circuit has been shown to be dopamine and NMDA-dependent (Matsuda et al., 2006). Furthermore, LTP mechanisms could interact with the necessary physiological mechanisms required for working memory, a cognitive function underlied by the PFC. It has been shown that protein synthesis, necessary for maintenance of LTP, is required for proper acquisition of spatial working

memory tasks (Touzani, Puthanveettil, & Kandel, 2007), while deficits in synaptic plasticity are observed in animal models of psychiatric disorders (Goto et al., 2010). Our results reinforce the importance of LTP in proper PFC function and propose that intracortical LTP could regulate the emotional control exerted by the PFC. Our study also shows that Rac1 cKO mice, with decreased inhibition, exhibit impaired LTP in the PFC. This defect is likely to arise from homeostatic changes in response to increased excitation. These changes include the decrease in the length, branches and density of mushroom type spines as well as decrease in the expression of NMDA receptor subunits and probably mediate the LTP impairment.

6.7. Effect of inhibition on LTP induction and maintenance

It is well known that the GABA system regulates the induction and maintenance of LTP. GABA blockade produces synaptic potentiation in vivo (Matsuyama, Taniguchi, Kadoyama, & Matsumoto, 2008), or enhances the EPSP-spike coupling in vitro (Staff & Spruston, 2003). Decreased GABAergic transmission usually results in increased network excitability and could have a ceiling or saturating effect for LTP, decreasing the potential for potentiation of synaptic responses. Furthermore, changes in GABAergic transmission could alter the rules for LTP induction (Meredith, Floyer-Lea, & Paulsen, 2003). Pathological conditions in mice that result in long-term decreased GABAergic transmission also affect the emergence of LTP (Jedlicka, Papadopoulos, Deller, Betz, & Schwarzacher, 2009; R. R. Wang et al., 2014). Our data also show that a chronic pathological condition, such as the developmental decrease in the number of interneurons, results in impaired LTP in the PFC. Our study also reveals that the decreased inhibition leads to network adaptations, such as reduced dendritic length and

spine density and decreased NMDA receptor subunit expression. Therefore, it is likely that chronic changes in GABAergic transmission trigger network-wide adaptations that eventually change synaptic plasticity.

6.8. The PFC as the site of co-morbidities of psychiatric/neurologic disorders

The PFC is a multi-tasking brain area, involved in several cognitive functions, such as working memory, attention, behavioral inhibition, emotional regulation, as well as long-term memory storage. Many of these functions are compromised in several neuropsychiatric/neurological diseases either as a result to the primary injury (such as in schizophrenia) or as a secondary result (such as in epilepsy, as discussed above, as well as in Parkinson's disease or Alzheimer's disease), leading to co-morbidities.

Furthermore, clinically, the presence of co-morbidities impairs or decreases the effectiveness of medication. Many co-morbid conditions find a common anatomical substrate in the PFC. For example, epilepsy and autism (Tuchman & Rapin, 2002), epilepsy and attention-deficit hyperactive disorder (Kaufmann, Goldberg-Stern, & Shuper, 2009; Parisi, Moavero, Verrotti, & Curatolo, 2010) epilepsy and anxiety (Hamid et al., 2011; Reilly et al., 2011), all have a common denominator in involving impaired PFC function. Therefore, understanding the physiology of PFC function and how it is affected under co-morbid conditions is critically important.

6.9. The role of Interneurons in Neuronal Synchronicity, Gamma-Frequency Oscillations and the specificity for induction of Persistent Activity as predicted by the PFC microcircuit

Cortical oscillations, particularly in the gamma-frequency, have been suggested to significantly contribute to several cognitive functions, such as selective attention,

perception. These oscillations are thought to reflect synchronous activity of rhythmically firing neurons (Jensen, Kaiser, & Lachaux, 2007). Activity of PV/FS interneurons has been found in several studies, both experimental and computational, to have a significant role in maintaining the above oscillations and neuronal synchronization (Börgers, Epstein, & Kopell, 2008; Börgers & Kopell, 2008; Cardin et al., 2009; Sohal et al., 2009; Vierling-Claassen, Cardin, Moore, & Jones, 2010).

Gamma oscillations have been shown to increase the mutual information between incoming synaptic frequency and output of action potentials (Sohal et al., 2009).

In our model, there is a bidirectional modulation of neuronal synchronicity by the FS interneuron. Decreasing the FS input results in increased synchronicity, while a "KO" simulated condition results in decreased synchronicity. Replacing the FS input with either the RS or IR neurons increased synchronicity, but past the levels of the control network. This suggests that the effects of FS input on neuronal synchronicity are complex. Small reductions of FS inputs (20%) result in both increased synchronicity and a small deviation in the firing rate and ISI variability, suggesting that this could be beneficial for the network activity and could possibly result in working memory enhancements. However, 40% or greater reductions in FS-mediated synaptic inputs result not only in increased synchronicity but also in increased firing frequency and decreased ISI variability, indicating a possible defect that could move the network activity toward epileptiform behavior. Therefore, as mentioned in (Yu, Liu, Wang, & Lee, 2004), "it is not the weaker or stronger but an appropriate synchronous state may be of more functional significance in sensory encoding."

Epilepsy is a condition characterized by excessive neuronal synchrony (Traub & Wong, 1982), suggesting that decreased interneuron function can also be associated with increased synchrony. Our results predict that decreases greater than 50% in the number of interneurons or GABAergic synapses lead to disruption in stimulus-specific persistent

activity induction. Therefore, any type of stimulus irrespective of neuromodulation could results in persistent activity, a condition that should greatly impair performance in working memory tasks and other PFC-dependent cognitive functions, such as attention and behavioral flexibility.

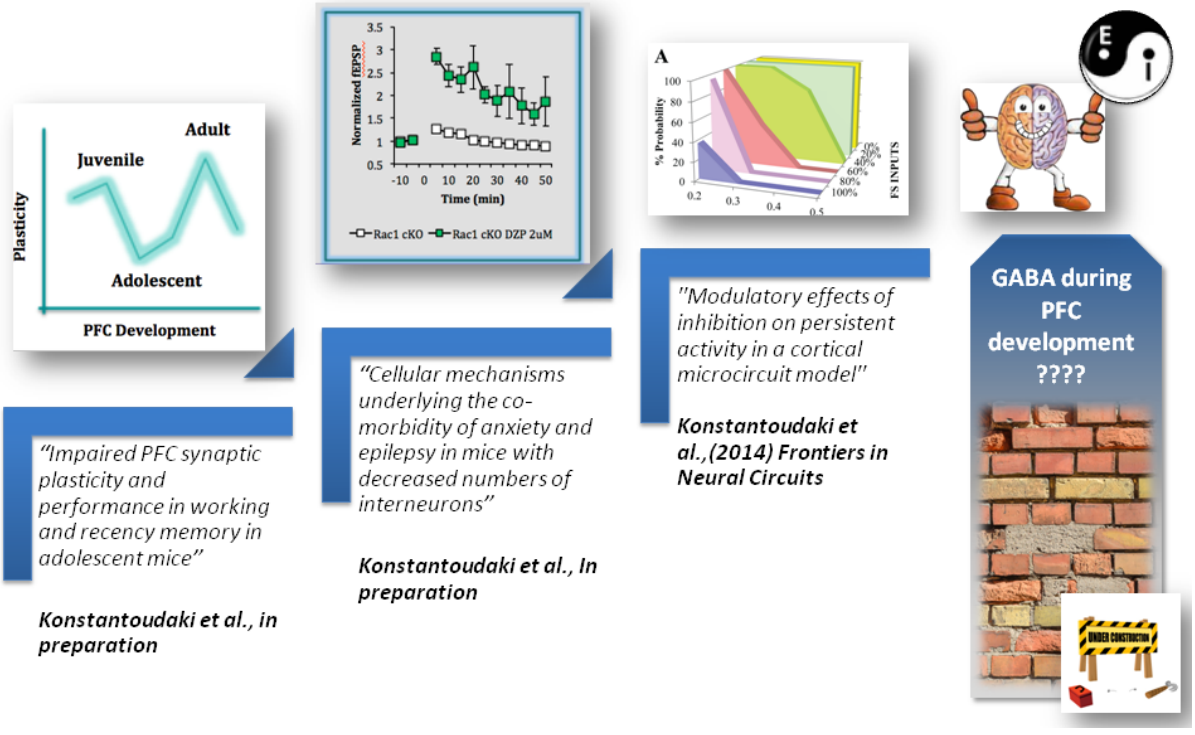
6.10. Closing Remarks: Excitation/Inhibition Balance - the "Yin and Yang" of the brain function

Excitation-inhibition balance (E/I balance) is a form of homeostatic plasticity that helps to maintain neuronal activity within a narrow, safe range. It refers to the relative degree of excitatory and inhibitory drive in a neural circuit, is established during development and is maintained in the mature nervous system. Even small changes in the balance between excitation and inhibition can result in runaway excitability (Chagnac-Amitai & Connors, 1989) disruption of sensory responses (Nelson, 1991) and alteration of experience-dependent plasticity (Takao K Hensch, 2005). And this is not the only reason why it is obvious that the adjustment in the relative strength of excitatory and inhibitory feedback onto pyramidal neurons is important for the delicate balance of cortical network activity. Most neurological disorders, such as schizophrenia, attention deficit hyperactivity disorder, anxiety-disorders, and autism emerge before adulthood; either in childhood or in adolescence, as this time periods exhibit great vulnerability, with special sensitivity to environmental factors. Recent evidence has associated the above pathological states with disrupted excitation/inhibition (E/I) balance in the cortex, and particularly in the PFC. Clearly, GABAergic interneurons play a critical role in shaping cortical maturation at various stages of development in the healthy brain, as they allow cortical networks to generate oscillations in the fast gamma range, a hallmark of mature circuits, thus enabling brain complexity in higher mammals. The PFC is involved in higher-order cognitive functions

(short-term and long-term mnemonic functions) as well as in emotional processes. Although age seems to play a significant role in both the normal function of PFC and the emergence of disease states, very little is known with regards to the age-dependent changes of behaviors involving the PFC and the underlying cellular mechanisms. What is well known from previous studies is that this higher-order brain region, unlike other primary cortical areas, exhibits delayed cortical development until young adulthood, during which it displays great vulnerability for the occurrence of disease states.

This dissertation contributed in the studies regarding the developmental effect of disrupted E/I balance in physiology and in behavior by providing evidence about its specific role in fundamental brain functions (long-term potentiation, persistent activity) and cognitive behaviors.

During this thesis, the PFC development and the effect of decreased inhibition on its functions both in transgenic mice and using computational models were studied.



With our results:

1. We enhanced the knowledge of the delayed development of PFC compared to other brain regions, concurrently revealing an additional period of increased plasticity
2. We found the differential effect of GABA in synaptic plasticity of PFC comparing with other brain regions
3. We identified the fundamental role of somatic inhibition provided specifically by the parvalbumin interneuron in persistent activity of PFC.

We conclude that in spite the great role of the PFC in executive functions and the substantial knowledge already existed, there are limited findings both for its development and the effect of GABA system in it. Our results contribute considerably in the knowledge of this unexplored field laying a solid foundation for further research.

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APPENDIX

Appendix

Table A1. Structural parameters of model neurons.

	Length (µm)	Diameter (µm)
Pyramidal Cell		
Soma	86.3	10.14
Basal Dendrite	150	1
Proximal Apical Dendrite	400	2.6
Distal Apical Dendrite	400	2.6
Axon	113.22	1.1
FS Interneuron		
Soma	27	29
Dendrite	22	7
Axon	115	1.5
RS Interneuron		
Soma	42	42
Dendrite	22	7
Axon	113.22	1.1
IS Interneuron		
Soma	27	27
Dendrite	22	7
Dendrite	22	7
Axon	113.22	1.1

Table A2. Active and passive ionic properties of pyramidal neurons.

Pyramidal Neuron, mechanisms	Soma	Axon	Basal dendrites	Proximal Apical Dendrite	Distal Apical Dendrite
Sodium conductance, S/cm^2	0.108	1.08	0.0108	0.0432	0.0216
Delayed rectifier K^+, S/cm^2	5.4e-3	5.4e-3	4.86e-4	2.16e-5	5.4e10-6
Persistent sodium, S/cm^2	18e-7	0	18e-7	54e-7	18e-6
sAHP, S/cm^2	0.025	0	2.5e-5	0.0025	-
A-type K^+, S/cm^2	7e-4	0	7e-4	7e-4	7e-4
N-type calcium, S/cm^2	2e-5	0	6e-6	6e-6	0.0001
T-type calcium, S/cm^2	6e-6	0	6e-6	6e-6	6e-7
CaR, S/cm^2	3e-8	0	9e-9	9e-9	15e-8
L-type calcium (a1D)	3e-5	0	3e-5	0	-
L-type calcium (a1C)	1e-5	0	1e-5	1e-5	3e-6
D-type K^+, S/cm^2	6e-4	0	0.0006	0.0006	0.0006
fAHP, S/cm^2	2e-4	0	2.2e10-6	2.2e-4	2.2e10-6
H-current, S/cm^2	9e-6	0	9e-6	9e-6	9e-5
Calcium diffusion model	Yes	No	Yes	Yes	Yes
C_M (µF/cm^2)	1.2	1.2	2.4	2.4	2.4
R_A (ohm/cm)	150	150	150	150	150
R_M (kΩ cm^2)	11	11	6	6	6

Table A3. Active ionic properties of FS interneuron model

FS interneuron mechanisms	Soma	Axon	Dendrite
Sodium conductance, S/cm^2	0.135	1.35	0.09
Delayed rectifier K^+, S/cm^2	0.036	0.018	0.0075
N-type calcium, S/cm^2	0.0003	–	–
D-type K^+, S/cm^2	0.0000725	–	–
H-current, S/cm^2	0.00001	–	–
A-type K^+, S/cm^2	0.0032	–	0.032
fAHP, S/cm^2	0.0001	–	–
Calcium diffusion model	Yes	No	No
C_M (μF/cm^2)	1.2	1.2	1.2
R_A (ohm/cm)	150	150	150
R_M (kΩ cm^2)	10	10	10

Table A4. Active ionic properties of RS interneuron model

RS interneuron mechanisms	Soma	Axon	Dendrite
Sodium conductance, S/cm^2	0.075	0.75	0.018
Delayed rectifier K^+, S/cm^2	0.018	0.009	0.009
T-type calcium, S/cm^2	0.003	–	–
H-current, S/cm^2	0.000002	–	–
A-type K^+, S/cm^2	0.035	–	0.00875
fAHP, S/cm^2	0	–	–
Calcium diffusion model	Yes	No	No
C_M (μF/cm^2)	1.2	1.2	1.2
R_A (ohm/cm)	150	150	150
R_M (kΩ cm^2)	40	40	40

Table A5. Active ionic properties of IS interneuron model

IS interneuron mechanisms	Soma	Axon	Dendrites
Sodium conductance, S/cm^2	0.015	0.15	0.075
Delayed rectifier K^+, S/cm^2	0.018	0.009	0.009
D-type K^+, S/cm^2	0.000725	–	–
N-type calcium, S/cm^2	0.001	–	–
fAHP, S/cm^2	0.00003	–	–
Calcium diffusion model	Yes	No	No
C_M (μF/cm^2)	1.2	1.2	1.2
R_A (ohm/cm)	150	150	150
R_M (kΩ cm^2)	20	20	20

Table A6. Input resistance values of the model neurons and those obtained from electrophysiological data

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	IR (Model)	IR (Experimental)
Pyramidal	91.3	80 ± 6.8 (Nasif et al., 2004)
FS	250.19	235 ± 68 (Zaitsev, 2005)
RS	487.75	582 ± 195 (Zaitsev, 2005)
IS	545.18	585 ± 137 (Zaitsev, 2005)

Table A7. Summary of synaptic connections in the microcircuit

Type of connection	Location	No. of synapses	References
Thalamocortical (incoming)	Proximal dendrite	120	Kuroda et al., 1998
Pyramidal (recurrent)	Basal dendrite	24	Thomson and Lamy, 2007; Peters et al., 2008
Autapses in Pyr	Basal dendrite	8	Lubke et al., 1996
Pyr -to-FS	Dendrite	12	Markram et al., 2004; Thomson and Lamy, 2007
Pyr -to-RS	Dendrite	14	Markram et al., 2004
Pyr -to-IS	Dendrite	7	Cauli et al., 1997; Markram et al., 2004
Autapses in FS	Soma	1	Bacci et al., 2003
FS -to-Pyr	Soma	15	Tamás et al., 1997a,b; Markram et al., 2004
RS -to-Pyr	Distal dendrite	12	Tamás et al., 1997a,b; Markram et al., 2004
IS -to-Pyr	Distal dendrite	10	Tamás et al., 1997a,b
IS -to-RS	Dendrite	2	Murayama et al., 2009

Equations of all biophysical mechanisms used

Almost all equations for the intrinsic and synaptic biophysical mechanisms have been published in Papoutsi et al, 2013, and have not been changed for the current model. Below, we present those equations that have been modified for use in our model.

The NMDA receptor on pyramidal neuron models and interneuron models.

The NMDA receptor (H. Wang et al., 2008; H.-X. Wang & Gao, 2009)

$$I = g \cdot (V - E_{rev}) \tag{1}$$

$$g = (R_{on} + R_{off)} \cdot 1(ohm^{-1}) \cdot B \tag{2}$$

$$B = B(V) = \frac{1}{1 + \exp(0.072(mV^{-1}) \cdot (-V)) \cdot (Mg / 3.57(mM))} \tag{3}$$

$$\frac{dR_{on}}{dt} = \frac{synon \cdot R_{\inf} - R_{on}}{R_{\tau}} \tag{4}$$

$$\frac{dR_{off}}{dt} = -\beta \cdot R_{off} \tag{5}$$

$$R_{\inf} = \frac{C_{\max} \cdot \alpha}{C_{\max} \cdot \alpha + \beta} \tag{6}$$

$$R_{\tau} = \frac{1}{C_{\max} \cdot \alpha + \beta} \tag{7}$$

Where, $E_{rev} = 0(mV), Mg = 1(mM), C_{\max} = 1(mM), \alpha = 4(ms^{-1}mM^{-1}), \beta = 0.015(ms^{-1})$

The above β=0.015 (ms^{-1}) is used in the pyramidal neuron model and the IS neuron models. For the FS and RS neuron models, β=0.02 (ms^{-1})

The AMPA receptor

$$I = g \cdot (V - E_{rev}) \tag{8}$$

$$g = (R_{on} + R_{off}) \tag{9}$$

$$\frac{dR_{on}}{dt} = \frac{synon \cdot R_{\inf} - R_{on}}{R_{\tau}} \tag{10}$$

$$\frac{dR_{off}}{dt} = -\beta \cdot R_{off} \tag{11}$$

$$R_{\inf} = \frac{C_{\max} \cdot \alpha}{C_{\max} \cdot \alpha + \beta} \tag{12}$$

$$R_{\tau} = \frac{1}{C_{\max} \cdot \alpha + \beta} \tag{13}$$

where $E_{rev} = 0(mV), C_{\max} = 1(mM), \alpha = 10(ms^{-1}), \beta = 0.11(ms^{-1})$

The above β value is used in the pyramidal neuron model. In the FS, RS and IS neuron model, β=0.18 (ms^{-1})

The equation for the fast sodium channel $I_{Na(fast)}$ for the FS neuron model has been previously published in (Sidiropoulou & Poirazi, 2012)