



# Deciphering the effects of microbial products on Peptide YY expression and secretion in human enteroendocrine L-cells

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Par

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Régulation de l'expression et de la sécrétion du Peptide YY par  
des produits du microbiote intestinal dans des cellules  
entéroendocrines humaines de type L

**Thèse présentée et soutenue à Jouy-en-Josas, le 4 septembre 2015 :**

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## Résumé détaillé

Le tractus gastro-intestinal est un organe essentiel pour l'hôte, permettant la digestion des aliments et l'absorption des nutriments nécessaires au développement et au fonctionnement de l'organisme chez les animaux. Il constitue ainsi un organe vital. Le tractus digestif participe également à la régulation d'un grand nombre de fonctions, telles que le développement et la maturation du système immunitaire, la régulation du métabolisme énergétique et lipidique, et le dysfonctionnement de certaines de ses fonctions sont associées à de nombreuses pathologies dont la prévalence a fortement crû depuis trente ans. Les différentes composantes de cet organe permettent d'assurer ces multiples fonctions.

Une composante importante du système digestif est l'ensemble des micro-organismes, bactéries, virus archées et eucaryotes qui le colonisent, appelé microbiote intestinal. Présent tout du long du tractus digestif, toutes les parties ne sont pas colonisées de manière similaire. La concentration en bactéries croît de l'estomac au colon, le microbiote colique représentant un écosystème d'extrême densité microbienne. Seuls quelques phyla sont représentés, les bactéries appartenant principalement aux Bacteroidetes et aux Firmicutes indiquant une adaptation aux conditions particulières, physiques (température, pH, degré oxydatif) que chimique (nutriments disponibles) et biologique (bactériocines, occupation des niches écologiques par d'autres espèces...). Cependant, une très grande diversité au niveau des espèces est retrouvée. Le microbiote intestinal occupe des fonctions primordiales dans le tractus intestinal, permettant notamment la digestion des fibres complexes, non dégradables par l'hôte, en sucres simples fermentés en acides gras à chaînes courtes (AGCC). Ces AGCC servent de substrat énergétique pour l'hôte. Le microbiote intestinal permet également la production de nutriments nécessaires à son hôte (vitamines, métabolisme d'acides aminés...) et a un rôle important dans la maturation du système immunitaire ainsi que dans la défense contre des pathogène, occupant une certaine niche écologique. Le microbiote intestinal a également été associé à la régulation de nombreuses autres fonctions de l'hôte, telles que le métabolisme (énergétique ou lipidiques), le fonctionnement intestinal (absorption/sécrétion, perméabilité, transit) ou encore le comportement.

Une autre composante importante du tractus digestif est son rôle endocrinien, permettant la régulation de nombreuses fonctions de l'hôte. En effet, le système digestif représente le système endocrinien de l'organisme, sécrétant plus de vingt hormones différentes dont la ghréline, la gastrine, la cholecystokinine, la sérotonine, le glucagon-like peptide 1 (GLP1) et 2 ou le peptide YY (PYY). Ces hormones régulent aussi bien des fonctions locales, telles que la sécrétion d'enzymes digestives, la régulation du pH, l'absorption des nutriments, des électrolytes et de l'eau ou le transit, que des fonctions périphériques telles que le métabolisme énergétique par le contrôle de la sécrétion d'insuline, lipidique ou d'autres tissus ou encore régulant les réponses inflammatoires. Un certain nombre de ces hormones participent également à la régulation du comportement, notamment alimentaire, agissant directement au niveau du cerveau, et plus particulièrement du noyau arqué de l'hypothalamus ou indirectement par le nerf vague.

Ces hormones sont sécrétées par des cellules spécialisées et rares de l'épithélium intestinal, les cellules entéroendocrines. Les cellules entéroendocrines représentent en réalité une famille de cellules pouvant être caractérisées par leur localisation et l'expression de différentes hormones spécifiques. Ces cellules proviennent de la différenciation des cellules souches situées en fond de crypte dans le lignage sécrétoire comprenant également les précurseurs des cellules de Paneth, des cellules caliciformes et des cellules tuft, puis par l'expression de facteurs de transcription permettant l'expression des gènes précurseurs des hormones ou permettant leur synthèse.

Les cellules entéroendocrines expriment également un grand nombre de récepteurs et transporteurs à leur surface, permettant la reconnaissance de différents éléments présents dans la lumière intestinal. La stimulation de ces récepteurs, principalement des récepteurs couplés à des protéines G (GPCR), induit une réponse sécrétrice de la cellule entéroendocrine. Ainsi, les éléments de la lumière intestinale permettent de réguler la sécrétion, et donc les niveaux plasmatiques, d'un grand nombre d'hormones. En particulier, les nutriments induisent la sécrétion dans un certain nombre de types de cellules entéroendocrines, permettant l'augmentation rapide après la prise alimentaire des niveaux des hormones anoréxigènes.

Les cellules entéroendocrines exprimant un grand nombre de récepteurs leur conférant une sensibilité au contenu intestinal, elles paraissent comme pouvant être un élément important

du dialogue entre le microbiote intestinal, composante importante de la lumière intestinale, et l'hôte. Les cellules entéroendocrines de type L, qui sécrètent le GLP-1, -2, le PYY et l'Insulin-like Peptide-5 sont principalement localisées dans la partie distale de l'iléon et le côlon, là où le microbiote intestinal est le plus dense. L'hypothèse émise est que des produits microbiens régulent le fonctionnement de ces cellules, et en particulier l'expression des gènes codant pour ces hormones ainsi que leur sécrétion.

Le GLP-1 a été particulièrement étudié pour son rôle dans la régulation de la sécrétion d'insuline, faisant de lui une cible particulière dans la recherche médicale sur le diabète. Le PYY a de nombreuses fonctions. De la même famille que le neuropeptide Y et le polypeptide pancréatique, il agit par l'intermédiaire d'une famille de récepteurs, les récepteurs Y. Le PYY est présent sous deux formes circulantes dans le plasma : une forme longue de 36 acides aminés (PYY 1-36) et une forme tronquée de 2 acides aminés en partie N-terminale (PYY 3-36). Ces deux formes ont des affinités différentes pour les récepteurs, le PYY1-36 activant les récepteurs Y1, Y2 et Y5 alors que le PYY 3-36 n'active que le récepteur Y2 et dans une moindre mesure Y5. Les récepteurs Y sont des GPCR couplés à des protéines Gi/o, et ont donc des effets inhibiteurs. Les principales fonctions du PYY 1-36 sont la stimulation de la motricité intestinale, l'inhibition de la sécrétion d'insuline induite par le glucose et l'inhibition de la réponse inflammatoire dans les macrophages localisés dans les tissus adipeux. Le PYY 3-36 permet d'inhiber la prise alimentaire par l'inhibition des neurones NPY, orexigènes, dans le noyau arqué, l'inhibition de la motricité intestinale, l'inhibition de la sécrétion d'électrolytes par les cellules coliques ou l'inhibition du métabolisme osseux.

Des modifications des niveaux plasmatiques de PYY ont été corrélées avec certaines pathologies, notamment des pathologies associées à des modifications du comportement alimentaire telles que l'obésité ou différentes formes d'anorexies. Il semble que le niveau de ces hormones soit particulièrement important dans ces maladies et l'obésité, puisque la modification de leurs niveaux, soit directement par injection de l'hormone ou par chirurgie bariatrique induisant une plus grande stimulation de ces cellules par des nutriments, permet de réduire la prise alimentaire de ces personnes. Ces pathologies étant également associées à des modifications du microbiote intestinal, ces résultats montrent une corrélation entre modification du microbiote intestinal et modification des niveaux plasmatiques du PYY,

appuyant l'hypothèse que le fonctionnement des cellules entéroendocrines de type L (cellules L) peut être affecté par le microbiote intestinal.

Cependant, très peu de liens fonctionnels entre produit microbiens et cellules L ont été établis dans la littérature jusqu'à présent. Le caractère dispersé des cellules entéroendocrines dans l'épithélium intestinal et l'absence de marqueurs externes permettant leur tri ont limité les études directes et la réponse à différents produits bactériens. Plusieurs lignées cellulaires issues de tumeurs ont été développées comme modèle pour l'étude *in vitro* des cellules entéroendocrines. En particulier, les cellules NCI-h716, issues d'un adénocarcinome colique, et les cellules HuTu-80, d'origine duodénales, sont des lignées humaines connues pour sécréter le GLP-1 et donc comme étant des modèles de cellules L. Ces deux dernières lignées ont donc été utilisées pour étudier *in vitro* l'effet de produits bactériens sur la production et la sécrétion de PYY. La caractérisation de ces cellules au niveau expression, par qRT-PCR, et protéiques par immunofluorescence, a permis de montrer l'expression de PYY et de Chromogranin A, un marqueur spécifique des cellules entéroendocrines, dans ces cellules. De plus, ces cellules expriment les facteurs de différenciations précoces spécifiques des cellules entéroendocrines *Neurogenin3* et *FoxA1* et ceux spécifiques des cellules L *Pax4* et *Pax6*. L'origine des cellules NCI-h716 et leur pattern d'expression des facteurs de différenciations en font le meilleur modèle de cellules L coliques, les cellules d'intérêt car le plus en contact avec les bactéries du microbiote intestinal.

L'objectif étant de trouver des produits bactériens régulant l'expression et la sécrétion de PYY dans les cellules L, des systèmes rapporteurs ont cherché à être développés. Un système rapporteur permet en effet par la mesure d'un paramètre simple et à moindre coût de mesurer une activité cellulaire, et donc de cribler un grand nombre de produits. Un premier système rapporteur pour mesurer l'expression du gène du *Pyy* a été envisagé. La structure du gène humain codant pour le *Pyy* est beaucoup plus complexe que le gène murin, le gène humain comprenant 3 exons supplémentaires aux quatre murins, et qui sont répartis sur plus de 40 kb. Face à la difficulté de connaître les régions importantes régulant l'expression de ce gène, une stratégie d'intégration d'un gène codant pour une protéine fluorescente, l'eGFP ou la mcherryFP, directement au locus du gène. Cette intégration est facilitée grâce aux outils récemment développés permettant d'induire des cassures doubles brins de l'ADN à des endroits précis et choisis, les Talen ou les Crispr/Cas9. Cependant, le développement d'un tel

système n'a pas abouti. Ainsi, l'analyse de l'expression du gène de *Pyy* a été effectuée par qRT-PCR, une méthode efficace mais ne permettant pas un criblage à haut débit.

Un deuxième système rapporteur a été développé permettant de mesurer indirectement la sécrétion. Celui-ci repose sur le fait qu'une augmentation de la concentration calcique cytosolique locale est nécessaire pour la fusion des granules de sécrétion avec la membrane plasmique, et donc la sécrétion des hormones. Des lignées exprimant de manière constitutive une protéine dont la fluorescence dépend de la concentration calcique, la GCaMP-5G ont été sélectionnées. Par vidéo-microscopie, ici en utilisant un microscope confocal à balayage, il est ainsi possible de suivre les réponses calciques à différents produits bactériens. Des outils d'analyse de reconnaissance et de mesure de fluorescence ont permis l'analyse automatisée des réponses. Le criblage d'une banque métagénomique, une technique permettant de trouver de nouveaux produits bactériens, a tout d'abord été envisagé. Le principe consiste à faire exprimer des gènes de bactéries du microbiote intestinal par une bactérie facilement cultivable en laboratoire, tel qu'*E. coli*, s'affranchissant ainsi des difficultés de mises en cultures des bactéries intestinales. Le criblage n'a pu être effectué du fait de la forte réponse induite par le surnageant d'*E. coli* lui-même, limitant la capacité à observer d'autres réponses induites par d'autres molécules produites spécifiquement par des clones métagénomiques. Cependant, ce système peut facilement être utilisé pour mesurer la réponse à des produits purifiés et a permis de décrire précisément les réponses induites par des molécules d'origine bactériennes.

De nombreuses études ont mis en avant le rôle des acides gras à chaînes courtes (AGCC) produit par la fermentation des fibres sur les niveaux plasmatiques de PYY. En effet, des régimes riches en fibres est associé à une augmentation à court et long terme des niveaux plasmatiques de PYY. Les AGCC sont produits par certaines bactéries spécifiques du microbiote par différentes voies métaboliques de la fermentation des sucres issus de la dégradation des fibres non digérées par l'hôte. Les AGCC sont principalement produits dans le côlon et trois sont principalement produits, l'acétate, le propionate et le butyrate, à des concentrations d'environ respectivement 60, 20 et 20 mM. Ces voies requièrent un arsenal enzymatique important qui n'est souvent pas possédé par une espèce uniquement, mais le métabolisme peut associer différentes espèces agissant chacune à leur niveau dans le

métabolisme. De plus, des régimes enrichis en fibres conduisent à des modifications du microbiote intestinal, favorisant les espèces capables de les utiliser comme substrat énergétique, et permettant ainsi une plus grande capacité métabolique. Ainsi, les régimes en fibres peuvent avoir un effet indirect sur la production de PYY en favorisant des bactéries produisant des molécules reconnues par les cellules L.

Cependant, l'administration d'AGCC directement dans le côlon induit la sécrétion de PYY par les cellules L, indiquant un rôle direct des AGCC produit par la fermentation des fibres. Un effet des AGCC a été démontré permettant d'expliquer en partie leur effet à long terme passant par l'augmentation du nombre de cellules L. Cependant, d'autres mécanismes peuvent être impliqués, notamment un effet direct sur les cellules individuelles et notamment sur la sécrétion. Les AGCC agissent sur les cellules eucaryotes par différents mécanismes décrits dans la littérature. Tout d'abord, ils sont internalisés par des transporteurs spécifiques, et peuvent être utilisés comme substrat énergétique par la cellule par  $\beta$ -oxydation. Un deuxième effet intracellulaire, concernant uniquement le propionate et le butyrate, et dans des proportions beaucoup plus faible l'acétate, est un effet inhibiteur de lysine déacétylases, et notamment d'histones déacétylases (HDAC). Cet effet conduit à une modification de l'acétylation des histones et de la structure chromatinienne, et donc conduit à une modification de l'expression d'un grand nombre de gènes. Enfin, des récepteurs couplés à des protéines G ont été identifiés comme récepteurs aux AGCC. FFAR2 et FFAR3 appartiennent à la famille des récepteurs aux acides gras (FFARs, free-fatty acid receptors). FFAR2 reconnaît l'acétate, le propionate et le butyrate avec la même affinité alors que FFAR3 reconnaît l'acétate avec une affinité beaucoup plus faible que le propionate et l'acétate. Il est à noter que ces différences affinités ne sont pas conservées chez les orthologues murins. Un troisième récepteur reconnaît le butyrate avec une affinité faible, GPR109a. Les GPCRs permettent souvent d'induire des réponses sécrétoires en modulant les flux calciques par la signalisation des petites protéines G. Les trois récepteurs sont couplés à des protéines Gi/o, qui inhibent l'AMP cyclase, et ont donc des effets diminuant la concentration d'AMP cyclique, et donc de calcium cytosolique. FFAR2 peut également être couplé à une protéine Gq, voie qui active la phospholipase C, enzyme clivant le phosphatidylinositol (4,5) bis-phosphate en diacylglycérol, activant la phosphokinase C, impliquée dans la régulation de l'expression d'un certain nombre

de gènes, et en inositol tris-phosphate, induisant la libération de calcium du réticulum dans le cytosol.

Les récepteurs FFAR2 et FFAR3 ont été impliqués dans plusieurs études montrant leur rôle sur la réponse sécrétoire aux AGCC, donnant des résultats controversés sur le rôle précis de chacun de ces récepteurs. De plus, ces études ont uniquement été réalisées sur des modèles murins, et ne permettent donc pas de déterminer précisément les réponses des cellules L humaines aux AGCC. L'utilisation des NCI-h716 et des HuTu-80 comme lignées modèles des cellules L ont permis de caractériser précisément les effets des AGCC sur l'expression et la sécrétion de PYY. Ces lignées expriment au moins un transporteur des AGCC, MCT-1 et les trois récepteurs aux AGCC, mais avec des niveaux différents en ce qui concerne FFAR2. *FFar2* est beaucoup plus exprimé dans les NCI-h716, reflétant le caractère plus colique de cette lignée que les HuTu-80.

Les trois AGCC principaux, l'acétate, le propionate et le butyrate augmentent significativement l'expression du *Pyy*, mais à des degrés variables. Le propionate et le butyrate augmentent très fortement l'expression du gène dans les cellules NCI-h716, par des rapports respectifs de 40 et de 120, alors que l'acétate a un effet beaucoup modéré, augmentant l'expression de 80% environ. De plus, l'effet du butyrate varie avec la concentration et le temps d'incubation avec le butyrate, sans saturation aux doses et temps testés (5mM ou 48h). La Trichostatin A, une drogue mimant les effets du propionate et du butyrate par ses effets inhibiteur d'HDAC, induit également une forte surexpression du *Pyy*, suggérant que l'effet médié par les AGCC passe par une inhibition des HDAC. De plus, les agonistes spécifiques des récepteurs FFAR2 et FFAR3, respectivement l'acide tiglic et le 1-MCPC, induisent une augmentation de l'expression du *Pyy*, mais à des niveaux similaires à ceux de l'acétate. La niacine, agoniste de GPR109a n'a pas d'effet. De plus, des effets similaires sont retrouvés dans cellules HuTu-80 avec des effets moindres. En particulier, aucune réponse significative à l'acétate n'est observée. Cependant, la différence importante d'expression du récepteur FFAR2 suggère que cette absence de réponse à l'acétate peut être due à la moindre stimulation du récepteur dans ces cellules. En effet, la surexpression transitoire du récepteur dans ces cellules permet d'induire une augmentation de l'expression de *Pyy* en réponse à l'acétate, indiquant le rôle essentiel de FFAR2 dans la réponse observée à l'acétate.



Ainsi, les AGCC modulent l'expression du *Pyy* par deux mécanismes distincts et complémentaires : d'une part la stimulation du récepteur FFAR2 qui induit une augmentation de l'expression du gène d'environ 80%, et d'autre part l'inhibition d'HDAC qui induit une très forte surexpression du gène, effet uniquement produit par le propionate et l'acétate.

Les trois AGCC induisent également des réponses calciques dans les cellules NCI-h716 sur exprimant GCaMP-5G, avec des effets variés. Les trois induisent des augmentations transitoires de la concentration calciques oscillantes, mais avec des amplitudes différentes, et pendant un temps plus ou moins long. L'acétate induit les oscillations les plus fortes et en plus grand nombre, le propionate produit un effet proche bien que légèrement plus faible alors que le butyrate induit que quelques oscillations de faible amplitude, puis une diminution de la concentration calcique est observée. Parmi les agonistes des récepteurs connus, seuls l'acide tiglic, agoniste de FFAR2, induit une réponse calcique similaire à l'acétate. Ces résultats sont en cohérence avec les connaissances de ces trois récepteurs, puisque seul FFAR2 est couplé à une protéine Gq, induisant une augmentation de la concentration calcique par libération du calcium réticulaire. L'utilisation d'inhibiteurs de la voie de signalisation des protéines Gq à deux niveaux : l'U73122 et le 2-APB ont permis de confirmer que l'inhibition de cette voie abolie la réponse calcique induite par les AGCC. De plus, dans les cellules HuTu-80, les AGCC n'induisent pas de réponse. Par contre, la surexpression de FFAR2 dans ces cellules permet une augmentation transitoire de la concentration calcique par tous ces récepteurs, démontrant l'importance d'une forte expression de FFAR2 dans la réponse des cellules L aux AGCC.

La mesure par ELISA de la sécrétion du PYY dans le surnageant pendant 2h par des cellules soumises aux AGCC a permis de confirmer l'augmentation de la sécrétion par rapport aux cellules non traitées d'environ 50%, même si la faible sensibilité par rapport aux concentrations mesurées n'ont pas permis de détecter une différence dans les niveaux de sécrétion en réponse aux différents AGCC. De plus, les cellules traitées au préalable par du butyrate ont des niveaux de sécrétion sans traitement supérieur d'un rapport d'environ 4 comparé aux cellules non traitées, montrant que la surexpression du gène induite par le butyrate dans ces cellules conduit à une augmentation de la production et de la quantité du PYY sécrétée. Par contre, la réponse aux AGCC dans les cellules traitées au butyrate diffère de

celle des cellules non traitées au préalable. Ainsi, l'acétate n'induit pas d'augmentation de la sécrétion, et le butyrate induit une diminution du niveau de sécrétion.

L'effet inhibiteur du butyrate sur la sécrétion a été montré comme étant dépendant de l'activation de la voie de signalisation Gi/o. Cet effet est produit par l'activation simultanée aux concentrations testées des trois récepteurs couplés à des Gi/o, et l'activation d'uniquement FFAR2 et FFAR3 par le propionate n'est pas suffisant pour induire cette inhibition. En effet, l'ajout de butyrate inhibe les oscillations induites par l'acétate, alors qu'une deuxième addition successive d'acétate ou de tampon contrôle ne modifie pas les oscillations induites par l'acétate. De plus, la toxine pertussis, inhibitrice de la voie Gi/o, inhibe cet effet inhibiteur du butyrate, qui est également observé par une décroissance de la concentration calcique dans les cellules après ajout de butyrate.

De plus, le butyrate induit également une inhibition à long terme sur la réponse à l'acétate, puisqu'après incubation avec du butyrate, la réponse calcique à l'acétate est réduite. Un mécanisme passe par la modulation de l'expression du récepteur FFAR2 par le butyrate, puisque son expression est réduite de 80% après 24h de traitement.

Ainsi, les AGCC modulent la sécrétion dans les cellules L, à la fois en activant à court terme la sécrétion par la stimulation de FFAR2 et de la voie Gq, mais également, et principalement par le butyrate, inhibe à long terme la sécrétion par l'activation cumulative de FFAR2, FFAR3 et GPR109a et de la voie Gi/o. Le butyrate permet donc à long terme l'accumulation de PYY pouvant être sécrété par deux mécanismes distinct : d'une part l'augmentation de l'expression du gène de *Pyy*, et d'autre part par l'inhibition de sa sécrétion.

Les AGCC semblent ainsi être des molécules particulièrement importantes dans la régulation de l'expression et la sécrétion du PYY, avec le butyrate qui semble un régulateur fort à la fois de la production et de la sécrétion de cette hormone. Les effets des AGCC sont médiés par des GPCRs, mais également par un effet inhibiteur d'HDAC du propionate et du butyrate qui permettent d'augmenter fortement la production de PYY. Cependant, la localisation de ces récepteurs dans ces cellules polarisées dans l'épithélium n'est pas connue et le modèle utilisé ne permet pas d'obtenir de caractériser ce modèle. Etant donné les grandes différences de concentrations en AGCC entre la lumière intestinale et la *lamina propria*, les cellules ne sont pas stimulées similairement si les récepteurs sont sur la membrane apicale ou basolatérale.

Les concentrations très élevées dans la lumière intestinale induiraient des stimulations permanentes de ces récepteurs, et donc n'induirait pas de variations de sécrétion lors de l'évolution de leur concentration, alors que la localisation basolatérale permettrait la reconnaissance et une réponse aux variations de concentrations d'AGCC dans la *lamina propria*, ces concentrations étant du même ordre de grandeur que les Kd de FFAR2 et de FFAR3.

De plus, il est à penser que les concentrations dans la lumière ne sont pas uniforme, mais qu'un gradient décroissant de butyrate existe dans les cryptes coliques. Les cellules L répondant de manière dépendante à la concentration de butyrate concernant la concentration de PYY, il est envisageable que lors de la migration des cellules L du fond de crypte vers le haut de crypte, elles soient soumises à des concentrations de plus en plus élevées de butyrate. Ainsi, elles produisent des quantités croissantes de PYY, leur permettant d'augmenter les quantités de PYY libérées en réponse à des stimulations. En parallèle, le butyrate par son effet inhibiteur sur la sécrétion diminue leur sensibilité. Ce mécanisme peut correspondre à une sensibilité accrue à des stimulations dans le milieu de crypte, là où les stimulations sont plus faibles, et une plus faible sensibilité pour les zones de plus forte excitation. Ainsi, le butyrate peut être considéré comme un produit bactérien participant à la maturation des cellules L, et modulant leur activité en fonction de leur localisation dans la crypte.

Une deuxième voie de communication entre les microbes et les cellules de l'hôte bien caractérisée passe par la reconnaissance de motifs conservés (MAMPs microbial associated molecular patterns) par une famille de récepteurs, les récepteurs de type Toll (Toll-like Receptors, TLRs). Ces récepteurs ont tout d'abord été décrits dans les réponses immunitaires, activant principalement la voie de signalisation NF- $\kappa$ B, un facteur de transcription induisant la surexpression et la sécrétion de cytokines pro-inflammatoires. Les TLR peuvent être divisés en deux groupes : les TLRs localisés à la membrane plasmique, TLR1/2, TLR2, TLR4, TLR5 et TLR2/6 qui reconnaissent essentiellement des éléments de membranes ou de parois de microbes, tels que la flagelline, les lipopolysaccharides ou les peptidoglycans ; et les TLRs localisés dans des endosomes, TLR3, TLR7, TLR8 et TLR9 qui reconnaissent des séquences nucléiques d'ARN ou d'ADN bactériennes ou virales.

La majorité des TLR sont exprimés par les cellules épithéliales de l'intestin, et plusieurs fonctions associés au maintien de la barrière intestinale ont été associées telles que la sécrétion de défensines par la stimulation de TLR9 dans les cellules de Paneth ou la sécrétion de sérotonine par les cellules entérochromaffines. Cependant, ni leur présence ni leur fonction sur les cellules L n'a été montrée à ce jour. Ces cellules pouvant avoir un rôle important dans le dialogue entre le microbiote et l'hôte, l'expression de TLR et leur sensibilité aux MAMPs associées peuvent participer à ce dialogue. De plus, l'analyse du promoteur du gène de *Pyy* montre la présence de domaines de liaison à NF- $\kappa$ B.

Afin de mesurer l'activité NF- $\kappa$ B dans ces cellules, des lignées NCI-h716 exprimant de manière stable un gène rapporteur, la SEAP, sous contrôle de 5 domaines de liaison à NF- $\kappa$ B ont été construites (lignées pnifty2-SEAP). Tout d'abord, l'expression des différents TLR a été mesurée par qRT-PCR, et a permis de montrer que tous les TLR à l'exception de TLR 4 et 8 sont exprimés dans ces cellules. L'utilisation d'agonistes spécifiques et des lignées pnifty-SEAP a permis de montrer que tous les agonistes des TLR détectés par qRT-PCR activent la voie NF- $\kappa$ B de manière significative, sauf les ligands de TLR 3 et de TLR 7. De plus, l'ensemble des ligands des TLRs, à l'exception des ligands de TLR3 et de TLR4 augmentent significativement d'un rapport de deux l'expression du gène de *Pyy*, confirmant l'hypothèse que l'activation de NF- $\kappa$ B module l'expression de *Pyy*. Cela laisse supposer que les ligands de TLR7, et de TLR8 qui est très proche de celui de TLR7 et peut stimuler ce récepteur, active la voie NF- $\kappa$ B de manière non détectable pour le système rapporteur pnifty2 SEAP, mais à un niveau suffisant pour induire l'expression de *Pyy*. De plus, cet effet est cumulatif au butyrate, les cellules traitées uniquement au butyrate pendant 48h ayant une augmentation de l'expression de *Pyy* d'un rapport de 320 alors que l'ajout de tout agoniste de TLR, y compris les agonistes de TLR3 et de TLR4 conduit à une augmentation de l'expression par un rapport entre 450 et 550 par rapport aux cellules contrôle. La réponse au ligand de TLR3 et TLR4 dans les cellules incubées avec du butyrate a été confirmée comme correspondant à une augmentation de la réponse NF- $\kappa$ B aux ligands des TLR dans les cellules incubées avec du butyrate, à l'exception du ligand de TLR8. Aussi, le butyrate augmente l'expression de la majorité des TLR, à l'exception de TLR6, le TLR le plus exprimé de base dans ces cellules, et de TLR8 qui reste non détectable. TLR4, qui était non détectable par qRT-PCR, l'est après incubation par du butyrate.

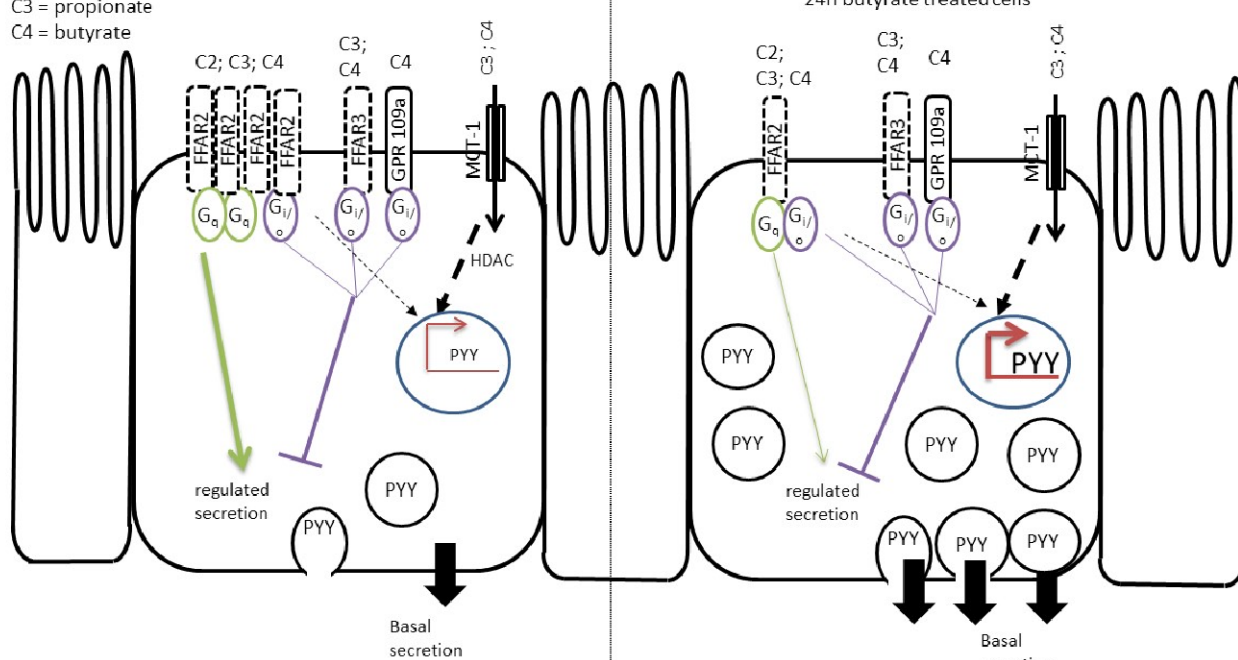
Les TLR sont donc des récepteurs pouvant participer à la régulation de l'expression du *Pyy*, par l'activation de la voie NF- $\kappa$ B participant à la régulation de ce gène. Cependant, la stimulation des TLR ne paraît pas avoir d'effet sur la sécrétion. En effet, la stimulation des TLR de la membrane plasmique n'induit aucune réponse calcique, et la stimulation des TLR endosomaux induit une réponse brève qui ne paraît pas spécifique car produite par les ligands de TLR3 et TLR8 qui n'induisent pas de réponse NF- $\kappa$ B.

Ainsi, un produit bactérien, le butyrate, module la réponse des cellules L aux MAMPs en augmentant l'expression des TLR ainsi que la réponse NF- $\kappa$ B associée à la stimulation de ces récepteurs. Le rôle de ces TLR dans les cellules L n'est pas complètement connu, même si ils paraissent comme pouvant participer à la modulation de l'expression du *Pyy*. Leur localisation, non déterminée, est importante considérant que la partie apicale de la cellule est en permanence en contact avec des MAMPs, et induiraient un signal constant alors qu'une stimulation basolatérale correspondrait à un franchissement microbien de la barrière intestinal, et donc à une potentielle infection. La voie NF- $\kappa$ B, pour la première fois décrite ici comme stimulant l'expression d'une hormone, le PYY, active de nombreuses voies de signalisation, et l'effet de son activation dans les cellules L reste à caractériser.

Les résultats de ces travaux ont permis de mettre en évidence le rôle des cellules entéroendocrines de type L dans le dialogue avec le microbiote intestinal par leur capacité à reconnaître plusieurs types de produits bactériens, à savoir les acides gras à chaîne courte et les MAMPs. La reconnaissance de ces produits bactériens permet de moduler l'activité de ces cellules, notamment concernant la production d'une hormone régulant plusieurs fonctions de l'hôte, le PYY, ainsi que sa sécrétion. L'utilisation de modèles cellulaires, et notamment des cellules NCI-h716 a permis de caractériser les mécanismes cellulaires associés. En particulier, le butyrate, un acide gras à chaîne courte, semble un élément important dans la régulation des cellules affectant fortement la production de PYY, mais également régulant l'expression d'un grand nombre de récepteurs, permettant de moduler la sensibilité des cellules à d'autres produits, que ce soit la diminuer comme par exemple en diminuant l'expression de FFAR2 qu'à l'augmenter comme c'est le cas pour l'expression des TLR.

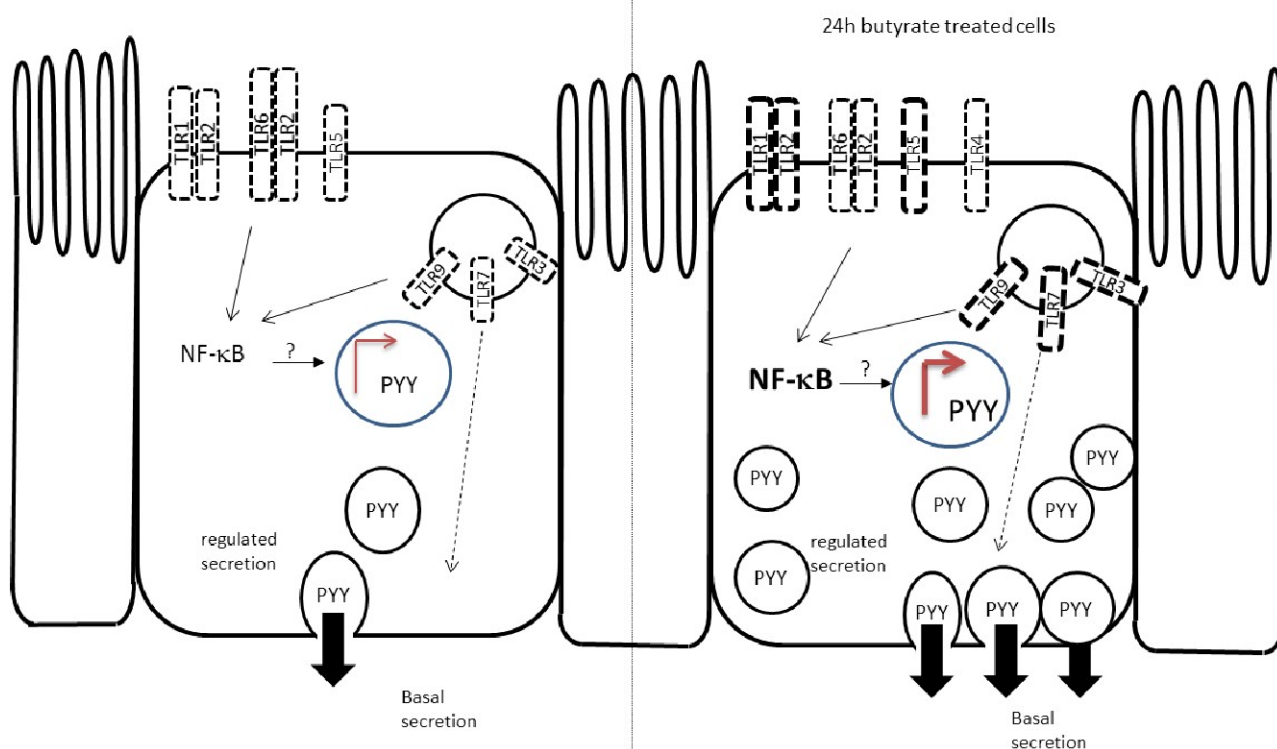
Le PYY de par ses nombreuses fonctions sur l'organisme peut donc être considéré comme une molécule importante du dialogue entre le microbiote intestinal et l'hôte. Ces résultats ont permis de mettre en évidence plusieurs mécanismes régulant son expression et sa sécrétion. Cependant, il est à penser compte tenu de ses effets sur la prise alimentaire, la motricité intestinale ou l'absorption d'électrolytes, qu'il peut participer à la régulation du contenu intestinal, et donc du microbiote intestinal. En effet, plusieurs études ont montré un lien entre transit intestinal et composition du microbiote intestinal. Les niveaux de PYY peuvent potentiellement correspondre à un équilibre permettant à un certain microbiote intestinal de se maintenir dans la niche intestinale. Ainsi, les produits bactériens contrôlent en partie les niveaux de PYY qui eux même régulent le contenu intestinal. Des perturbations de cet équilibre, tant du point de vue du microbiote que du PYY ou d'autres facteurs importants intervenant dans ce dialogue et non étudiés ici participent au développement de pathologies associées à des perturbations du métabolisme ou du comportement alimentaire et à des dysbiose telles que l'obésité. Une meilleure compréhension des mécanismes impliqués dans le dialogue pourront permettre de mieux comprendre ces pathologies et envisager des pistes thérapeutiques rétablissant un équilibre dans le dialogue microbiote/hôte.

C2 = acetate  
C3 = propionate  
C4 = butyrate



Cells express low levels of PYY and SCFAs strongly increase PYY secretion

Cell expressing high levels of PYY with less sensitivity to SCFAs



L-cells express functional TLRs and their activation increases Pyy expression

Butyrate increases MAMPs sensitivity

Résumé Graphique : Représentation de cellules entéroendocrines, de leurs récepteurs et des principaux effets des acides gras à chaîne courte (haut) et des ligands des TLR (bas) sur l'expression et la sécrétion de PYY dans les cellules L non traitées (gauche) ou traitées 24h au butyrate (droite)







**Abbreviations:**

1-MCPC	1-Methylcyclopropane Carboxylate
2APB	2-Aminoethyl Diphenylborinate
5-HT	Serotonin
AgRP	Agouti-Related Peptide
$\alpha$ -MSH	$\alpha$ -Melanocyte Stimulating Hormone
ARC	Arcuate nucleus
BMP	Bone Morphogenetic Protein
cAMP	cyclic Adenosine Monophosphate
Cas9	Crispr associated protein 9
CCK	Cholecystokinin
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DAG	Diacylglycerol
DPP-IV	Dipeptidyl peptidase-4
EC	Enterochromaffin cells
ECL	Enterochromaffin-like cells
EEC	Enteroendocrine cells
eGFP	enhanced Green-Fluorescent Protein
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FFAR	Free Fatty Acid Receptor
GIP	Glucose dependent Insulin-tropic Peptide
GLP	Glucagon Like peptide
GPCR	G-Protein Coupled Receptor
IBD	Inflammatory Bowel disease
IP3	Inositol-trisphosphate
LGR5	Leucine-rich Repeat-containing G-protein coupled Receptor 5
LHA	Lateral Hypothalamic Area
LPS	Lipopolysaccharide
MAMPs	Microbial Associated Molecular Patterns

NF- $\kappa$ B	Nuclear Factor - $\kappa$ B
NPY	Neuropeptide Y
NTS	Nucleus Tractus Solitarii
PKC	Protein Kinase C
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PP	Pancreatic Polypeptide
PVN	Para Ventricular Nucleus
PYY	Peptide-YY
RT-qPCR	Reverse Transcriptase quantitative Polymerase Chain Reaction
SCFA	Short Chain Fatty Acid
TALEN	Transcription Activator-Like Effector Nuclease
TLR	Toll-Like Receptor
TR	Taste Receptor
TSA	Trichostatin A
UPR	Unfolded Protein Response

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# Introduction



## The gastrointestinal tract

### The digestive system, vital functions for the host

#### *Components of the digestive system*

Human being relies on energy and nutrient intake for its development and functioning that have to be tightly controlled. The digestive system is composed of several organs permitting food intake, digestion and transformation into nutrients that are then assimilated and expulsion of compounds and toxins that are not absorbed. Regulation of these processes is autonomous, but signaling mainly from the brain can modulate these functions. Moreover, it also participates in the regulation of nutrient availability and storage in the liver and in the adipose tissues as well as the cross talk with the brain, participating in control of food intake. The digestive system is divided in two functional groups of organs: the gastrointestinal tract consisting of tissues in contact with foodstuffs and accessory organs that participate in the regulation of the food digestion and the host metabolism.

Accessory organs are the salivary glands, the pancreas, the liver, and the gallbladder. Salivary glands and acinar cells of the pancreas secrete digestive enzymes that participate in the food breakdown. Gallbladder stores bile produced by the liver and releases it under the control of hormones in response to nutrients entry in the duodenum and increases lipid absorption. Pancreas is also composed of endocrine cells localized in Langerhans islets that secrete insulin, glucagon, pancreatic polypeptide and somatostatin that regulate glucose homeostasis, food intake and intestinal motility.

The gastrointestinal tract is composed of five main parts. The buccal cavity participates in the first digestion steps through mechanical and chemical breakdown of food. Esophagus allows transit of food from the buccal cavity to the stomach. The stomach secretes enzymes, mainly proteases, acidifies and mixes mechanically the bolus to increase digestion process. The small intestine is divided in three parts, the duodenum, the jejunum and the ileum, which are the main absorptive surfaces and regulate transit. Finally, the large intestine composed of the caecum, the colon and the rectum that mainly store non absorbed elements, re-absorb water and electrolytes and regulate stool expulsion. Another important part of the digestive system that can be considered as an additional organ by itself is the important and dense population of microbes colonizing the gastrointestinal tract called microbiota. Microbial density is high in the colon and densities in the proximal part of the gastrointestinal tract except in the buccal cavity are much lower (Savage 1977) (Figure 1). Microbiota is important in the regulation of digestion as well as regulation of immunity and the protection against pathogens. However, even if most of these bacteria are commensal or symbiotic, meaning they are not harmful for the host, they must be kept in the lumen of the gastrointestinal tract to protect the organism from their colonization and from an over-induction of the immune system.



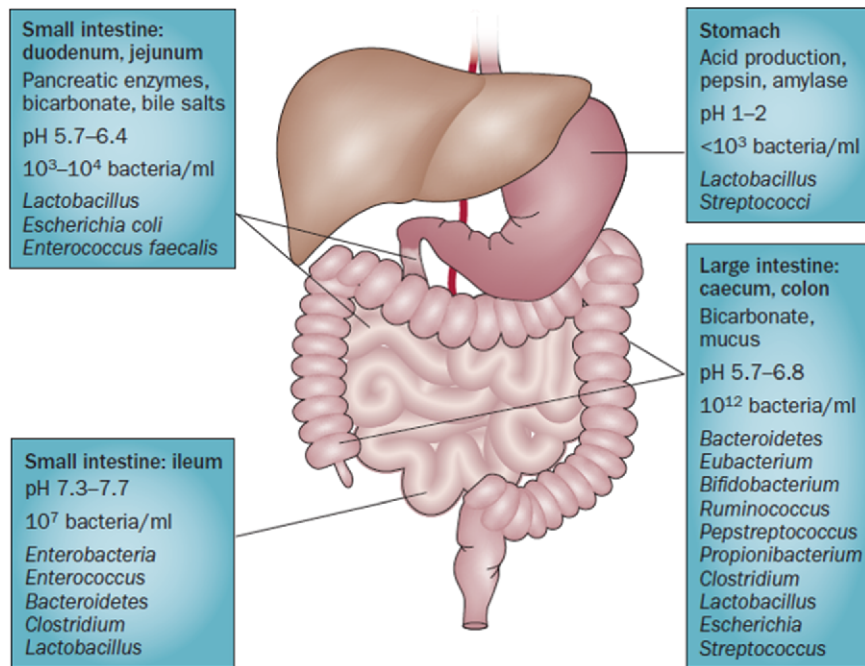


Figure 1 : Main host secretion and bacteria genus in the different parts of the gastrointestinal tract, adapted from Aron-Wisniewsky 2012

Lumen is composed of many different elements including degraded food, products secreted the digestive systems participating in foodstuff digestion and in the intestinal barrier, and the gut microbiota

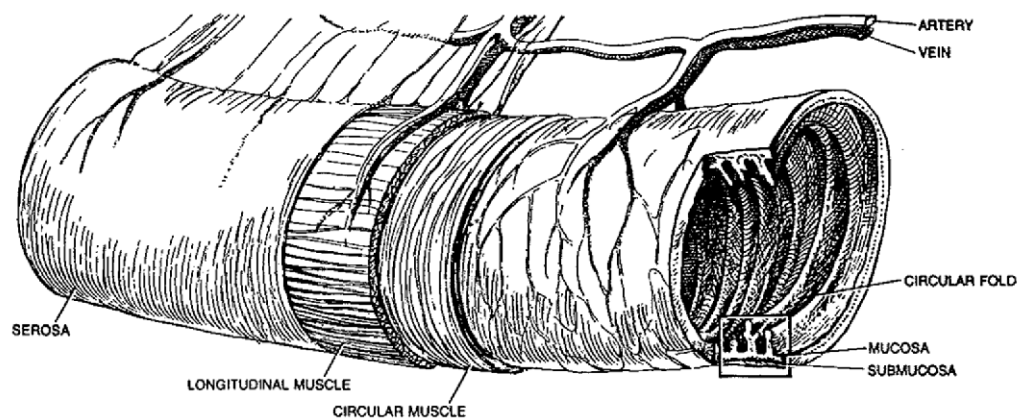


Figure 2 : Representation of different layers surrounding intestinal lumen (Johnson LR Physiology of the gastrointestinal tract, 2<sup>nd</sup> edition, 1987)

The gut wall is composed of several layers enabling the different functions of the gut: barrier with the lumen, absorption of nutrients and their delivery in the blood circulation, contractions enabling gut motricity, neuronal communications

### *Structuration of the gut wall*

To achieve this function as well as absorptive and motility functions, small and large intestines are composed of a superposition of different layers with specific functions (Figure 2). The inner layer is the mucosa consisting of a single epithelium layer with a specific 3D architecture increasing surface exposure to intestinal lumen supported by a lamina propria composed of extra cellular matrix and stromal cells as well as immune cells that participate in the regulation of microbial population and sense potential barrier ruptures as well as blood and lymph vessels for transport of absorbed nutrients. Outer this mucosa is a submucosal layer that permits irrigation of mucosa by larger blood and lymph vessels and is also composed of a plexus composed of neurons controlling mucosal secretion and sensing hormones secreted by intestinal cells. This layer is further externally surrounded by two muscular layers, an inner one composed of circular smooth muscle and an outer composed of longitudinal smooth muscle with another plexus between both of them that controls muscle contractions. These two layers induce contractions that regulate the luminal content, by churning and propelling it to more distal parts. This function is important to remove luminal waste, including dead cells, toxins and non-digestible elements as well as part of the microbiota. The two plexuses compose the enteric nervous system, which autonomously regulates muscle contractions as well as enzymatic secretion, but is also linked to the brain by the vagal nerve. These layers are further surrounded by a thin layer, the serosa delimiting the organ. This complex structure is necessary for the different functions of the intestine: absorption of nutrients that enter circulation, transit of luminal content from proximal part to distal parts, neuronal and humoral communication with other organs including the brain through the vagal nerve. Luminal content is composed of a great variety of elements including microbiota, food stuffs and nutrients when digested, but also secreted host products including bile acids, enzymes and mucus. Conditions vary between different parts of the digestive tract: pH is very low in the stomach, increases from the duodenum to the ileon from 6 to 7.4 and is between 5.8 and 7 in the colon (Evans 1988). Similarly, whereas oxygen is present in the proximal parts, its concentration is very low in colon enabling anaerobic bacteria growth due to consumption by first bacteria colonizing the intestine.

### *Gut microbiota*

Specific micro-environment in the colon allows the development of an important population of bacteria as well as other microorganisms including viruses, archaea, yeast and other eukaryotes. Most of the studies focused on bacteria as they represent the most important population, composed of up to  $10^{12}$  bacterial cells per gram of luminal content, outnumbering by one log the number of human cells (Luckey 1970). In the past, studies on gut microbiota functions were limited to the low number of bacterial species that could be cultivated. Advances in sequencing technologies enabled a new approach to study gut microbiota based on DNA extraction, high throughput sequencing and *in silico* analysis (Suau 1999, Eckburg 2005, Ley 2006). Two main methods have been used: sequencing of variable regions of ribosomal 16S, giving information about the species present in the studied samples and metagenomics sequencing giving access to the entire metagenome sequences. Gut bacteria mainly belong to two phyla, Bacteroidetes and Firmicutes. A few other phyla are also represented such as Actinobacteria and Proteobacteria. Thus, phyla diversity in the human microbiota is low, corresponding to adaptation to this specific environment that represents the intestinal track. However, diversity at the species level is much higher, as it is considered that a healthy human harbors about one thousands of different species (Tap 2009). More precisely, species annotation remains difficult

due to the great number of unknown bacteria which lead to consider the number of bacterial genes as a read-out of gut microbiota diversity. Human microbiota metagenome is composed of about six hundred thousand different genes per individual. Even if main features and functionality of microbiota is conserved between individuals, the gut microbiota is unique to each individual (Gill 2006, Human Microbiome Project consortium 2012, Qin 2010). Bacterial colonization of the gut is simultaneous to birth, and evolves during the first years of life, shaping and maturing the immune system to stabilize after weaning and access to complex food. Microbiota is supposed quite stable afterwards, but is influenced by environment, diet, infections, inflammatory responses including stress and antibiotic treatments. Humans can be clustered independently of diet, origin or health status in three groups named enterotypes, corresponding to enriched proportion of specific genus. One group has higher proportion of *Bacteroides*, another has increased proportion composed of *Prevotella* and finally the last one was associated with high proportion of *Ruminococcus* and archaea, mainly *Methanobrevibacter smithii* (Arumugan 2011). However, among this great diversity of microbiota, functional analysis of sequenced genes reveal a relative homogeneity in representation of main functions, consisting of a minimal core metagenome (Human Microbiome Project 2012, Quin 2010, Turnbaugh 2009). However, some specific functions may have different representation between individuals; mainly functions carried by low abundant species. Thus, some specific functions may only be present in some individual gut microbiota (Arumagan 2011).

Commensal bacteria colonize this specific ecological niche which is the gut thanks to specific pH condition (pH neutral to mildly acid), anaerobia, and energy mostly coming from fibers undigested by human enzymes and other residues from digestion as well as mucins. Moreover, cross talks with the host are important to maintain a homeostatic response in regard of these colonizing bacteria. Indeed, the host recognized microbes by a wide range of mechanisms, including Toll Like Receptors (TLRs), and does not induce an excessive inflammatory response in the gut. Microbiota is essential for the immune system establishment by expressing different compounds that can be sensed by immune cells to induce their stimulation and maturation. Reciprocally, the immune system and, more generally, the host shape the microbiota, resulting therefore in a balanced cross talk (Hasegawa 2014, Nicholson 2012). However, some modifications, mainly a loss of microbiota genetic diversity, called dysbiosis induces a stronger inflammatory response and has been associated with some pathologies as diverse as obesity or inflammatory bowels disease (Shen 2013, Cotillard 2013, Le Chatelier 2013). Microbiota participates also in the host protection being a barrier to pathogens as it occupies this highly competitive ecological niche and secretes a wide range of bacteriocins regulating also its own ecology. However, some commensal bacteria can be opportunistic pathogens, meaning that under particular conditions, they can have harmful properties if not present at the right place (for example after crossing the epithelial barrier) or have an increased population or ultimately when the host is somehow immuno-deficient. Microbiota is also a source of energy for the host as it ferments fibers into short chain fatty acids (SCFAs) and gases. Indeed, whereas human genome codes for a small number of highly-specific substrate enzymes that can breakdown some oligosaccharides, whereas the microbiome encodes for a wide diversity of such enzymes (Flint 2008), enabling degradation of more complex fibers, including plant cell-wall constituents. Interestingly, one bacterium is not equipped with all the enzymes required for the entire polysaccharide degradation, but relies on a symbiotic degradation process where enzymes from different bacteria cooperate to entirely degrade polysaccharide into sugars that are further fermented.

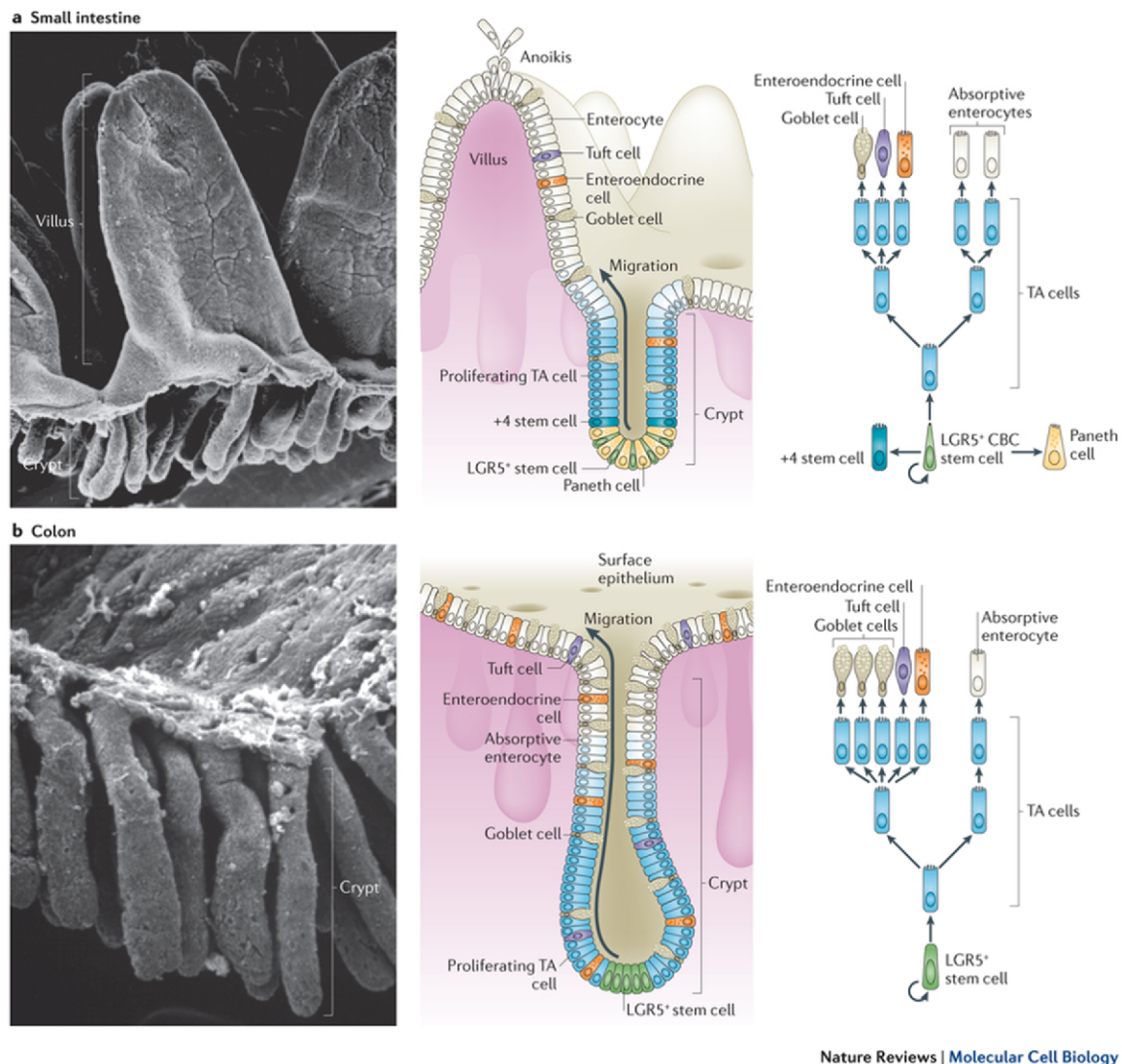
Microbiota is dependent on carbon and energy source derived from fibers. Changes in quantity and origin of fiber intake have important effects on microbial population by promoting the proliferation of bacteria able to use this new substrate. Indeed, in humans and in mice, a modification of diet rapidly modified the microbiota composition (Leitch 2007). Diet intervention with enriched fiber intake has

been associated with increased genomic diversity of the microbiota, especially for obese individuals having a low microbial diversity (Cotillard 2013). On the contrary, lipid rich diets have been associated with microbiota alteration, modifying also gene abundance according to their functions (Hildebrandt 2009). Interestingly, microbiota changes observed after diet modifications were reversed when returning to original diet, indicating that the gut microbiota is resilient. Moreover, microbiota modifications differ amongst individual and is depend on complex regulatory processes including bacteria-bacteria (and other microorganisms) and microbiota-host cross talks (Walker 2011), highlighting the importance of the host in regulating his own microbiota. Being the organ in contact with the microbiota, the gastrointestinal tract has a great importance regarding microbiota shaping, and the intestinal epithelium has a great importance in sensing and modulating it by different ways.

## Intestinal epithelium

The intestinal epithelium is composed of a single layer of cells. It has a specific three dimensional architecture composed of crypts and villi in the small intestine and only crypts in the colon enabling the increase of the absorptive surface on contact with lumen. Intestinal epithelium is composed of five cell types originating from stem cells localized at the bottom of the crypts, including absorptive enterocytes, goblet cells, enteroendocrine cells, tuft cells and Paneth cells, the latter being absent in the colon. The different types of cells are present in different proportions depending on the localization, the most abundant cells being the absorptive enterocytes, representing about 80% of total cells whereas enteroendocrine cells represent less than 1% of total epithelial cells in the intestine (Barker 2014). Comparisons of small intestine and colon structures as well as different cell types are represented in Figure 3.

This epithelium is a barrier delimiting the outside (the lumen) to the inside of the host organism. This active barrier is able to transport specific elements and to secrete a wide range of diverse molecules including mucins and bacteriocins. This barrier is of great importance: it allows entry of ingested and digested nutrients, water, electrolytes while it protects from other elements such as non-digested fibers, toxins, microbes. Transport through the epithelium goes through two main pathways: transcellular transport and paracellular transport. Moreover, some immune cells, namely the dendritic cells can extend cellular extensions through the intestinal epithelium, allowing bacterial sampling (Coombes 2008), but we will not discuss further their role.



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Figure 3: Small Intestine and Colon 3D architecture, cell type proliferation and differentiation, adapted from Barker 2014

Small intestine structure composed of crypts and villi highly increases absorptive surface. Colon epithelium only form crypt structures. Small intestine and colon epithelium are composed of similar cell type, all emerging from stem cells localized at the bottom of the cells, except Paneth cells only present in the small intestine epithelium that reside in the crypts close to stem cells.



### *Enterocytes, Transport and cell junctions*

Intracellular transport is mainly mediated by enterocytes. It is dependent of two different processes: transport from the lumen (internalization) and export to circulation. Apical membrane has an extended surface due to highly structured microvilli forming a brush border. Many transporters for ions, sugars, fatty acids, peptides and vitamins are present on this surface to allow intake of these elements. Their regulations are often dependent to a sodium gradient between lumen and cytosol and have been subject to extensive literature describing many nutrient-specific transporters (Burzle 2013, Gulec 2014, Kellet 2008, Reboul 2013). Moreover, some of these elements need to be metabolized before secretion to the circulation (Stumple, 2001). As an example, lipids are internalized directly as fatty acids or in structures with biliary acids, namely micelles, and are then metabolized to triacylglycerol and then form chylomicrons with cholesterol and phospholipids. Chylomicrons are secreted by enterocytes to intestinal lymph and blood circulation. (Wang 2013, Giammanco 2015). Colonocytes mainly absorb bacterial products including short chain fatty acids and vitamins, electrolytes and water as other nutrients are already absorbed in the small intestine. However, depending on conditions, colonocytes can also secrete in the lumen some electrolytes (Binder 2005, Geibel 2005, Pouokam 2011) and net absorption of electrolytes depends on the balance between secretion and uptake. Aquaporins enable transport of water through the membrane in different epithelial cells (Ma, 1999), even if water is mainly diffusively transported paracellularly. Transcellular transport can also be mediated through cellular endocytosis and further exocytosis to the lumen, called transcytosis (Bu 2010, Moon 2014). Transcytosis is dependent on stimulation of receptors at the membrane and can be used from apical to basolateral membrane transport or inversely to secrete elements into the lumen and allows transport of diverse large elements, including whole bacteria, drugs and immunoglobulins (Figure 4).

Ionic and small molecules are also able to cross the junctions of the epithelium through what is called paracellular transport (Liang 2014, Fasulo 2013), but represent the minority of the absorbed nutrients. This process is highly regulated in healthy tissues (Rodgers 2011). Different paracellular junctions have been reported in intestinal epithelial cells: tight junctions, adherens junctions, desmosomes and gap junctions; tight junctions and adherens junctions being the two most important ones for intestinal epithelium integrity. They form the limit between the apical and basolateral membranes of epithelial cells. These junctions are composed of different transmembrane molecules that have different characteristics, Occludin and Claudin for tight junctions and E-cadherins and catenins for adherens junctions. Junctions are anchored to filamentous-actin (F-actin) by zonula occludens (ZO). Cytoskeleton tension and contraction enable precise regulation of maintenance of the epithelial structure (Lee 2015, Rodgers 2011). Importantly, barrier must be maintained as increased permeability is associated with inflammation, due to leakage of endotoxins and microbial molecules and even whole bacteria that can be highly detrimental for the host in the case of opportunistic pathogens (Garrett 2010).

### *Goblet cells and Mucus*

These cell junctions are of high importance in the barrier function of the epithelium, and maintenance of these junctions is highly regulated. However, another important mechanism devoted to their barrier function is the secretion and the formation of a thick layer of mucus. Mucus is mainly composed of glycosylated proteins, namely mucins, and more particularly the O-glycosylated Muc2 in the colon. It forms a continuously renewed gel covering the whole epithelium that is thicker and denser in the colon.

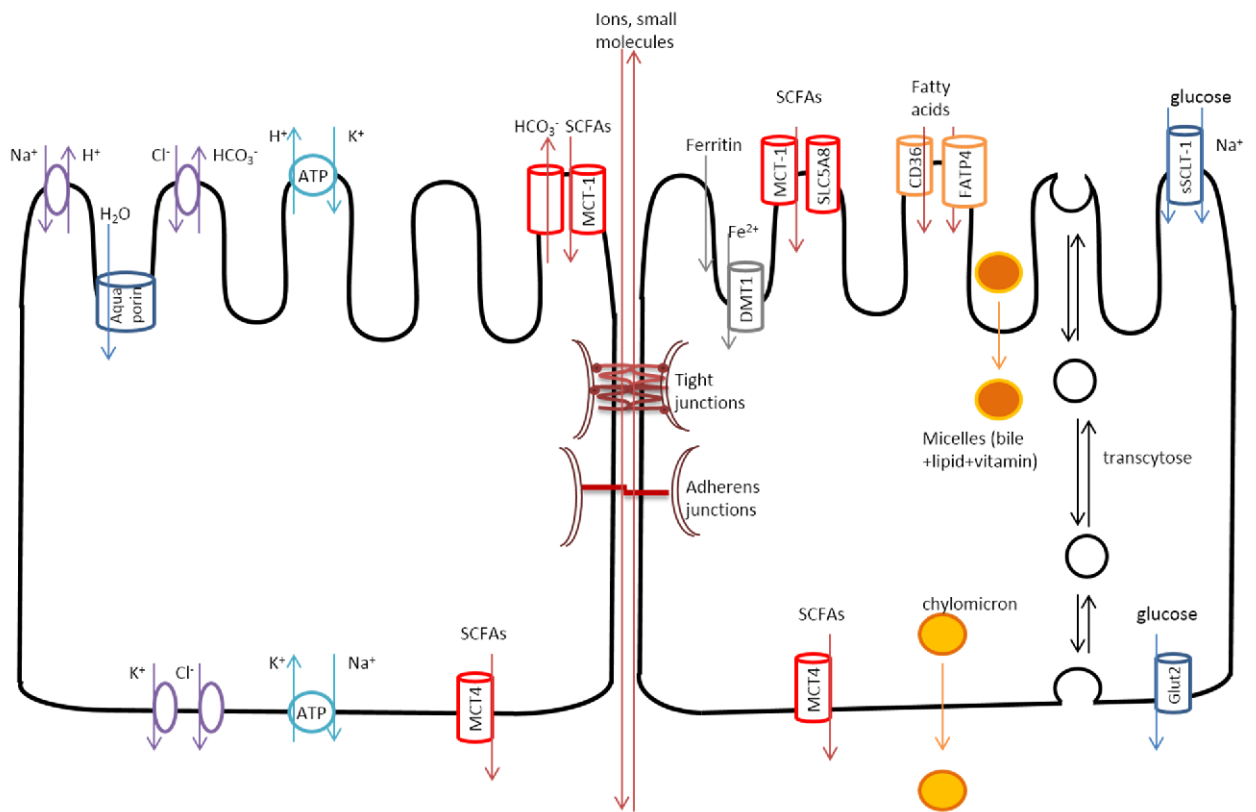


Figure 4 : Representation of main nutrient transports through intestinal epithelial.

Right cell represent main pathways present in small intestine involved in the transport of fatty acids, glucose and ions and left cell represent main transports in colonocytes. Transcellular transport is regulated by tight junctions to allow diffusive transport or different size molecules

In the colon, mucus can be divided in two layers, the inner one, which is denser, continuously and rapidly renewed and is limiting bacterial contact with epithelial cells and the outer one, which is less dense and enhance the development of some bacteria able to use mucins as an energetic substrate (Johansson 2011, Figure 5). The mucus is continuously renewed, leading to an efficient removal of bacteria and toxins. The formation and maintenance of mucus is mainly achieved by the secretion by goblet cells of its main component, Muc2, but implies other components, including CLCA1, FCGBP, ZG16 and AGR2. In addition to its important role in constitution of mucus barrier, goblet cells have recently been shown to transport luminal antigens to dendritic cells of the lamina propria permitting an efficient antigen presentation to T lymphocytes (Pelaseyed, 2014).

Many studies highlight the importance of the mucus layer to avoid direct contact between bacteria and epithelial cells and consequently avoiding intestinal inflammation. Indeed, in mice models, DSS, a molecule often used to induce colitis has a strong effect on mucus homogeneity (Johansson, 2010), and mice with damaged mucus (*muc2* <sup>-/-</sup>) have much higher inflammation (Van der Sluis, 2006). In human, ulcerative colitis is associated with more penetrable inner mucus (Gersemann), and therefore increasing bacterial direct contact with epithelial cells and local inflammation.

In addition, mucus layer is also a reservoir of high concentration of immunoglobulin A (IgA) and host antimicrobial peptides, that also participates in the maintenance of the nearly sterile environment observed in the inner layer (Mantis 2011, Santaolalla 2012). Part of the antimicrobial peptides is secreted by a specific intestinal cell type, Paneth cells.

#### *Paneth cells*

Only present at the bottom of small intestine crypts, Paneth cells secrete antimicrobial peptides enabling modulation of bacterial population. Whereas murine Paneth cells secrete a variety of  $\alpha$ -defensins, human Paneth cells mainly secrete only two  $\alpha$ -defensins as well as other antimicrobial components such as lysozyme and secretory phosphatase A2. These antimicrobial peptides are important components of innate immunity as they participate in defense against luminal pathogens and shape the microbiota (Clevers 2013). Secretion of antimicrobial peptides is observed in non colonized intestine from germ-free models or prenatally, but Paneth cells are sensible to microbes, notably expressing some Toll-like-receptors, and respond to microbes by increasing antimicrobial peptides secretion (Ayabe 2000, Rumio 2009, Tanabe 2005). Another function of Paneth cells is its role to maintain stem cell niche at the bottom of small intestine crypts.

#### *Tuft cells*

Tuft cells are a particular cell type recently described, representing less than 0.4% of the intestinal epithelium. They have an unusual shape, with a highly acetylated tubulin structure forming a brush-like phenotype. Tuft cells express a number of chemoreceptors, including taste receptors and secrete opioids (Gerbe 2012). However, specific marker of Tuft cells DCLK1 was described recently, and little is known about their clear functions in the intestinal epithelium (Gerbe 2011).

#### *Enteroendocrine cells*

Enteroendocrine cells represent a family of cell subtypes characterized by their localization, shape and hormonal secretion profile. Even if recent models have shown that hormonal expressions are not entirely exclusives in single cell, even in matured cells (Egerod 2012, Habib 2013), a classification of



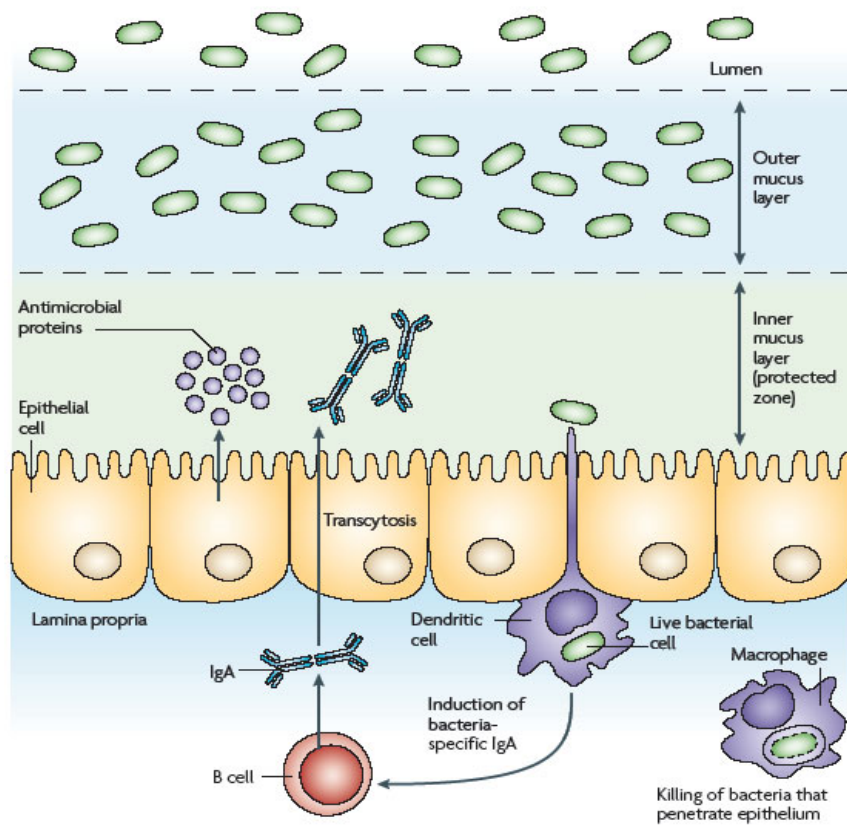


Figure 5 : Mucus layers, a barrier to protect epithelium from bacteria (adapted from Hooper 2009)

Mucus in the colon is composed of an inner mucus layer limiting exposition of epithelial cells to bacteria and a less dense mucosal layer which represent a niche for bacteria.

cells can still be achieved (Rehfeld 1998, Rindi 2004, Gunawardene 2011, Table 1). Table 1 : Classification of main types of enteroendocrine cells, main secreted hormones, localization, and main known receptors expressed at their surface (Furness 2013)). A description of the role of the main secreted hormones will be discussed later. The functions of the secreted hormones depend on their localization along the gastrointestinal tract that might correlate with the variety of molecules they sense vary locally, and some hormones have local functions. Only D cells and enterochromaffin cells (EC), expressing respectively somatostatin, and serotonin (5-HT) and substance P, are present all along the gastrointestinal tract. Stomach enteroendocrine cells include P/D1 (called X/A cells in rodents), ECL cells, G cells, secreting respectively Ghrelin, Histamine and Gastrin (Stengel, 2012). Found only in the proximal small intestine, I, K, M and S cells secrete cholecystokinin (CCK), glucose dependent insulinotropic Peptide (GIP), Motilin and Secretin. In the colon, L-cells secrete PYY, GLP-1, GLP-2, oxyntomodulin and Insulin-like Peptide 5 (Insl-5) (Grosse 2014, Habib 2013) and N cells, present as well in the small intestine secrete Neurotensin (Buffa 1978). Another specific population has enteroendocrine characteristics but do not migrate and stay close to the stem cell niche at the bottom of crypts. This cell population expresses a wide variety of hormones as well as the receptor LGR5, marker of stem cells in the intestinal epithelium. These cells are supposed to proliferate as they express low levels of Ki.67 but their clear functions are still unknown (Sei 2011).

Enteroendocrine cell sub-populations are characterized by the expression of wide range of receptors enabling the detection of luminal content or the response to paracrine stimulation (Raybould 2010, Reimann 2012). Main role of enteroendocrine cells is to secrete their specific hormones in response to receptor activation. Hormones are sorted to secretory granules by carboxypeptidase E (McGirr, 2013, Hosaka 2005) along with granins including secretogranin III and Chromogranin A, the former being extensively used as a specific marker of enteroendocrine cells in intestinal epithelium. Fusion of secretory granules is depending on classical pathways implicating SNAREs (Li, 2014, Takahashi 2010) and calcium signaling, similarly to other endocrine cell types like pancreatic  $\beta$ -cells or neurons (Burgoyne, 2003), a mechanism acquired during cell differentiation.

## Intestinal stem cells

Even if the colon and the small intestine epithelium display some differences, self-renewal of epithelial cells is a major property of both epithelia. Except Paneth cells residing in the base of the small intestinal crypts, all three other lineages amplify and differentiate while migrating from the crypt to the top of villi, indicating an important cell population in the bottom of the crypt that has high turnover (Barker 2007). In intestinal epithelium, position of the cell is referred by the number of cells separating it from the cell at the bottom of the crypt. These stem cells, specifically stained by LGR5 and localized in position +1 to +6, are able to indefinitely self-renew as well as to engage differentiation into all epithelial cell lineages. LGR5 is a receptor for R-spondins, known activators of the Wnt pathway that induces intestinal epithelium proliferation (Carmon 2012). Interestingly, LGR5 positive cells sorted and cultivated *in vitro* with specific factors are able to form mini-guts that include all intestinal epithelial cell types (Sato 2009) and reproduce 3D architecture with crypt- and villi-like structures. Moreover, Paneth cells seem to have important functions to maintain stem cells cycling, as removal of Paneth cells results in loss of LGR5 positive cells. Moreover, formation of mini-gut was highly improved when LGR-5 positive cells were grown with Paneth cells (Sato 2011).

Cell	Products	Luminal receptors	Locations	Principal effects
A (X-like) cells and subtypes	Ghrelin, nesfatin-1	T1R1–T1R3; T2Rs	Stomach	Appetite control, growth hormone release
ECL cell*	Histamine	Closed cell	Stomach	Stimulation of gastric acid secretion
G cells	Gastrin	LPAR5; GPRC6A	Stomach	Stimulation of gastric acid secretion
D cells	Somatostatin	LPAR5; GPRC6A	Stomach, small intestine (and pancreas)	Inhibition of gastrin release (stomach); modulation of insulin release (pancreas)
Enterochromaffin cells	5-HT. 5-HT is also contained in subgroups of I, K and L cells	FFARs 2, 3; TRPA1; toxin receptors; TLRs	Stomach, small and large intestine	Facilitation of intestinal motility reflexes and secretion; triggering of emesis and nausea in response to toxins
I cells	CCK (5-HT)	T2Rs; FFA1; GPR120; LPAR5; CaSR; TRPA1; TLRs	Proximal small intestine	Activation of gallbladder contraction and stimulation of pancreatic enzyme secretion
K cells, and subtypes	GIP	GPR119, GPR120; FFAR1	Proximal small intestine	Stimulation of insulin release
L cells, and subtypes	GLP-1, GLP-2, PYY, oxyntomodulin (5-HT)	T2Rs; T1R2–T1R3; FFARs 1–3; GPR119, LPAR5, GPR120; CaSR	Distal small intestine, colon	Stimulation of carbohydrate uptake, slowing of intestinal transit, appetite regulation, insulin release
M cells	Motilin <sup>‡</sup>	Bile receptors	Small intestine	Initiation of migrating myoelectric complex in pig, dog and human
N cells	Neurotensin <sup>§</sup>	FFARs	Small and large intestine	Inhibition of intestinal contractions
P cells	Leptin	Nutrient receptors	Stomach	Appetite regulation, reduction of food intake; leptin might also be in chief cells
S cells	Secretin	Acid receptor	Proximal small intestine	Reduction of acidity in upper small intestine by stimulation of bicarbonate release
<p>Several of the enteroendocrine cell types, notably A, K and L cells, have subgroups or gradients along the intestine that contain different combinations of products; subgroups of I and L cells contain 5-HT.<sup>158,159</sup> Moreover, overlaps between N and S cells and I, K and L cells are observed. *ECL cells do not contact the lumen. <sup>‡</sup>Bile is an effective stimulus for motilin release, but the receptor type is not identified. <sup>§</sup>Short and medium chain fatty acids cause neurotensin release, but the receptor type of N cells is not identified. Abbreviations: 5-HT, serotonin; ECL, enterochromaffin-like; FFAR, free fatty acid receptor; TLR, Toll-like receptor.</p>				

*Table 1 : Classification of main types of enteroendocrine cells, main secreted hormones, localization, and main known receptors expressed at their surface (Furness 2013)*

Importantly, Paneth cells express and secrete factors maintaining stem cell characteristics, Wnt3, EGF and DLL4 which is a Notch ligand. However, the colonic epithelium lacks Paneth cells, and mechanisms to support cycling and differentiations are less well understood. Paneth like cells have been hypothesized to participate in the maintenance of stem cells in the crypt. Colonic organoid culture has been developed, but necessitate more factors to induce stem cell cycling and survival, including Wnt3 A, an activator of Wnt signaling secreted by Paneth cells in the small intestine (Sato, 2011). Interestingly, small intestine stem cells grown in colonic tissue induced small-intestinal like epithelium, including Paneth cells differentiation, indicating differences between small intestine and colonic stem cells (Fukuda, 2014). On the contrary to many stem cells, LGR5 positive cell division is symmetric, and both daughter cells have stem cells characteristics. Differentiation only occurs in migrating cells that escape the crypt bottom.

### *Cell differentiation*

Cells escaping the crypt bottom, called transient-amplifying cells enter a proliferative differentiation migration stage leading to fully differentiated cells on the top of the crypts and on villi for small intestine cells. Transient-amplifying stem cells express C-myc, an important factor for cell division (Muncan, 2006). Generally they divide two to three times before entering differentiation into either secretory lineage or absorptive lineage during migration. Modification from a cycling undifferentiated to a differentiation process is mainly due to a gradient of Wnt from the bottom to the top of the crypts and a reverse gradient of the Bone Morphogenetic Protein (BMP) (Kosinski 2007). This protein is of TGF- $\beta$  family, and inhibits cell proliferation by inhibiting Wnt signaling and is implicated in cell differentiation (Reynold 2014). Thus, BMP and Wnt maintain two distinct compartments along the crypt: a proliferative one associated with strong Wnt signaling and a differentiating one associated with BMP (Figure 6) Figure 6: Stem cell niche, proliferation and differentiation areas in colonic crypt (from Wakefield 2013).

Notch is the other important factor maintaining cycling stem cells. It also inhibits formation of secreting cells whereas it allows differentiation into absorptive cells. Inhibition of Notch decreases the number of absorptive cells whereas its overexpression increases the number of absorptive cells (Fre 2005, Stanger 2005). Importantly, Notch signaling activates Hairy/Enhancer of Split (Hes)-1 which represses expression of ATOH1 (Math1 in mice), a transcription factor required for secreting cell differentiation (Jensen 2000, Yang 2001, Kazanjian 2011). Absorptive cells, called enterocytes represent up to 80% of total epithelial cells and are issued from cells expressing a high level of Notch. They develop specific apical microvilli, increasing the surface by which they can absorb luminal content. Further differentiation of enterocytes is modulated by development of adherens junctions and ERK signaling to induce formation of microvilli (Chartier 2011, Boucher 2011).

On the contrary, transient-amplifying cells expressing low levels of Notch differentiate into secreting cells and prevents further cell cycling, mainly due to the expression of Atoh1. Secreting cells include goblet cells, enteroendocrine cells, tuft cells and Paneth cells in the small intestine. Paneth cells have a specific fate as unlike other intestinal cells, they do not migrate to the top of the crypt but are maintained in the crypt by ephrin B1, (Batlle 2002). Inhibition of Wnt on top of the crypt induces Ephrin B1 expression. It binds to its receptor, EphB3, which is expressed by Paneth cells and inhibit their migration. Further differentiation factors are implicated to differentiate into distinct cellular lineages. In mice, depletion of the transcription repressor *gfi1* induces an increase of enteroendocrine cells and a decrease of the number of Paneth and goblet cells (Shroyer 2005). *Gfi1* inhibits the expression of the transcription factor *neurogenin3*, required for the differentiation of enteroendocrine cells (Jenny 2002, Bjerknes 2010). Further differentiation into Paneth cells requires

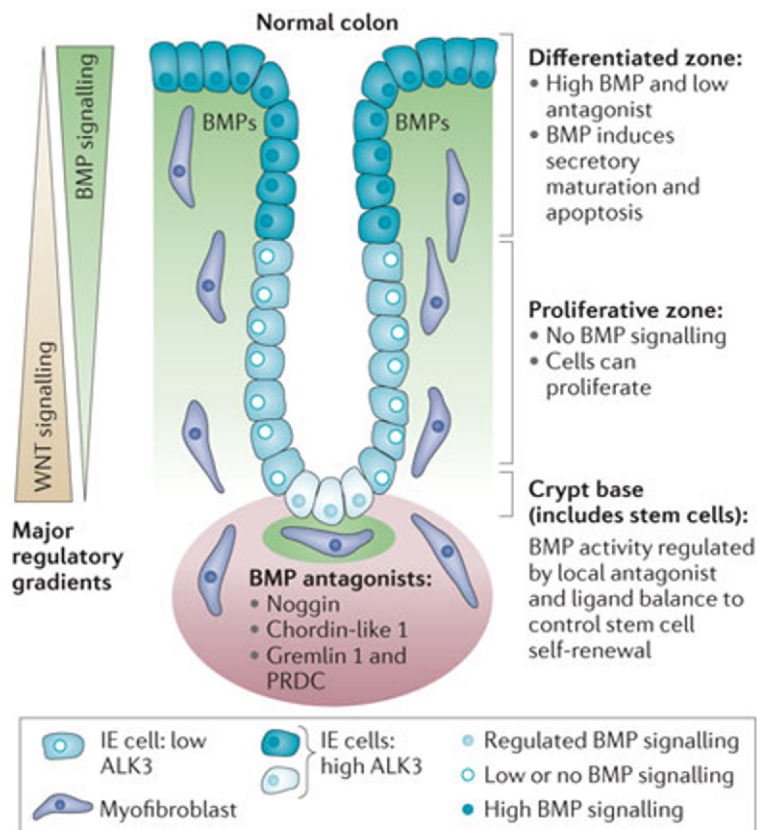


Figure 6: Stem cell niche, proliferation and differentiation areas in colonic crypt (from Wakefield 2013)

Stem cells divide at the bottom of the crypts. Migration to the top of the crypt induces their differentiation. Proliferation and differentiation are sequential and organized through opposite gradients of Wnt (for proliferation) and BMP (for differentiation). During migration, cells are exposed to different concentrations of WNT and BMP and go through allowing first to proliferate and then differentiate

sox9 expression, a transcription factor expressed in cells at the bottom of crypts, as mice depleted for Sox9 have no Paneth cells (Mori-Akiyama, 2007). Using also k.o. mice models, Klf4, a zinc finger transcription factor, was shown to be necessary for goblet cells differentiation (Katz, 2002). Tuft cell differentiation is controversial. Requirement of Atoh1 is unclear as depletion of this gene, depending on studies increases or depletes presence of Tuft cells expression (Gerbe 2012) (Figure 7).

#### *Enteroendocrine cells differentiation*

Further differentiation of enteroendocrine cells requires the expression of different transcription factors to induce precise hormonal production. Most studies were done using mice models depleted for expression of specific genes and comparing numbers of different cell types stained by specific markers. Neurogenin3, downstream of Math1, is required for enteroendocrine cell differentiation in the intestine. Mice lacking neurogenin3 had no enteroendocrine cells whereas mice over-expressing neurogenin3 had increased number of enteroendocrine cells and decreased number of goblet cells, except in stomach where EC, ECL and X/A cells were still present, due to different mechanisms of structure and differentiation between intestines and stomach (Jenny 2002, Lopez-Diaz 2007). Expression of neurogenin3 is needed for early differentiation and is then extinguished, as only cells present in +5 +8 localization expressed neurogenin3 (Bjerkness 2006). The transcription factor insm1 has been implicated in development of specific enteroendocrine features including the secretory machinery, as its depletion induces a lack of ChromograninA. However, its depletion only partially affected hormone expression with an abolition of expression of neurotensin and substance P but not of 5HT, CCK or PYY (Gierl 2006). Neurogenin3 induces expression of NeuroD by directly binding to its promoter and increasing its expression (Huang 2000). NeuroD is expressed in all enteroendocrine cells but is required only for secretin expression in S cells and CCK in I cells, as depletion had no effect on number of L, N, K and D cells. Depletion of another transcription factor, Arx, inhibits G, L, I, K, S and N cell formation while increases D and P/D1 cell number. Moreover, it does not affect the number of ChromograninA positive cells, meaning that it is required only for late enteroendocrine differentiation (Du 2012). Moreover, Arx is only transiently expressed in Neurogenin3 positive cells and is no longer present in mature enteroendocrine cells (Beucher 2012).

Foxa1 and Foxa2, which are also implicated in terminal differentiation of goblet cells, are activators of preproglucagon gene, encoding GLP-1 and GLP-2 in L-cells. Depletion of Foxa1/2 in mice results in loss of L-cells and decreases the D cell number, but has no effect on I, S, G, EC and K cell number, indicating that Foxa1/2 are specifically required for L-cells and D-cells differentiation (Ye 2009). Pdx1 has been implicated in the proximal differentiation in the duodenum and is necessary for development of G cells in stomach (Offield 1996). Nkx2.2 is required for EC, K, I, G, D cell maturation, GLP-1 expression, but neither PYY, secretin, substance P nor chromograninA expression, and depletion of Nkx2.2 induces a strong over-expression of ghrelin. Moreover, mice depleted for expression of Nkx2.2 have reduced expression of Pax6 and NeuroD, but no modification of expression of Pax4 or Pdx1 nor of early differentiation factors Neurogenin3 or Math1, indicating that it may be implicated in early stages of differentiation (Desai 2008). Mice depleted for Pax6 have no D-cells and a lower number of G-cells and K-cells whereas depletion of Pax4 decreases the number of EC, S, K, I and PYY positive cells and abolishes D-cells formation (Larsson, 1998) but increases number of G, D, P/D1 and GLP-1 positive cells. Figure 6 represents the main transcription factors that have been implicated in enteroendocrine differentiation and indicates their effect on development of specific cell types based on effect of their depletion in mice.



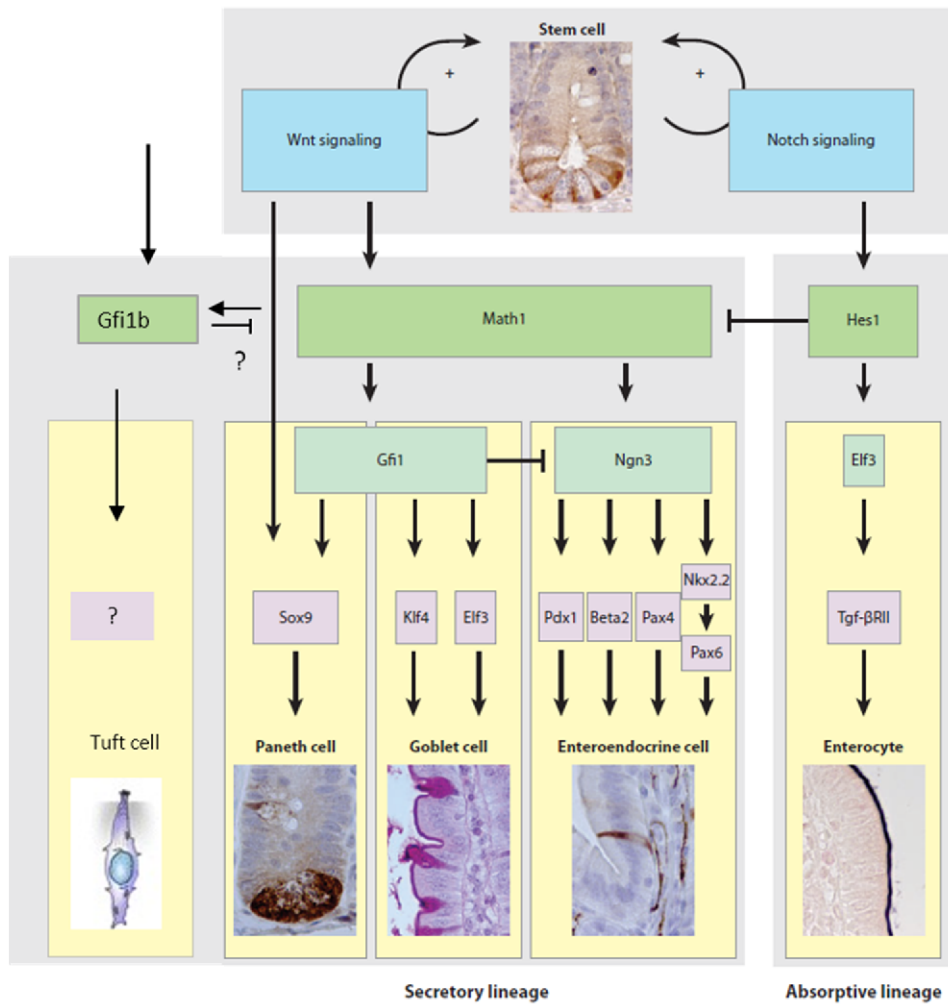


Figure 7 : Differentiation of intestinal epithelial cells (Adapted from Van der Flier 2009)

The formation of the five types of cells in the intestinal epithelium requires precise control of expression of specific differentiation factors deciding the fate of cells. A first important division is the expression of *Math1* leading to differentiation into secretory cells

Interestingly, even if L-cells are defined as a single enteroendocrine cell type co-expressing PYY and GLP-1, expression of these two hormones relies on different differentiation mechanisms, including differential dependency on expression of Pax4 or Pax6. Moreover, the expression pattern of these two hormones are different along the intestine, as PYY is more expressed in a proximo-distal gradient, with the highest expression in the rectum whereas GLP-1 is similarly expressed from the distal jejunum to the rectum (Roth 1990, Eissele 1992). Precise mechanisms inducing expression of these transcription factors are poorly known, and other parameters, including environmental factors, are implicated in enteroendocrine cell differentiation. In *in vitro* models of mini-gut expressing all types of intestinal epithelial cells, addition of Short Chain Fatty Acids (SCFAs), mainly present in the colon, increases the number of L-cells. Moreover, effect of SCFAs on transcription factor expression between mouse and human organoids is different, indicating that this environmental factor may signal for an increase of specific cell type formation (Petersen 2014). Moreover, many of the transcription factors found to be implicated in enteroendocrine cell differentiation have also been implicated in pancreatic endocrine cell differentiation, as these cells share many features.



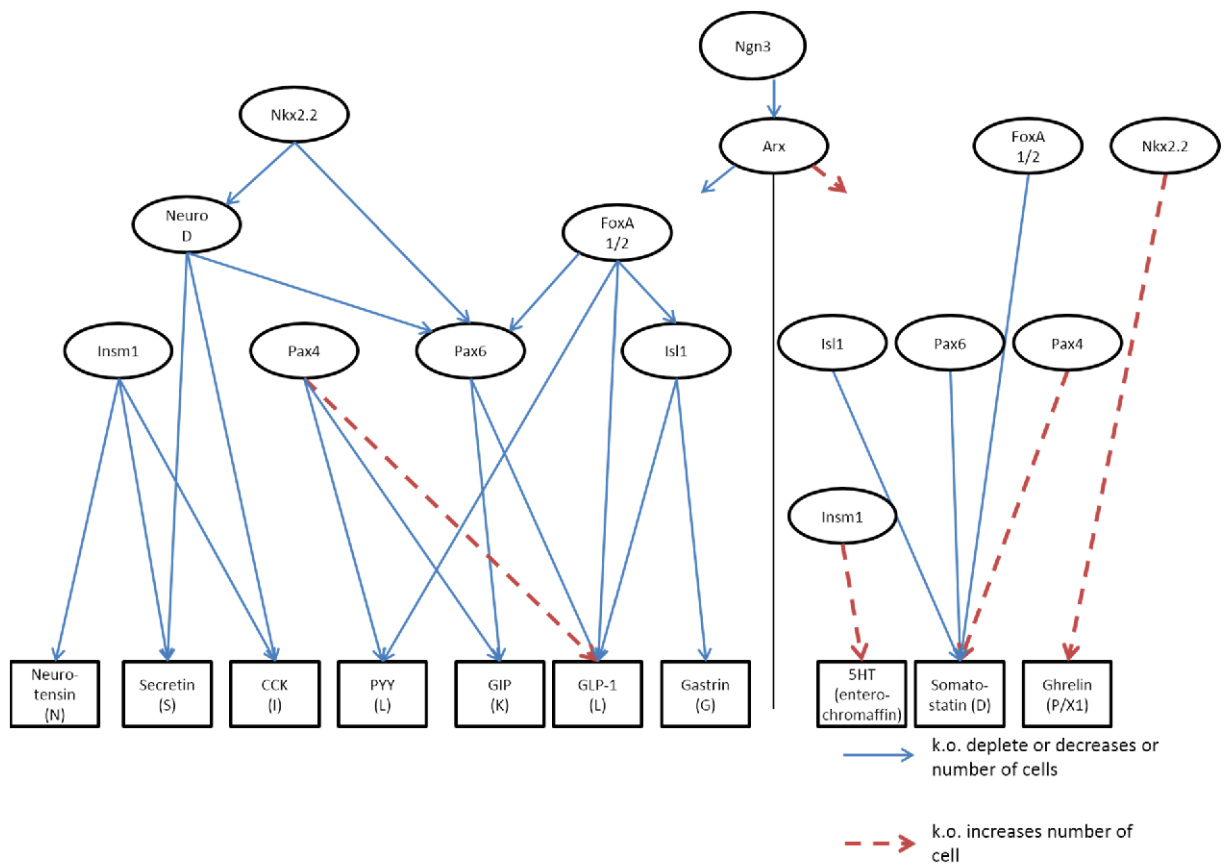


Figure 8 : Transcription factors implicated in enteroendocrine cells

The expression of specific hormones require the expression of a pattern of transcription factors. Depletion in mouse models of specific differentiation factors reduces or even abolish cell types, while increases the number of other cell types. Blue arrows represent therefore transcription factors required for enteroendocrine cell formation whereas red arrows indicate potential inhibitory effect of transcription factor expression on enteroendocrine cell formation.

## Enteroendocrine cells

### Role of enteroendocrine cells

Gut hormones, which production is precisely localized throughout the gut by expression of the different transcription factors act in a paracrine or endocrine way, through specific receptors. Main functions of these hormones are to regulate intestinal functions: enzyme production and secretion, gastric emptying and gut motility, electrolyte absorption, cell proliferation... Other main function is to regulate insulin secretion by  $\beta$ -cells in the pancreas and participate in food behavior control, regulating satiation and satiety. Gut peptides have also some functions on other tissues, implicating regulation of bone metabolism for PYY, GLP-1, GLP-2, 5HT, GIP and ghrelin, (Wong 2012, Gershon 2013), or adipose tissue inflammation (Gogebankan 2015, Challa 2012) (Figure 9)

#### *Gastric hormones*

Gastric hormones are mostly associated with regulation of secretion of gastric enzymes, gastric emptying and food intake. Ghrelin is a gut hormone reported to have an orexigenic effect both acting on hypothalamus and on reward circuits, the two main centers implicated in regulation of food intake. It requires post translational modification to be active (Wren 2001, Kamegai 2001, Yang 2008). It also has effects elsewhere, due to expression of its receptor, the Growth Hormone Secretagogue Receptor (GHSR) 1a, in other tissues. In particular, it has been shown to inhibit insulin secretion, increase gut motility, increase gastric acid secretion and decrease fat oxidation. Ghrelin levels increase during the fasting and decrease rapidly after food intake (Muller, 2015), and its secretion is regulated both by nutrients that inhibit its secretion and by neuronal and endocrine signaling that increases its secretion during the fasting.

Gastrin and histamine have similar roles, histamine being though to act downstream of gastrin, and increase acid secretion by parietal cells. Histamine is secreted by ECL cells in response to the gastrin, secreted from the antral G cells (Schubert 2010, Dimaline 2014) and acts in a paracrine way on parietal cells. Gastrin is secreted in response to a pH increase and the surface tension.

Somatostatin has inhibitory effects by coupling a family of receptors, the somatostatin receptor, expressed in parietal cells, in the brain and in the pituitary gland, where it inhibits growth hormone secretion and has an orexigenic effect (Luini 1986, Rai 2015). Moreover, somatostatin also inhibits PYY secretion from L-cells (Rigamonti 2011). Acid secretion is also inhibited by another small intestine hormone, GIP, which also has an incretin effect.

#### *Enzymatic secretion regulation*

Enzymes and bile acids are needed to ensure the digestion of proteins, carbohydrates and lipids. They must be secreted from the stomach, the pancreas, the gallbladder and the duodenum in a regulated manner depending on food presence. Nervous signaling associated to food intake is a first signal inducing release of these enzymes, but gut hormones are also important in the control of their secretion (Jaworek, 2010). CCK and secretin have been implicating in pancreatic secretion and gallbladder contraction regulation (Sabbatini, 2010, Murphy 2008, Berna 2007, Afroze 2013, Castillo

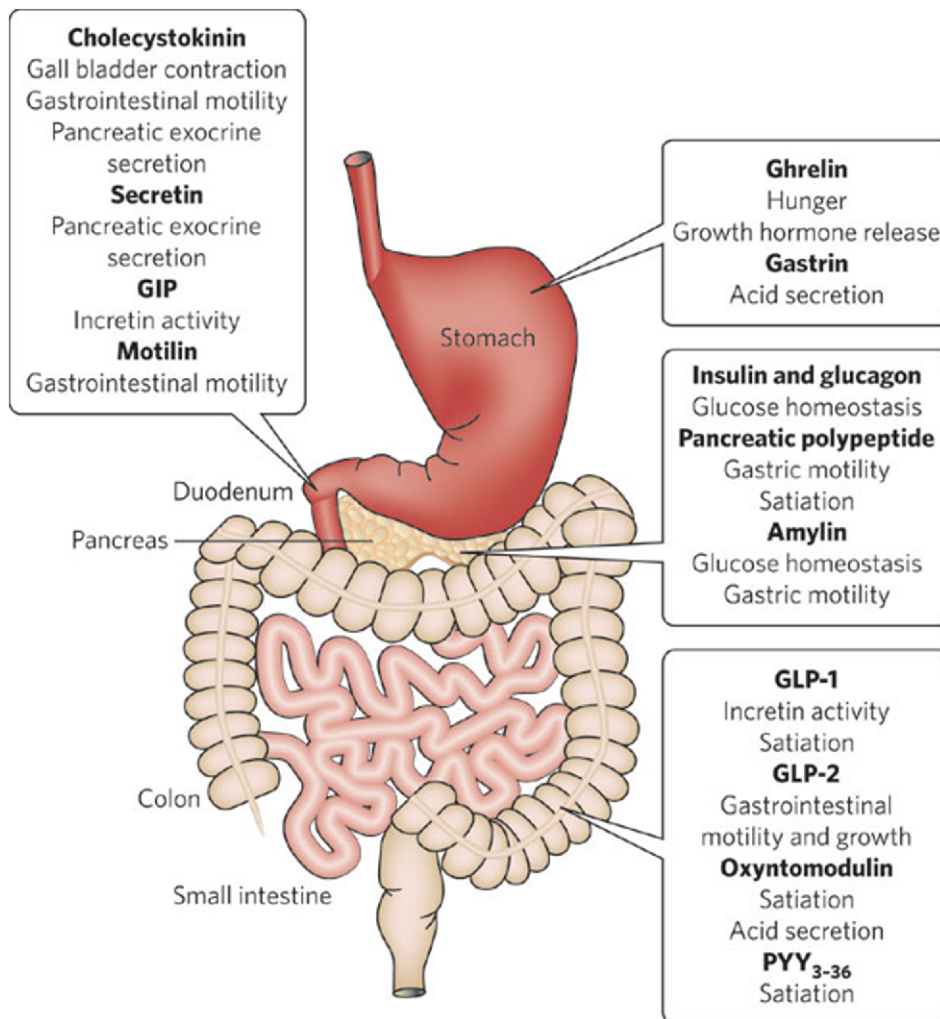


Figure 9 : Localization and role of main gut hormones (From Murphy 2006)

Gut epithelium secretes a wide range of hormones that are implicated in the regulation of many physiological functions, regulating digestion, energy metabolism and food intake

2004). Expression of CCK receptors on pancreatic acinar cells enables a direct response to CCK and induced secretion. Gastrin, acting on the same receptor as CCK (CCK-R2) may also participate in these functions. CCK is mainly secreted in response to nutrient sensing by I-cells through expression of many GPCRs. Moreover, CCK receptors are expressed in many different tissues, implicating CCK in many other functions discussed later. One important function of CCK is also regulation of secretion of other hormones, including distal gut peptides like PYY and GLP-1 as discussed later.

Secretin receptors are also expressed in acinar and ductal epithelial cells, stomach smooth muscles and gastric glands as well as in hypothalamus (Siu, 2006). Secretin induces secretion of bicarbonate and water in response to a decrease of pH, regulating therefore acidity in the gastrointestinal tract. In the NTS, secretin participates in maintaining synaptic activity of some neurons (Yamagata, 2008).

Motilin, which shares similarity with ghrelin and is recognized by receptors of the same family as ghrelin has also been reported to induce secretion of pepsin in the stomach (Fiorucci 1993). On the contrary, distal hormones like PYY inhibits gastric and pancreatic enzyme secretion (Lundberg 1982, Yang 2002) similarly to the related pancreatic peptide PP.

### *Gut motility regulation*

Food must be processed all along the gastrointestinal tract and toxins and non digestible elements must be eliminated. After digestion and processing in the proximal part, nutrients must be absorbed in the small intestine, necessitating decreased transit time. A first important step is the gastric emptying, enabling further processing of nutrients in the duodenum. Ghrelin, serotonin and motilin increase gastric emptying, whereas postprandial hormones CCK, GLP-1 and PYY decrease gastric emptying (Chelikani, 2006. Broad, 2014, Chelikani 2004)

In the fasted state, gut motility is cyclic and is called the Migrating Motor Complex (MMC), consisting of a quiescent phase, a phase of low and irregular contractions and a short phase of higher contractions that propagates from proximal to distal intestine. The latter is also associated with gallbladder emptying. This motility enables sweeping of intestinal content to more distal part (Takahashi, 2012).

Serotonin secreted from duodenum, through sensing by 5-HT receptors in the enteric nervous system, is implicated in the regulation of the second phase of the MMC, peristaltic reflex and increased motility (Li, 2011). However, precise origin of serotonin is controversial as serotonin is secreted by afferent serotonergic neurons and EC cells. Motilin secretion is responsible for the short last phase of high motility, and infusion of motilin provokes a response similar to the third phase of the MMC. Motilin is mainly secreted in response to gallbladder emptying, and altered gallbladder emptying decrease motilin associated high motility phase (Stolk, 2001, Sanger 2012). Of note, studies on the role of motilin are based on non-rodents models, mainly dogs and clinical experiments as mouse motilin is a pseudogene. After food ingestion, motilin secretion is alternatively regulated, being inhibited by Pancreatic Polypeptide (PP) and activated mostly by gallbladder emptying phases and mechanical stress. However, it participates to a lesser account into regulation of gut motility after food intake. Some of gut hormones that are secreted after food intake, mainly GLP-1, PYY and CCK, have been implicated in the regulation of gastrointestinal motility, with different effects depending on their localization. CCK increases small intestinal transit whereas GLP-1 and PYY induce the ileal brake, a slowing of ileal transit time enabling increased time for nutrient absorption (Ko 2011, Castillo 2004, Schwizer 1997, Cuche 2000, Cherbut 1998). However, these hormones have controversial effects on colon motility, due to different regulating mechanisms implicated, depending on different receptors activation, as discussed later in the case of the effects of PYY.

### *Incretin hormones*

GIP and GLP-1 are the two main incretin hormones, increasing insulin secretion by  $\beta$ -cells in the pancreas, the cell population responsible for insulin secretion (Dupre 1973, Kreymann 1987).  $\beta$ -cells are mainly sensitive to glucose, but secretion is also modulated by incretins. Both GIP and GLP-1 are secreted after food ingestion, and more particularly in response to glucose and proteins. However, both hormones are rapidly inactivated by cleavage of their two N-ter amino-acids by Dipeptidyl Peptidase-4 (DPP-IV). They bind to different receptors that both activate secretion by increased cyclic AMP concentration. They share many functions, including insulin secretion,  $\beta$ -cells number increase by limiting apoptosis and increasing proliferation. They also have distinct roles: only GLP-1 has effects on food intake and gastric emptying (Seino 2010) whereas GIP inhibits gastric acid secretion. Moreover, they also have effects on other pancreatic cells. GLP-1 decreases glucagon expression in  $\alpha$ -cells, a hormone enabling glucose production in the liver stimulating glycogenesis in the liver (Ramnana 2011) whereas GIP increases its expression (Hare 2009, Meier 2003). Some other peptides participate in insulin secretion, having an effect on  $\beta$ -cells survival or modulating their response to glucose, including CCK and PYY (Irwin 2013, Boey 2007).

### *Food intake control*

Several hormones have been implicated in the regulation of food intake via different signaling pathways, either directly acting on arcuate nucleus in the hypothalamus or through afferent vagal nerves. Ghrelin is the main hormone increasing food intake, even if recently, Insulin-like-peptide 5 has been shown to exert similar effects (Gross 2014). CCK has been demonstrated to decrease food intake, and administration of analogs or resistant forms decrease food intake in animals and humans (Konturek 2004, Camilleri 2015). Moreover, CCK also regulates PYY activity on food intake by increasing the expression of its receptor on vagal afferent neurons (Degen 2007, Burdyga, 2008, Irwin 2013). Oxyntomodulin and GLP-1 have also been associated with decreased food intake through activation of vagal GLP-1 receptors (Abbott 2005, Cohen 2013). Moreover, indirect pathways are implicated in regulation of food intake including gut motility and gastric emptying as mechanoreceptors of stomach distension modulate food intake increasing satiation (Marciani 2015, Rolls 1998). Modulation described before of gastric emptying can therefore be part of regulation of food intake.

## Production and secretion of hormones

These hormones regulate many physiological important functions and their circulating concentration must be precisely controlled both temporally and spatially. Hormonal levels result of a balance between production and secretion of hormones and degradation or inactivation. Degradation of these hormones can be mediated by several enzymes, including DPP-IV (CD26) for GLP-1 and GIP, metalloendopeptidases for PYY (Abbott 1994, Mentlein 2009, Addison, 2011), or hormone inactivation by intracellular uptake by specific transporters and further degradation, like serotonin. Regulation these inhibitors or use of hormones that have longer half-life are used in therapeutic strategies when level of these hormones are low and higher levels may be beneficial.

### *Regulation of hormonal production*

On the other side of the regulation of hormonal concentrations, production and secretion of these hormones is dependent on activity of enteroendocrine cells. Regulation of secretion by enteroendocrine cells has been highly studied and many mechanisms implicated have been described. However, regulation of hormone production is less known, mainly due to the fact that enteroendocrine cells are sparse cells, limiting studies on gene expression in these cells. Hormonal gut peptide production is mainly dependent on expression and translation of the gene coding for the proprotein and proteases inducing cleavage of this proprotein into final peptide (Brakch 2002). As a same proprotein can be processed differently into different hormones, precise expression of proteases is required for production of the right hormone at the right place (Rehfeld 2008). As an example, proglucagon is differently processed in L-cells and pancreatic  $\alpha$ -cells, needing expression of different proteases (Rouillé 1995) (Figure 10). Moreover, some posttranslational modifications can be required for functionality as for ghrelin requiring an octanoyl function (Yang 2008). Non peptidic hormones like serotonin in EC cells and histamine in ECL cells are synthesized from aminoacids (respectively tryptophan and histidine). Their production is therefore dependent on enzymes regulating their synthesis, and increase production necessitates increased levels of limiting hormone. Main known regulations of hormone production is based on modulation of the expression of transcription factors and increased epithelium expression of hormone, but this can be associated with two distinct mechanisms to increase hormone production: hormone production per enteroendocrine cell and increased number of enteroendocrine cells. Moreover, increased protein production required to increase secretion often induces endoplasmic reticulum (ER) stress. High protein production in ER is often associated to an increase of unfolded protein production due to a lack of chaperones. Sensing of increased production of unfolded or misfolded protein is mediated by a conserved protein, the Unfolded Protein Response (UPR). UPR reduces protein production, increase recruitment of chaperones and increase misfolded protein degradation while possible for the cell. However, high levels of UPR induce RE stress which can lead to cell apoptosis. However, to our knowledge, ER stress has not been studied in intestinal enteroendocrine cells, whereas it has been associated with other secreting cells and has been implicated in some diseases like type II diabetes in pancreatic  $\beta$ -cells (Montane, 2014) or with IBD in goblet and Paneth cells (Luo, 2015). Regulation of hormone production can be important to permit increased response by higher secretion levels, but are often long-term regulation due to the kinetic of hormone production and sorting. Gut peptides are in majority rapidly secreted after meal ingestion. Thus, important control of secretion in these cells is required to increase thei levels at the right time to ensure their physiological functions associated with food intake: delayed gastric emptying, decreased gut motility, increased gastric acid secretion, satiation feeling, insulin secretion...

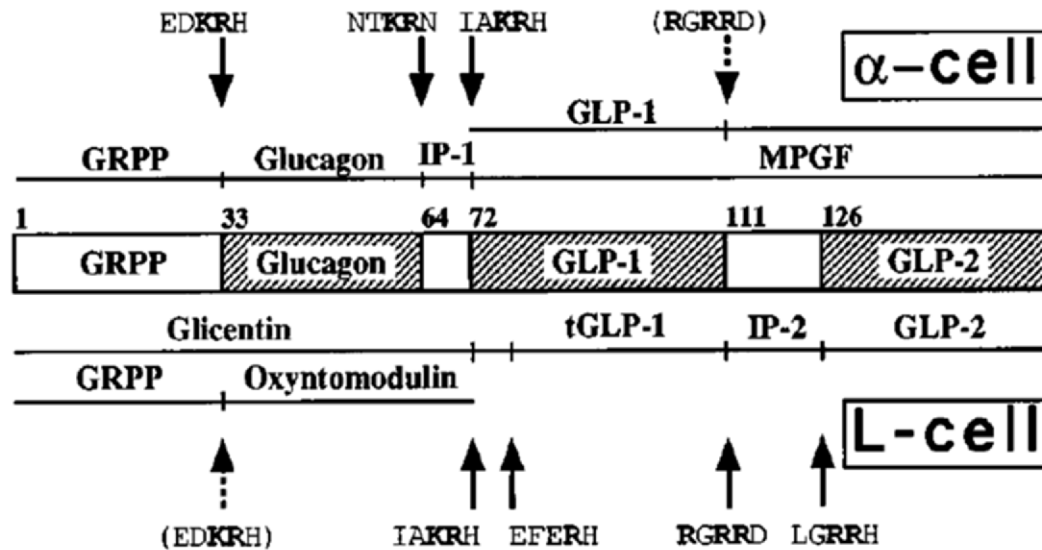


Figure 10 : Differential processing of proglucagon in colonic L-cells and pancreatic  $\alpha$ -cells (from Rouillé 1995)

Proglucagon can be cleaved into different peptides, and different processing occurs in different type of cells. The upper part represents the two peptides obtained in  $\alpha$ -cells, namely glucagon and MPGF, the major proglucagon fragment (which can be further be processed to GLP-1). The lower part represents cleavage to glicentin, GLP-1 and GLP-2, the first one being then processed to oxyntomodulin and GRPP (glicentin related polypeptide). Proteases cleave at different sites indicated by the arrows.

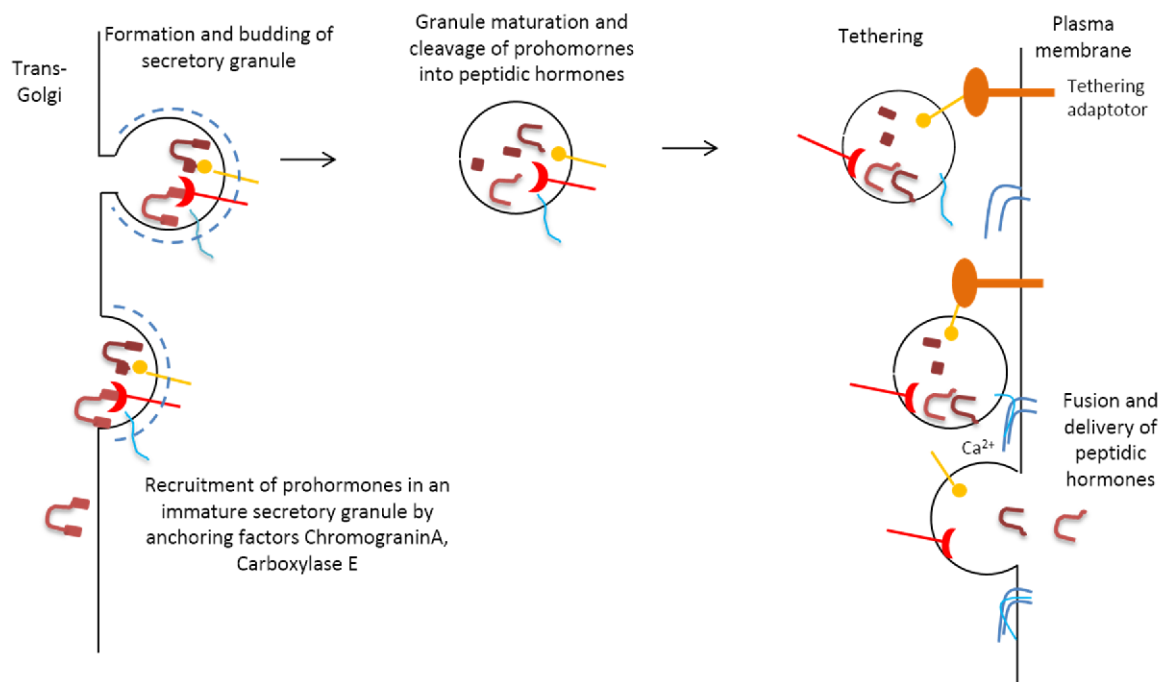


Figure 11 : Main steps of sorting, processing and secretion of gut hormones

Prohormone are produced and matured in the trans-Golgi network, sorted and matured into secretory granules before addressing to the plasma membrane. Local increase of cytosolic calcium concentration leads to fusion of the secretory granule membrane with plasma membrane and secretion of its content.



### *Regulation of secretion in enteroendocrine cells*

Like in other endocrine cells, secretion of gut hormones is highly regulated, depending on a specific cellular pathway, the regulated secretory pathway. Hormones are stored in secretory vesicles or secretory granules that are localized in proximity with plasma membrane where hormone must be secreted (Bohorquez 2010). Specific signaling depending on calcium induces granule fusion with plasma membrane and therefore secretion.

Prohormones are sorted into budding granules from the trans-Golgi Network by membrane bound receptors interacting with a sorting signal present in the prohormone. They therefore escape constitutive secretory pathway and lysosomal addressing pathway. Some of the proteins tethering prohormones have also enzymatic functions and are implicated in the processing of the prohormone (McGirr 2013, Bataille 2007), in particular the prohormone convertases and the carboxypeptidase E. ChromograninA, which is often used as a specific marker of enteroendocrine cells in intestinal epithelium participates in the formation and sorting of secretory granules (Elias 2012, Montero-Hadjadje 2009) (Figure 11)

Secretion can be induced by several signals, but all induce a local rise of cytosolic calcium concentration which is necessary for fusion of secretory granule with plasma membrane. Indeed, membrane fusion necessitates association between a v-SNARE localized on granule membrane and t-SNAREs present at the plasma membrane (Bonifacino, 2004, Takahashi 2010). Assembly of the SNARE protein complex enable docking of the granule to the plasma membrane and further membrane fusion require calcium. Insulin secretion mechanisms in  $\beta$ -cells have been well described (Wang, 2009). Because of similarities between pancreatic endocrine cells and enteroendocrine cells, some hypothesized that mechanisms regulating secretory granule exocytosis are shared. Indeed, in a model of L-cells, GLUTag cells, a same v-SNARE, VAMP2, was implicated in (Li 2014).

Secretion is regulated by external signals that induce a rise of calcium concentration to induce secretion (Dolensek 2011). Resting cytosolic calcium concentration is low, of about 100nM and can be increased mainly by two different main sources: the outside of the cell where calcium concentration is about 1mM, and endoplasmic reticulum which is an important store of calcium. Other sources like mitochondria and lysosomes are also implicated in regulation of calcium concentration. Calcium concentration is tightly regulated by ionic channels, allowing fast entry of calcium into the cytoplasm due to the polarization of cell, and pumps, expulsing calcium to ER or to outside the cell to decrease cytosolic calcium concentration (Toescu 2012).

Enteroendocrine cells are sensors of luminal content concerning open enteroendocrine, and they also express receptors on their basolateral membrane to sense paracrine and efferent neuron signaling. Main sensing is mediated by a super family of receptors, the G- protein coupled receptors (GPCRs) (Figure 12). Enteroendocrine cells express a variety of GPCRs recognizing most of nutrients including fatty acids (FFAR1, FFAR2, FFAR3, GPR120, GPR119), peptides and amino-acids (GPR93, CasR), bile acid (TGR5), endocannabinoid receptor, muscarinic receptors... (Diakogiannaki 2012, Sykaras 2012, Choi 2007, Reimann 2012, Maruyama 2002). They are called seven trans-membrane receptors as they share a similar structure composed of seven transmembrane domains, with their C-terminus being extracellular. These receptors bind to small G proteins, and activation of the receptor by binding to its specific ligand induces activation of a small G protein. Small G proteins are important signaling mediators.



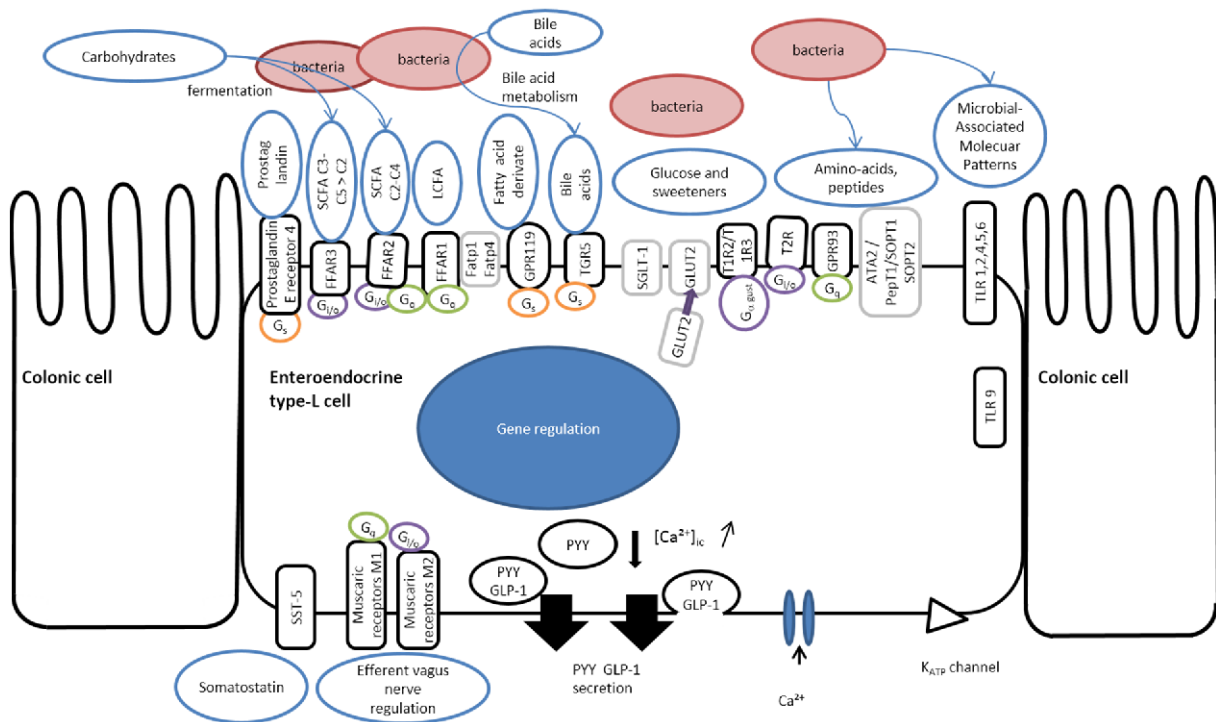


Figure 12 : Enteroendocrine cells, sensors of luminal content

Representation of main receptors and transporters implicated in the secretory response of enteroendocrine cells. Most of the receptors are supposed to be localized at the apical membrane, but some receptors are also important in the sensing of neighbor cell signaling.

They bind to GTP when activated, and are inactivated by hydrolyzing the GTP into GDP. G protein coupled to GPCR is a trimer and upon activation, small subunit  $\alpha$  is able to detach from the trimer. GPCR are characterized by type of  $G\alpha$  they can bind. Rapidly, four main types exist with different functions:  $G\alpha_s$  activates adenylyl cyclase, responsible of formation of cAMP from ATP, an important second messenger activating Protein Kinase A (PKA), implicated in gene expression regulation and secretion (Seino 2005).  $G\alpha_i/o$  on the contrary inhibits adenylyl cyclase and has therefore an opposite effect to  $G\alpha_s$ . However,  $G\alpha_i/o$  has also a weak effect on the activation of phospholipase C (PLC), which is the main pathways downstream of  $G\alpha_q$ . Phospholipase C hydrolyzes phosphatidylinositol (4,5) diphosphate into inositol-trisphosphate (IP3) and diacylglycerol (DAG). DAG regulates many cellular pathways including gene expression through PKC activation. IP3 is recognized by a specific receptor on ER resulting in opening of calcium channel and therefore induce cytosolic calcium concentration rise. The last main G protein type is  $G\alpha_{12/13}$  which activates Rho pathway, mainly implicated in cytoskeleton dynamic. Gustducin is a specific type of G protein associated to a particular family of GPCR, taste receptors which activates IP3 and increases cAMP concentration. Taste receptors are GPCRs functioning in homodimer or heterodimer associated with perception of the five tastes in the gustatory system, but are also expressed in the enteroendocrine cells and are implicating in the sensing of luminal content (Margoleske 2007, Jang 2007). After activation, GPCR are often inactivated by internalization, and either addressed to lysosomes where they are degraded or recycled to the plasma membrane and reactivated to sense luminal content (Zhang 1996, Roed 2015).

Another important pathway regulating calcium concentration and that is associated with secretion is activation of cation pumps or channels that can be associated with transporters. Many transporters, including glucose transporter SGLT1, amino acid transporter SLC38A2, necessitate sodium entry for molecule transport. This influx of sodium induces a cell depolarization leading to the opening of calcium channels, and thus a quick increase of calcium concentration (Yee 2011, Tolhurst 2011). A second mechanism implicated in glucose sensing is the transport through GLUT2 which induces an inactivation of  $K^+$  ATP sensitive channel. Inactivation of this channel also induces membrane depolarization, and therefore opening of calcium channels (Reimann 2002, Reimann 2008).

Then, secreted hormones can have either a paracrine or endocrine activity. Some epithelial cells express receptors to specific hormones and therefore respond to their signaling by secreting themselves other hormones (Dubé 2002, Afroze 2013). Enteroendocrine cells also activate neighbor cells including afferent neurons and immune cells that express receptors to these hormones (Zhang 2012, Bohorquez 2014, Irwin 2013). Finally some hormones enter the circulation and directly act on specific organs expressing their receptor (Figure 12).

## L-cells

Because of their localization and the pattern of peptidic hormones they secrete, L-cells appear to be important cells. GLP-1 in particular, because of its incretin effect, has been extensively studied, and different GLP-1 analogs or inhibitors of DPP-IV, the peptidase responsible for its inactivation have been tested and used for diabetes treatment (Owen 2013, Heppner 2015), and some molecules are now used to treat type II diabetes. Moreover, several models have been developed to study L-cells, including *in vitro* murine cell lines STC-1, GLUTag and human cell line NCI-h716 (Abello 1994, Geraeds 2009, De Bruine 1993). These cell lines are tumoral cell lines, meaning that even if they share important characteristics with the cell type they model, they have some important drawback including incomplete differentiation as some also express other gut peptides like CCK, genomic instability with increased number of chromosomes for all three cell lines. Moreover, they are not polarized like epithelial cells and are cultivated alone whereas this type of cell is normally scarce in the epithelium and therefore in contact with many other cell types. However, these models were until recently the only model to directly study cellular response of these cell, and remain unique tools to study hormonal production and secretion pathways. Models of transgenic mice expressing a GFP specifically in L-cells have been developed recently (Bohorquez 2010, Reimann 2008). Use of primary culture and microscopy allowed direct study of these cells, whereas primary culture of intestinal epithelium from wild type mice or from humans could not give access to cellular processes implicated in cell response, due to the scarcity of L-cells and the lack of external markers of L-cells. More recently, models of culture of *in vitro* gut, called organoids or mini-gut may allow more precise studies on enteroendocrine cells, using molecular tools to express specifically in L-cells reporter systems (Petersen 2014).

Cell lines modelling L-cells have been mostly studied for expression and secretion of GLP-1, as GLP-1 was highly expressed in these cells and modulation of GLP-1 levels is seen as a good strategy for diabetes treatment. As GLP-1 and PYY colocalize in same secretory granules, it is assumed that their secretion is similarly regulated (Nilsson 1991, Habib 2013). Moreover both hormones are secreted at low levels during fasting and plasma levels highly increase after food intake. Mechanisms inducing PYY and GLP-1 secretion shortly after food intake have been demonstrated, implicating CCK as L-cells can not directly sense arrival of nutrient due to their localization (Degen 2007, Ko 2011). However, whereas both hormones have short half-life in the circulation, PYY levels stay high up to five hours whereas GLP-1 plasma levels decrease rapidly in the two hours after food intake (Adrian 1985, Herrmann 1995), indicating that different regulations between these hormones may exist (Anini 1999, Gibbons 2013). Sustained higher levels of PYY after food intake can be explained by two different mechanisms. Firstly, stores of PYY per cell may be more important or can be rapidly increased in response to food intake by high levels of PYY production, whereas GLP-1 is rapidly depleted from L-cells after induced secretion. Secondly, PYY epithelium concentrations are higher in the distal part of the cell on the contrary to GLP-1, and late PYY secretion may be due to delayed nutrient arrival to this part (Figure 11).

## Peptide YY

Even if GLP-1 was the most studied of the hormones secreted by L-cells, due to its incretin effect and possible use for type 2 diabetes treatment, PYY also has a broad range of effects including regulation of food intake, gut motility and gastric emptying. Moreover, PYY is mainly secreted in the distal ileon and colon, where microbiota is the densest. Therefore, potential regulation of PYY expression and/or secretion by modulation of microbiota using prebiotics or probiotics can be hypothesized. Of note, modification of PYY levels have been observed in different diseases, with a clear functional association, like obesity or anorexia nervosa, making regulation of PYY levels potentially interesting as a new therapeutic strategy.

## The NPY family and their receptors

The gut hormone PYY belongs to a family of hormones including two other ones: neuropeptide Y (NPY), and pancreatic polypeptide (PP). They share a same size, 36 amino-acids, and a similar structure with an alpha helix in C-ter and a 3D hairpin structure (Keire 2000) (Figure 15). PYY and NPY have the highest percentage of similarity, with 70% of common residues whereas PP have only 50% common residues with NPY. Moreover, both NPY and PYY can be matured by cleavage of the two N-terminal residues by the peptidase DPP-IV, modifying their activity (Keire 2002, Mentlein 1993). These peptides are mainly implicated in the control of food intake, but with different effects as NPY is considered as an orexigenic hormone whereas PYY and PP have anorexigenic effects by acting on receptors located at different places. The NPY family is conserved among vertebrates, PP being the less well conserved gene and some functional duplications of this gene have occurred (Ding 1997). Among vertebrates, they share similar tissue expression and functions (Larson 2003, Lundell 2002).

### *NPY receptors*

These hormones are recognized by a specific family of receptors that is also conserved among vertebrates. The family of Y receptor (YR) is composed of 4 receptors in humans, Y1, Y2, Y4 and Y5 and 5 in rodents, y6 being the other one (Larhammar 2004). All of these receptors mainly couple to Gi/o proteins, and therefore signal by decreasing cAMP (Ewald 1988). Thus they can be considered to have inhibitory activities when stimulated. Moreover, it was reported that Y1 and Y4 can have a pertussis independent effect (Misra 2004). PYY and NPY are recognized by Y1, Y2 and Y5. Y1 and Y5 recognize the terminal parts of PYY and NPY whereas Y2 is specific of the loop part of the peptides. Truncation of the two first residues by DPP-IV abrogates their affinity to Y1 and strongly decreases affinity to Y5. Y4 recognize mostly PP and has only weak affinity for PYY (Cabrele 2000, Pedragosa-Badia 2013).

YRs are expressed in the brain, with the highest expression being in the hypothalamus, but also in other tissues including the bones, the adipose tissue, the gut (muscles and epithelium), the vagal nerve, some immune cells, lingual epithelial cells (Ferrier 2002, Lin 2004, Parker 1999).

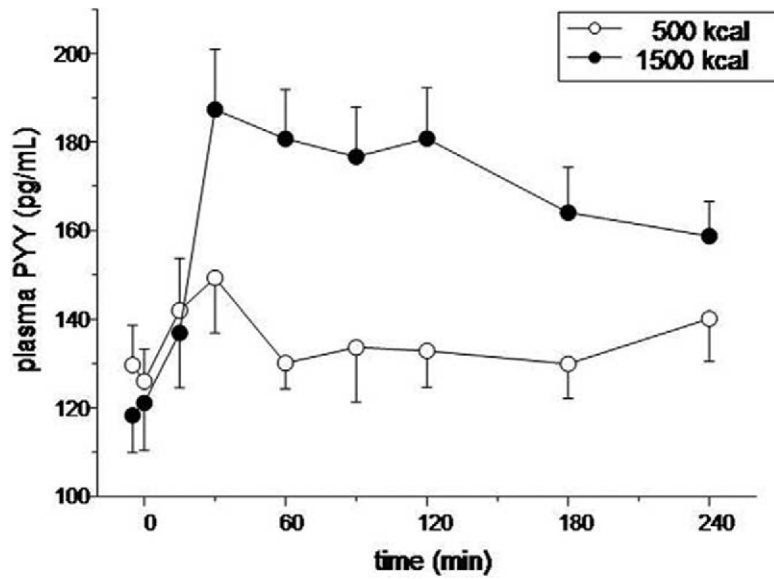


Figure 13 : Time course of PYY plasma level after food intake in response to two different caloric loads (Degen 2005)

PYY plasma levels rise just after food intake at different extend depending on caloric load, and remain high for several hours after food intake.

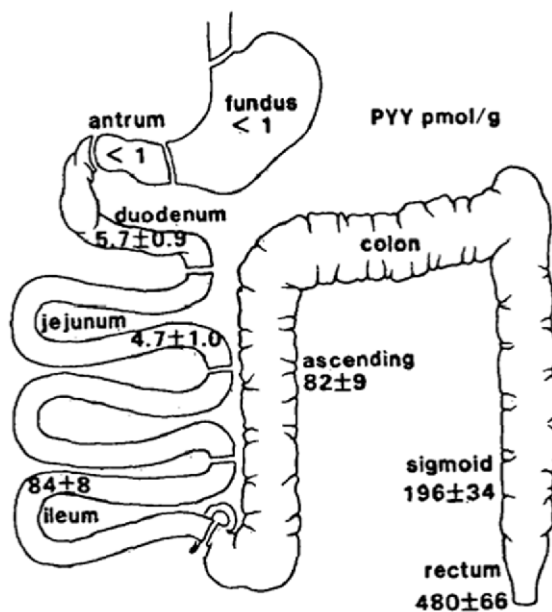


Figure 14: Distribution of PYY along the human gastrointestinal tract, in pmol/g (Adrian 1985)

### *PP, NPY and PYY*

PP is expressed in F-cells in the pancreas and its plasma concentration rises after food intake. It has mainly been described to signal for satiety in humans and mice (Batterham 2003, Shi 2013), but also to have paracrine effects on pancreatic cells. Indeed, it can inhibit glucagon and somatostatin secretion (Aragon 2015, Kim 2014). In mice, PP decreases food intake similarly to PYY 3-36 but also potentially by another mechanism. Different mechanisms from PYY 3-36 are implicated as in mice depleted for Y4, PP had no effect on food intake whereas PYY effect was unchanged (Shi et al, 2013).

NPY is mainly expressed in the arcuate nucleus (ARC) in the hypothalamus, as well as in the cerebral cortex and brainstem. It is also expressed in peripheral nervous system and has effects on different peripheral tissues. Moreover, it was reported to be directly expressed in adipose tissue. It has mainly been implicating in energy homeostasis and food intake (Caberlotto 2000, Sitticharoon 2013, Kuo 2007, Yang 2008). Central increase of NPY level, induced ever by secretion from arcuate neurons or directly by intracerebroventricular administration, induces hyperphagia as well as increases adiposity. Peripheral NPY also increases adipose tissue formation and inhibits lipolysis. It has also been implicated in regulation of bone formation, both centrally and peripherally as NPY receptors are expressed in osteoblasts. Expression of NPY in the ARC is limited to a specific population of neurons, most of them also expressing AgRP that integrate peripheral signal (Kohno 2012).

PYY is mainly secreted by L-type enteroendocrine cells. Its expression increases in a proximal-distal gradient with very low expression in duodenum and jejunum, higher in distal ileum and colon. Maximum localization is reached in rectum (Adrian 1985) (Figure 14). It is rapidly processed to PYY 3-36 by DPP-IV, considered to be the active form of PYY. PYY is then rapidly degraded. It is mainly secreted after food intake in response to caloric ingestion similarly to other gut peptides. However, its levels remain high for several hours, about 6h in humans (Figure 13, Figure 14). PYY is also secreted in islets of Langerhans in other cells than  $\beta$ -cells in mice (Choi et al, 2012, Sam et al, 2012), but functions of the PYY secreted in the pancreas are not known. PYY results from the cleavage of a 97 amino acid prohormone in humans and 98 amino acid one in mice. Prohormones between the two species share 75% identity but central region coding for the mature PYY is very highly conserved (only two differences, 95% of identity) whereas the N and C-terminal parts are much less conserved (30% identity) (Figure 15). Of note, the third amino acid localized just after the site of cleavage by the DPP-IV is not conserved between mice and humans being respectively an alanine and an isoleucine, but importance of this difference concerning cleavage by DPP-IV is not known.

Difference between human and mouse gene structure is important as human gene is encoded on 7 exons on 51,732 bp on chromosome 17 whereas mouse gene is encoded 4 exons on 1183 bp on chromosome 11. Mouse exons are quite similar to last four human exons and prohormone is encoded only on the last three exons. However, human and mouse *Pyy* genes may be differently regulated due to these extended exons part in humans (Figure 15).

PYY is also secreted in the saliva by taste cells along with other hormones such as leptin, adiponectin, glucagon, GLP-1 and CCK, and receptors have been found in taste cells and afferent taste nerves. However expression in these cells is much lower than in the colon. (Acosta et al, 2011, La Sala 2013). Only PYY 3-36 could be detected, and increased salivary PYY 3-36 reduced food intake. However, different mechanisms from circulating gut PYY may be involved to induce satiation (Hurtado et al, 2013), however, studies on salivary PYY are still limited and need further development to precise the role of PYY secreted in the saliva.

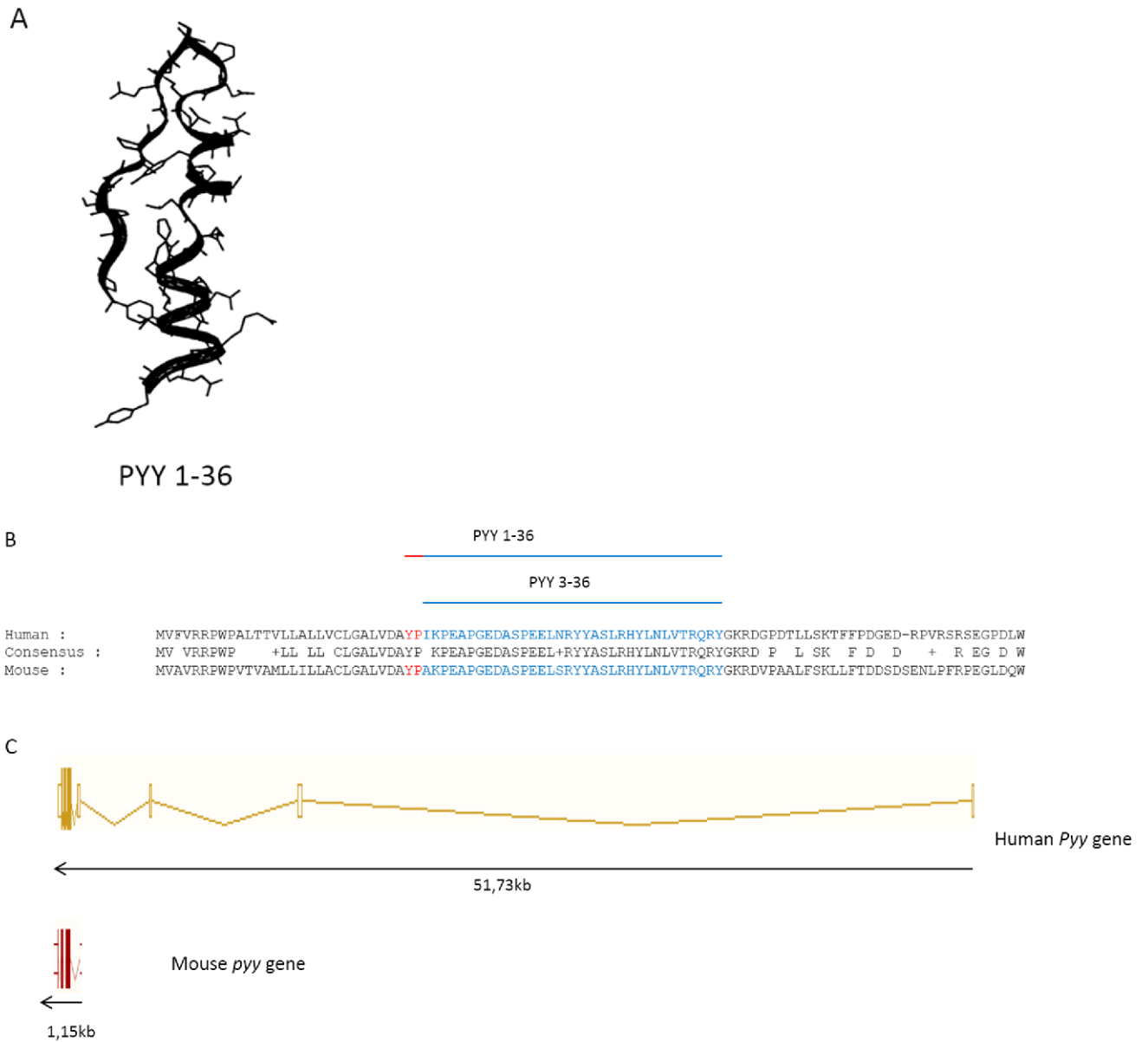


Figure 15 : Structure of PYY protein, and sequences

A: 3D structure of PYY(1-36) present a structure similar to PP and NPY, composed of an hair-pin and a C-terminal  $\alpha$ -helix

B: Alignment of human and mouse preproPYY amino acid sequence show the highly conserved sequence of the region of PYY(1-36), but N-ter and C-ter parts are less conserved

C: Representation of human and mouse Pyy gene structure, human gene being composed of 7 exons spanning over 50,000 bases whereas mouse gene is composed of only four exons similar to the last four human exons

PYY in the circulation is processed into PYY 3-36 that has different effect than PYY 1-36 due to different affinities with receptors, PYY 3-36 activates with high affinity Y2 and with low affinity Y5 whereas PYY 1-36 activates with equal affinity Y1, Y5 and Y2 (Pedragosa-Badia 2013, Berglund 2003, Keire 2000). The truncated form accounts for about 30 to 40% of total PYY (Grandt 1994, Penin 2008, Mentlein 1993) indicating that both forms are present in the circulation and can have different functions.

## Functions of PYY

### *Regulation of food intake by PYY*

Direct administration of PYY in human and rodents reduces food intake (Batterham 2002, Adams 2004, Degen 2005 ) and is associated with modulated activity in several brain area associated with response to food intake (De Silva 2011, Batterham 2007). Moreover, mice deficient for PYY become obese and have hyperphagia (Boey 2006) whereas mice overexpressing PYY are resistant to diet induced obesity (Boey 2008).

Hypothalamus and more precisely the ARC is an important region for food intake control as it represents localization of first order neurons, responding to different peripheral signaling such as hormones (leptin, insulin) and nutrients (Kohno, 2012), integrating the signals and modulating other cerebral region activities. ARC is localized close to the blood brain barrier that can be crossed by PYY (Nonaka, 2003). Two main family of neurons are present: NPY/AgRP (Agusti related peptide) neurons, also secreting the neurotransmitter GABA, and POMC/CART neurons, secreting  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH). Activation of NPY/AgRP has orexigenic effect whereas activation of POMC/CART neurons has anorexigenic effects. These first-order neurons send axonal projections to second order neurons in other hypothalamic areas where they have antagonist roles:  $\alpha$ -MSH stimulates melanocortin receptors 3 and 4 in order to activate satiation and satiety pathways in the paraventricular nucleus (PVN) whereas AgRP is an antagonist of these receptors (Loh 2015). NPY/AgRP neurons also inhibit POMC/CART activity. Moreover, NPY and GABA also promote food intake by inhibiting the parabrachial nucleus (PBN) activation. PBN is normally activated by specific neurons of the NTS secreting glutamate and that are themselves under the control of sensory signaling from the vagal nerve and the raphe nuclei secreting serotonin. PBN activation is also related to nausea signaling, inducing reduced food intake, which can be inhibited by NPY/GABA signaling. (Wu 2012). NPY/AgRP neurons have also projections to the lateral hypothalamic area (LHA) where orexins and melanin concentrating hormone are secreted, and ventromedial nucleus also implicated in control of food intake.

The form of PYY is important for it is only PYY 3-36 that reduce food intake. Administration of PYY(1-36) had an effect on food intake only in rats expressing DPP-IV (Unniappan 2006). In the hypothalamus, PYY 3-36 inhibits NPY secretion through Y2 expressed in these neurons and increases POMC expression in POMC neurons (Batterham 2007, Shi 2013). Combined effects of the decreased secretion of the inhibitory signal AgRP and the increased secretion of POMC is responsible for activation of melanocortin receptor 4 localized on neurons, thus decreasing food intake and increasing energy expenditure when activated (Huszar 1997). These neurons have projection to the PVN (paraventricular nucleus) and modify the signaling of neurons by increasing signaling corresponding to



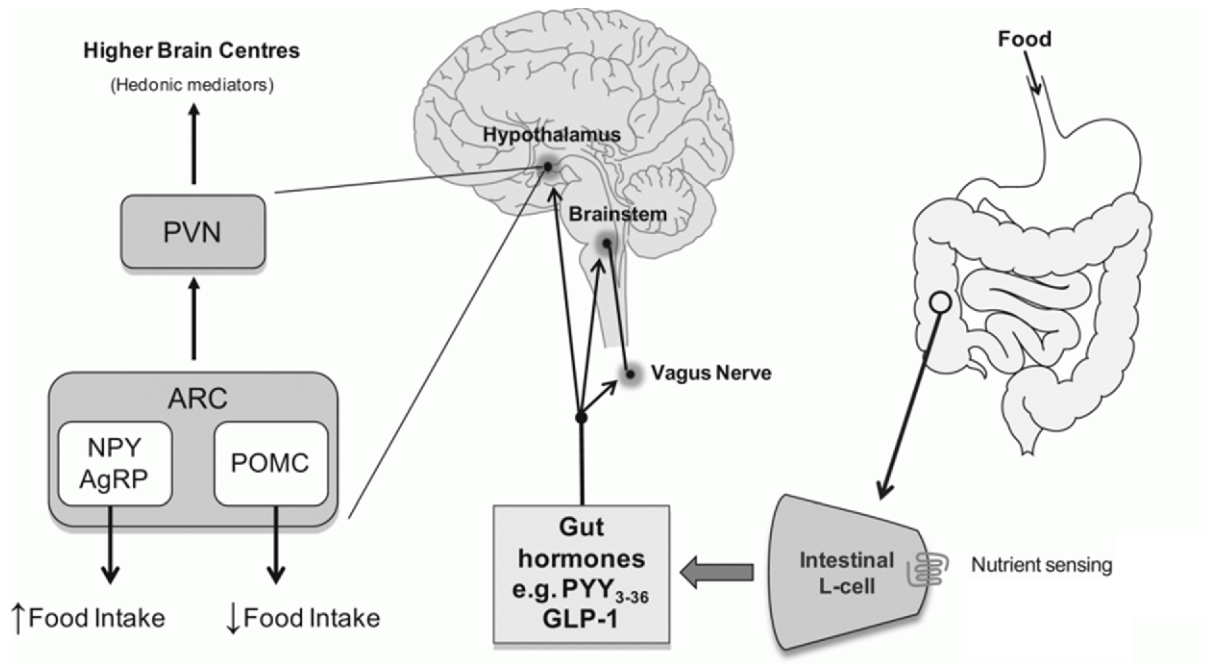


Figure 16 : Regulation of food intake by PYY and GLP-1 through modulation of arcuate nucleus activity (adapted from De Silva 2012)

PYY and GLP-1 are secreted after food intake and inhibit NPY/AgRP neurons in the arcuate nucleus, both directly and through the vagus nerve. Inhibition of NPY/AgRP neurons leads to inhibition of food intake through activation of different brain areas.

satiety (Schwartz 2000). Indeed, administration of PYY is associated with an increased activity in this region (Acuna-Goyalo 2005). Moreover, NPY/AgRP neurons have also projections to the lateral hypothalamic area, but have an inhibitory effect on these neurons. Lateral hypothalamic area is known as a feeding center as its neurons produce two orexigenic neuropeptides, orexin and melanin concentrating hormone, and their inhibition participates in the anorexigenic effect of PYY. On the contrary, direct central administration of PYY1-36 in the brain has an opposite effect. In that case, PYY has a similar role as NPY in the regulation of food intake, which is orexigenic, consistent with the fact that both hormones bind same receptors (Morley 1985).

Direct sensing of PYY in the ARC is thought to happen as PYY can cross the brain blood barrier, and this barrier is weaker close to the ARC (Nonaka 2003). Peripheral sensing of PYY through the vagal nerve is also involved and may be important considering the short half-life of PYY and the fact that its ablation reduces PYY 3-36 induced satiety (Abbott, 2005). Y2 receptors can also be found in the nodose ganglia, and discharges of afferent nerves can be associated with PYY 3-36 administration (Iwasaki 2012). The Nucleus Tractus Solitarii (NTS) is an important brain region that relays information from the vagal nerve to different centers, including PVN, the parabrachial area and the ARC (Figure 16).

Another important regulating center in control of food intake are the reward circuits localized in the cortex, as they determine not only quantity of what is eaten but also the type of aliments, and they react rapidly to eventually create aversion for what is eaten. PYY and several other hormones decreasing food intake decrease reward circuit activity implicated in regulation of food intake (Gao 2007, Batterham 2007, Weise 2012). However, mechanisms implicating in the regulation of these circuits by PYY are still unknown, even if seems to need signaling from the homeostatic center hypothalamus.

#### *Intestinal motility, nutrient absorption*

Another important role of PYY that is also associated with food intake is control of gut motility and gastric emptying. Indeed, mechanic sensing by vagus nerve is an important message for satiation, and is even the first to develop in infants (Crespi et al, 2014). PYY has been described as one of the hormones inducing the ileal brake that happens in response to entry of nutrients in ileum, where L-cells are present (Maljaars 2008, Lin 2003). Ileal brake consists of a decrease of intestinal motility, inhibition of gastric emptying and inhibition of pancreatic enzymatic and gastric enzyme secretion. Ileal brake is therefore supposed to permit increased absorption in the small intestine. Direct administration of PYY decreases motility and consequently increases gut transit time (Savage 1987). Motility is controlled by two layers of smooth muscles that express Y receptors (Ferrier 2002, Misra 2004). The contractions of these two layers are also dependent on enteric neurons. Ileal administration of PYY decreases gastric motility, either in innervated or denervated tissue and decreases gastric contractions (Cuche 2000). Moreover, PYY effect on motility is dependent on its isoform and activation of different receptors. Y2 receptor is only present on circular smooth muscle and enteric neurons whereas Y1 is localized on both smooth muscle layers and on neurons (Misra 2004). Activation of Y2 or Y1 in neurons by PYY 3-36 inhibits motility (Wang 2010, Tough 2011), whereas activation of Y1 by PYY(1-36) directly induces muscle contractility (Ferrier 2000, Ferrier 2002). Thus, PYY has two opposite effects on motility depending on different signaling: a direct pathway which increases muscle contractions and motility and a neuron mediated pathway inhibiting contractions and motility. Its effects are therefore associated with decreased motility by a distal regulation, *i.e.* in the proximal part of the intestine, corresponding to the gastric and small intestine decreased motility and decreased gastric emptying observed during an ileal brake, and increased motility by local regulation, *i.e.* mainly in the colon.

Y receptors are expressed on intestinal epithelial cells (Goumain 1998). Thus PYY has direct actions on these cells which include decreased histamine secretion by ECL cells, and therefore decreases gastric acid secretion (Yang 2002, Cloarec 1989, Bado 1993), and decreased pancreatic enzymes secretion (Adrian 1985). In the colon, PYY has an important role in controlling water reabsorption and electrolyte intake (Quin 1995, Nakanishi 1996, Cox 2002) by decreasing basal short circuit currents created by chloride secretion. In the colon and small intestine crypt,  $\text{Na}^+$  and  $\text{Cl}^-$  ions are absorbed in an electroneutral way, depending on exchange with a proton and a bicarbonate, but  $\text{Cl}^-$  is also secreted, depending on cAMP activation (Sandle 1998). Activation of Y2 receptor has an antiseecretory activity of electrolytes (Cox 2008), which is thus associated with increased absorption of electrolytes. Regulation of electrolyte secretion is a way to control water and nutrient absorption maintaining osmotic gradient. PYY participates therefore in the regulation of water absorption by colon (Sandle 1998), and PYY 3-36 has anti diarrhea effects (Moriya 2010) through activation on Y2 receptor.

PYY has also been implicated in lipid metabolism in intestinal epithelial cells (Grenier 2012) as basolateral administration of PYY on polarized Caco2 cells decreases formation of chylomicrons and regulates expression of several enzymes implicated in lipid homeostasis.

### *Metabolism*

Even if PYY is not considered as an incretin hormone compared to GLP-1,  $\beta$ -cells express Y1 receptors, and PYY has been implicated in the regulation of insulin secretion. PYY 1-36, by binding to Y1 receptor on these cells, inhibits glucose induced insulin secretion, but has no effect on basal insulin secretion (Nieuwenhuizen 1994, Bertrand 1992). This mechanism is important for maintaining insulin tolerance by avoiding hyperinsulinemia in response to high glucose concentration. Mice deprived of PYY or obese people with low PYY levels have hyperinsulinemia (Boey 2006), whereas chronic administration of PYY 3-36 to mice improves insulin tolerance (Van den Hoek 2004). Moreover, Y1 activation in  $\beta$ -cells by PYY 1-36 also increases cell survival and proliferation (Sam, 2012, Liu 2006). Y1 receptor is also expressed on pancreatic  $\alpha$ -cells, indicating a potential role of PYY1-16 on regulation of glucagon secretion.

$\beta$ -cells are not sensitive to PYY 3-36, but it participates indirectly in the regulation of insulin secretion through hypothalamic control of insulin secretion (Greeley 1988). Moreover, L-cells also express Y2 receptor and secrete GLP-1 in response to PYY 3-36. Thus, administration of PYY 3-36 induces insulin secretion through GLP-1 secretion (Chandarana 2013). PYY 3-36 also impacts insulin function by increasing glucose uptake by muscle and adipose tissue in response to insulin (Van den Hoek 2007) but precise mechanisms are not deciphered yet.

The Y receptor family is expressed in other tissues, but it is not known if physiological PYY is able to reach these tissues, and these receptors are also activated by two other hormones, NPY and PP. For example, expression of receptors Y1 and Y2 was shown on bones and a role of NPY has been proposed (Shi 2012). However, PYY is thought to also regulate bone mass formation as PYY k.o. mice have increased bone mass whereas mice overexpressing PYY have impaired bone mass (Wong 2012).

Thus, PYY can be implicated in the regulation of a great number of different physiological functions. Deregulation of PYY levels has been associated with some diseases, with the highest evidences concerning diseases related to food intake behavior. Obese patients have decreased levels of fasted and postprandial PYY (Le Roux 2006, Brennan 2012, Boey 2006). Interestingly, after bariatric surgery, considered as the most effective therapeutic strategy used today to treat morbid obesity resulting in sustained weight loss, PYY plasma levels are increased (Werling 2012, Peterli 2012, Mumphrey 2013).

Inversely, anorexia nervosa and aging related anorexia are associated with an increase of PYY plasma levels (Rigamonti 2011, Pfluger 2007, Moss 2012). PYY levels are also increased in patients with Inflammatory Bowel Diseases (Schmidt 2005, Moran 2012, Hirotani 2008). Of note, DPP-IV expression is also affected in these patients (Moran 2012), but more studies are needed to determine the association between these diseases and PYY levels.

These diseases are all associated with modification of microbiota, suggesting that microbiota may affect directly or indirectly PYY plasma levels and/or secretion in response to food intake. Different bacterial products have been associated with regulation of PYY production or secretion, including luminal compound metabolisms.

## Bacterial products implicated in cross talk with EEC

### Functional metagenomics to search for new bacterial genes regulating PYY expression and secretion

The objective of this thesis was to study the regulation of PYY expression and secretion by bacterial products to highlight the importance of crosstalk between gut microbiota and enteroendocrine cells. We hypothesized that PYY expression and or secretion may be modulated by different bacteria or bacterial products. Indeed, PYY is mainly produced and secreted in the gut regions where microbiota is the densest. Moreover, PYY plasma levels are found modified in several pathologies associated with modification of microbiota, and changes of microbiota due to diet modifications or surgical intervention like gastric bypass were also correlated with modification of PYY plasma levels (Pfluger 2007, Chandarana 2011, Furet 2010). Modification of microbiota at different stages of life could also be associated with different PYY plasma levels as neonates as well as elder people were reported to have increased PYY plasma levels (Adrian 1986, Siahianidou 2005, Moss 2012). Moreover, bacteria that modulate expression or secretion of PYY can be of particular interest and serve as a strategy to treat the diseases associated with deregulation of food intake, either by modifying its presence by probiotics or prebiotics or by finding the active molecule responsible for its activity on PYY regulation. An approach to find new bacterial genes regulating host functions is based on functional metagenomics, presented in a published review of literature presented in annex (Larraufie 2015).

Rapidly, this method consists in expressing genes from bacteria that may not be cultivable at the bench by integrating part of their genome in a fosmid transformed in an easily cultivable bacteria like *E.coli*. Each clonal *E.coli* part of a metagenomic library has a fragment of about 40kb of heterologous DNA corresponding to about 40 genes. Library is screened on a reporter system that allows easy determination of an activity like enzymatic activity or fluorescence, and clones are selected on their activity (Lakhdari 2010). Further analysis of the clone of interest by different techniques are required to find out the genes responsible for the activity and the bacterial species associated, and can lead to description of pathway implicated in the regulation of the detected activity.

Adapting this methodology, we searched to develop an approach to target *Pyy* expression and PYY secretion by the construction of two different reporter systems and assessed feasibility of screening.

### Short chain fatty acids

#### *SCFAs production in the colon*

The major bacterial-derived metabolites present at high concentration in the colon are short chain fatty acids (SCFAs), and more particularly the non-branched SCFAs: acetate (C2), propionate (C3) and butyrate (C4). Gut bacteria ferment polysaccharides, oligosaccharides and peptides in specific SCFA depending on the substrate. In the colon, non-digestible carbohydrates mainly derived from plant cell walls are the main source of SCFA production, and represent the main source of energy and carbon for endogenous bacteria (Flint 2008). However not all bacteria have the genetic background to degrade these complex polysaccharides into simple sugars. Specific enzymes, called CAZymes for carbohydrate

active enzymes, are active on specific substrates and several different ones are needed conjointly for fiber degradation. Simple sugars are then used in metabolic pathways needed for the formation of pyruvate, a central molecule in multiple metabolism pathways. Depending on sugar length used as a substrate, two main pathways are used by gut bacteria: the glycolytic pathway for six carbons sugars and pentose phosphate pathway for five carbons sugars. These pathways end up by the production of pyruvate, which will then be used to produce all types of SCFAs, as well as lactate, methane, carbon dioxide, dihydrogen and hydrogen sulfide (Figure 17). Not all bacterial species can transform pyruvate into one of the SCFAs, and formation of any of the pyruvate-end product is dependent on multiple bacterial enzymes as well as environmental conditions. Butyrate and acetate are both dependent in the production of acetyl-Co-A. Oxidative conditions are necessary for acetate production, whereas a low redox potential is required for butyrate (Pryde 2002, Macfarlane 2003, Louis 2014). Propionate is mainly derived from succinate but to a lesser extend from lactate. Many species do not express all the enzymes required for the production of the final products and required intermediate molecules from other bacteria. Acetate can be used by some other bacteria for butyrate production (Duncan 2004).

#### *Bacteria implicated in SCFAs production*

SCFAs bacterial producers were first determined from cultivable bacteria by directly measuring SCFA production, and closely related bacteria were assumed to possess similar activities. Recently, sequencing of whole bacterial genomes and metagenome allowed to search *in silico* for genomes including genes coding for enzymes related to SCFA production. These studies demonstrated that production was species dependent, but only some genera were associated with propionate and butyrate production (Louis 2009). Acetate is an end product of most bacterial fermentation that is increased by specific bacteria through acetogenesis. Butyrate production is principally done by Firmicutes and main producers belong to the *Lachnospiraceae* (including *Roseburia* spp and *Coprococcus* spp.), *Eubacteriaceae* (including *Eubacterium* spp.), *Clostridiaceae* and *Ruminococcaceae* (including *Faecalibacterium* spp.) (Pryde 2002, Vital 2014). Propionate production is less restricted than butyrate and can include some Bacteroidetes such as *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* as well as some Firmicutes such as species from the *Negativicutes*, *Ruminococcaceae* and *Lachnospiraceae* families (Reichardt 2014).

Other SCFAs are also produced in the colon, but at much lower concentrations (Cummings 1987), including formate (C1), valerate (C5), caproate (C6) and branched fatty acids isobutyrate, 2-methylbutyrate and iso-valerate. Most of them are dependent on branched amino acid fermentation such as valine, leucine and isoleucine (Cummings 1981, Macfarlane 1992).

#### *SCFAs transport*

SCFAs production is important in the proximal colon and decreases to the distal colon, whereas its concentration remains high in any part of the colon. Of note, ratio between main SCFAs remains stable throughout time and localization and is about 6:2:2 for acetate : propionate : butyrate (Cummings 1987) with highest concentration of acetate in the lumen being about 60mM. Most SCFAs are transported into colonocytes and only a small account is found in the feces. Butyrate is directly used by colonocytes as energetic substrate, whereas a more important part of propionate and acetate is transported to the circulation. Acetate and propionate are then mainly metabolized in the liver where they are both used as an energetic substrate through  $\beta$ -oxydation and for *de novo* lipogenesis for cholesterol and fatty acids productions (Knowles 1974). Remaining acetate and





propionate in the circulation can be used as energetic substrate by other tissues, mostly adipose and muscle tissues and in the brain. Gut luminal SCFAs are mainly transported through plasma membrane by the SCFAs transporters namely MCT1 (monocarboxyl transporter 1) and sodium dependent transporter SLC5A8. Exit from colonocytes is mainly mediated by two other transporters, MCT4 and MCT5 (Ritzhaupt 1998, Goncalves 2011, Lecona 2008). Acidic form of SCFAs can also diffuse through plasma membrane. However colon pH is about 2 units higher than SCFAs pKa that is about 4.8, thus acid form of SCFAs represents only 1% of total SCFAs, suggesting that diffusive transport may only account for a very small part of total transport.

#### *SCFAs, Inhibitors of HDAC*

Intracellular SCFAs have also specific function. One important function of butyrate with high affinity and propionate to a lesser extent is the inhibition of lysine/histone deacetylases (K/HDAC) (Candido 1978). HDAC are important in the regulation of gene expression as they participate in the modelling of DNA through modification of histone induced DNA compaction. Histones are proteins forming complexes with DNA, namely nucleosomes. Different modifications of histone have been reported, increasing or decreasing affinity with DNA and therefore DNA compaction. Among them, lysine acetylation decreases interaction with DNA by removing the positive charge associated with the amine function replaced by a neutral acetylamine function. HDAC have thus a compacting effect on DNA, inducing gene expression repression. Butyrate, has thus an important role in the regulation of some genes (Davie 2003, Demary 2001, Siavoshian 2000).

#### *SCFAs Receptors.*

SCFAs are also recognized by three GPCRs, FFAR2 (also called GPR43), FFAR3 (GPR41) and GPR109a (Brown 2003, LePoul 2002, Thangaraju 2009). FFAR2 and FFAR3 share many similarities and belong to the same family as FFAR1 (GPR40) and FFAR4 (GPR120), which recognizes medium-long chain fatty acids, (Hara 2008). FFAR2 and FFAR3 are activated by SCFAs present at micromolar concentration whereas GPR109a, which mainly recognizes niacin, only recognizes butyrate with millimolar affinity. FFAR2 can bind Gq and Gi/o small proteins whereas FFAR3 and GPR109a can bind only Gi/o small proteins. Even if FFAR2 and FFAR3 have mice orthologs, differences in affinity and order of affinity for SCFAs and known agonists have been described between species (Figure 18. Human FFAR2 has equal affinity for acetate, propionate and butyrate and very low affinity for other SCFAs whereas mouse FFAR2 order of affinity is acetate > propionate = butyrate. Human FFAR3 has equal affinity as FFAR2 for propionate, a slightly lower affinity for butyrate and much less affinity for acetate whereas mouse FFAR3 has very high affinity for propionate and similar affinity for acetate and butyrate (Hudson 2012, Hudson 2013, Le Poul 2002, Schmidt 2011).

FFAR2 is expressed in immune cells whereas FFAR3 has a broader but weaker expression, with a higher expression in adipose tissue (LePoul 2002, Brown 2003). Both receptors are expressed in human and mouse intestinal epithelium, with higher expression in the colon. FFAR2 and FFAR3 are specifically expressed in enteroendocrine cells in the intestinal epithelium, with higher expression in PYY immunoreactive cells (Karaki 2006, Karaki 2008, Nohr 2013, Tazoe 2009). However, whereas expression is similar in mouse colon, FFAR3 is much less expressed in human L-cells than FFAR2 (Tazoe 2009, Tolhurst 2012). GPR109a is also expressed in human intestinal epithelial cells, and its expression is not limited to enteroendocrine cells (Thangaraju 2009). However, precise localization of these receptors on polarized cells is unclear (Tazoe 2009, Karaki 2008). Monitoring of FFAR localization is



needed as SCFAs concentrations are two to three order of magnitude higher in the lumen than in the lamina propria, and therefore receptors would be differently activated depending on their localization

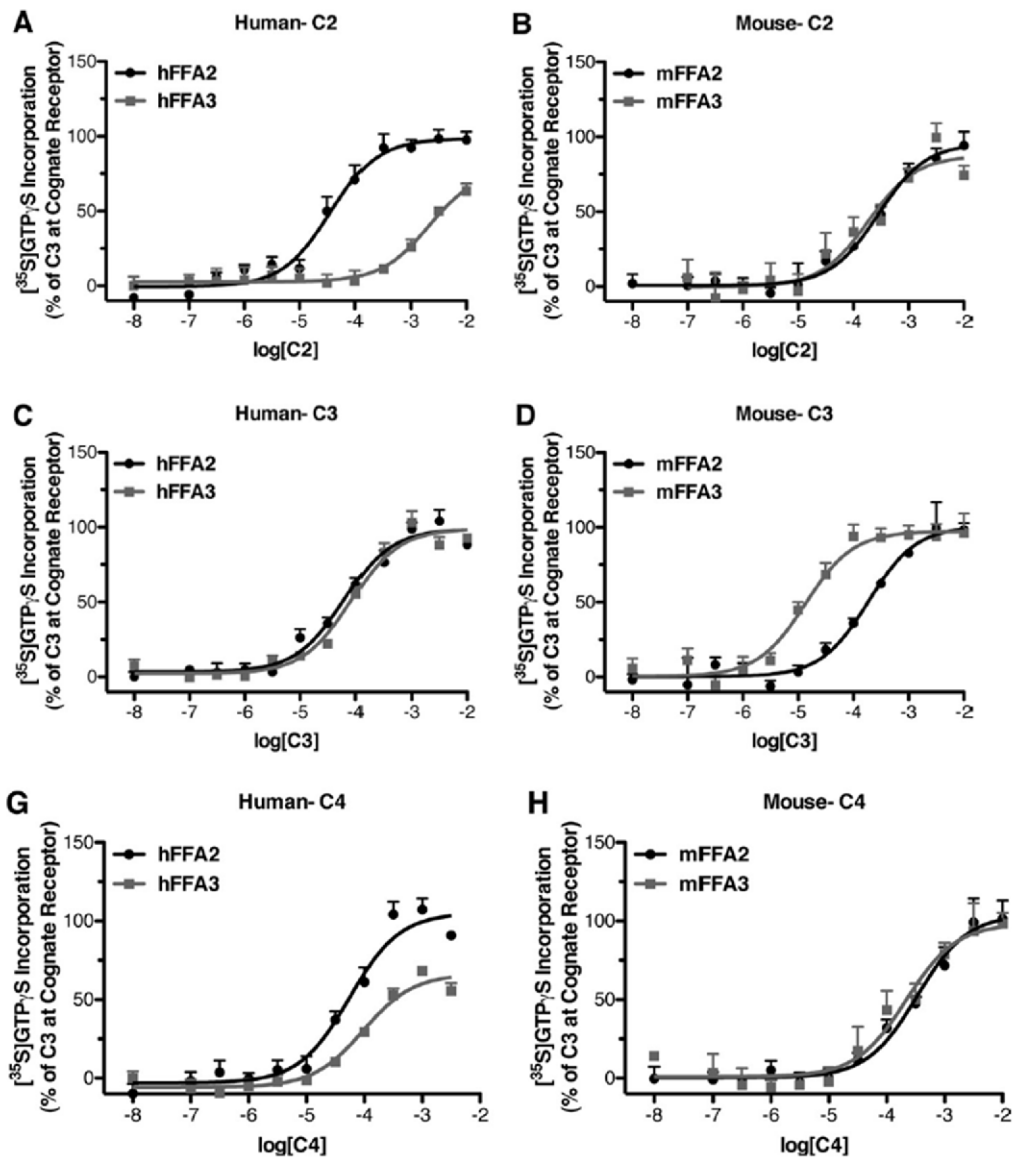


Figure 18: Comparison of human and mouse FFAR2 and FFAR3 affinities for acetate, propionate and butyrate (from Hudson 2012)

Measurement of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  incorporation by FFAR2 or FFAR3 on isolated membranes in response to addition of different concentrations of different SCFAs, comparing activation of heterologous receptors between humans and mice.

### *Effect of fiber rich diets and SCFA administration on PYY plasma levels*

Fiber rich diets increase SCFAs concentration in the colon. Use of different fiber sources in humans or in rodent models could show their influence on PYY plasma concentration (Arora 2012, Cani 2009, Delzenne 2005, Parnell 2012, Reimer 2012, Zhou 2008). Long term effects could be associated with modification of microbiota (De Filippo 2010, Neyrinck 2012, Wu 2011), including increased proportion of *Prevotella* spp., increased Firmicutes and decreased *Bacteroides*. Effects of fibers on PYY is dependent on their degradation by microbiota as in germ free animals PYY plasma levels were not affected whereas colonization with minimal microbiota enabling fermentation induced a PYY rise (Samuel 2008). However, in this study, only one fiber rich diet was tested and other effects due to presence of minimal microbiota per itself were not assessed.

Change of diet and increased colonic SCFAs concentration induced long term effects, including increased plasma levels of PYY and higher PYY content in colonic tissues, but the precise mechanisms implicated in these responses could not be precisely determined. Higher gene expression in tissue can be a consequence of either an up regulation of gene expression per cell or increased number of enteroendocrine cells producing the hormone. As L-cells are sparse in the epithelium, mRNA content per cell could not be assessed except in rodent models expressing specific markers for L-cells or in cellular models. SCFAs were shown to increase the number of L-cells in organoid models (Petersen 2014), but with differences between human organoids and mouse organoids concerning modulation of transcription factor expression. Similarly, diet supplemented with non-digestible carbohydrates increased number of L-cells in rats (Cani 2007, Kaji 2010) and mice (Everard 2011). Mice lacking FFAR2 or FFAR3 have been used to test the importance of these receptors in the response to SCFAs. In axenic mice, effect of colonization enabling fiber fermentation was reduced in *ffar3*<sup>-/-</sup> mice compared to *ffar3*<sup>+/+</sup> littermates, indicating that *ffar3* may be implied in the response of L-cells to SCFAs. Interestingly, in this study, SCFAs concentration was more important in the feces of *ffar3*<sup>-/-</sup> mice compared to *ffar3*<sup>+/+</sup> mice, indicating that *ffar2* may also be implicated in regulation of SCFAs absorption (Samuel 2008).

Expression of PYY has not been tested in cellular models, except promoter activity of rat PYY in human epithelial kidney cells (HEK) in which PYY expression was increased by SCFAs at different concentrations. However, the conclusions of this study are limited by the fact that HEK cells do not express receptors similarly to EEC and the use of orthologous promoter (Zhou 2008). As *Pyy* gene structure is very different between humans and rodents, conclusions concerning regulation of *Pyy* expression from rodent may not be relevant for humans. Expression of *proglucagon*, the GLP-1 and GLP-2 prohormone is increased in rodent cell line STC-1 models (Zhou 2008) but decreased in human cell line NCI-h716 (Cao 2003) by butyrate. As both models are cell lines, differences of SCFA effect may be cell line dependent or species dependent due to different promoters (Nian 1999). Enteroendocrine cells being secreting cells, most studies were interested in regulation of secretion in these cells.

### *Regulation of secretion by SCFAs*

Direct administration of SCFAs in human or rat colon rapidly increased PYY plasma levels (Cherbut 1998, Chambers 2014, Freelend 2010), indicating that SCFAs can directly induce secretion. Interestingly, rectal administration of acetate in humans did had a much stronger effect than intravenous administration, suggesting that acetate is sensed in the lumen of the colon rather than from the circulation (Freelend 2010). Moreover, *in vitro*, administration of propionate on human colonic crypts isolated from biopsies increased PYY secretion during a 2h incubation test. However, only non-physiologic concentration of propionate could induce this effect as only concentrations higher than 100mM had an effect (Chambers 2014). Similar results were published in the same time

using murine colonic crypts, but secretion was stimulated by much lower propionate concentrations, as low as 1mM (Psichas 2015). In STC-1, propionate or butyrate had no effect on secretion of PYY, but only submillimolar doses were tested (100 $\mu$ M) (Hand 2012). Effect of SCFAs on secretion in L-cells has been mainly studied by assessing the secretion of GLP-1. As PYY is co-secreted with GLP-1, it can be assumed that GLP-1 secretion is associated with PYY secretion (Habib 2013).

An elegant strategy to sort L-cell population for further studies is to express specifically a fluorescent protein with a specific L-cell promoter such as the proglucagon promoter (Reimann 2008). Incubation of these cells with single SCFA at millimolar dose increased GLP-1 secretion. L-cells isolated from *ffar2*<sup>-/-</sup> or *ffar3*<sup>-/-</sup> mice had a decreased secretory response to acetate and propionate compared to L-cells from WT mice (Tolhurst 2012). In another study, mice lacking *ffar2* had similar levels of circulating PYY in the portal vein, but direct administration of propionate in the colon decreased PYY induced secretion. Moreover, basal secretion by colonic crypt from *ffar2*<sup>-/-</sup> mice during two hours was similar to secretion by colonic crypt from WT mice, but secretory response to propionate was decreased in k.o. mice (Psichas 2015). These studies therefore indicated that both *ffar2* and *ffar3* may be implicated in the responses to SCFAs in mice. However, *ffar2*<sup>-/-</sup> mice had lower expression of *ffar3* compared to *ffar2*<sup>+/+</sup> mice (Tolhurst 2012), indicating potential regulation of the expression of these two receptors by each other. Thus, even if effect of SCFA at millimolar dose increased secretion in L-cells, precise pathways need to be determined, as well as validity of this effect in humans as only higher doses of propionate could induce a secretory response. Moreover, a third receptor can be implicated in the response to SCFAs in the intestine, GPR109a, and has been hypothesized as being implicated in the response to SCFAs in L-cells (Thangaraju 2009, Kaji 2014).

SCFAs regulate PYY plasma levels through different pathways, which may be different between species due to different receptors affinity, signaling pathways and promoter regulation. One of our objective in this project was to decipher the precise mechanisms involved in SCFAs effects on L-cells in humans. We took advantage of described cell lines modelling enteroendocrine cells to monitor precisely the effects of SCFAs on PYY production and secretion by these cells, and could use cellular tools to demonstrate implication of different mechanisms implicated in the response of L-cells to SCFAs.

## MAMPs and TLRs

### *Role and structure of TLRs in the recognition of MAMPs*

A well described communication between microbes and host cells is through recognition of Microbial Associated Molecular Pattern by the Toll-Like Receptors (TLRs). This system was first identified as an important mechanism for innate immunity. TLRs allow recognition of potential pathogens in immune cells and non-immune cells and induce a pro-inflammatory response by activating NF- $\kappa$ B pathway and secreting cytokines. Other families of receptors implicated in the innate response to pathogens have been identified and form a super family of pattern recognition receptors including additionally to TLRs Retinoic acid-inducible gene like receptors (RLR) and Nucleotide-binding oligomerization domain (NOD) and NOD-like receptors (NLR) (Medzhitov 2007, Akira 2006). Whereas RLRs and NLRs are intracellular receptors sensing compounds that cross the plasma membrane, TLRs are membrane receptors ever localized on the plasma membrane sensing extracellular content or on endosome membrane sensing endocytic content. TLRs family is composed of ten different functional receptors, TLR1 to TLR10, in humans and 11 in mice, with high similarity between most of them. However, mouse

TLR8 is not functional, have no TLR10. TLR11, TLR12 and TLR13 are expressed in mice but not present in humans. Heterologous receptors recognize similar patterns, enabling functional characterization of TLRs in mice. However, function of TLR10 which was much recently described is less known, but seems to have inhibitory effect on TLR2 dependent inflammatory response (Oosting 2014). Rapidly, TLRs can recognize mainly two families of molecules: TLR1, 2, 4, 5 and 6 recognize membrane associated molecules including lipoproteins, peptidoglycans, lipopolysaccharide and flagellin whereas TLR3, 7, 8 and 9 recognize bacterial and viral DNA or RNA patterns. Recognition of these molecules is associated with localization of receptors: TLR1, 2, 4, 5 and 6 are plasma membrane receptors whereas TLR3, 7, 8 and 9 are mainly present in endosomes. TLR1 and TLR6 require to be associated with TLR2 in heterodimers to recognize respectively triacyl lipoproteins and diacyl proteins (Kumar 2009, Kawai 2007) (Figure 19).

Activation of TLRs induces well characterized signaling cascades leading to activation and translocation into the nucleus of transcription factors NF- $\kappa$ B and AP-1. Rapidly, two main pathways have been described: recruitment and activation of adaptors TIRAP and MyD88 or recruitment of TRIF. Both of them induce NF- $\kappa$ B activation. All TLRs except TLR3 can activate MyD88 pathway whereas only TLR3 and TLR4 can activate TRIF pathway. Other non-canonical pathways have been described and regulate different responses. (Kawai 2007, Kawasaki 2014). NF- $\kappa$ B is a dimer composed of two subunits of a family of five subunits, p65, RelB, C-Rel, P50 (which precursor is p105) and P52 (which precursor is p100). The main form activated after TLR activation is p50-p65 heterodimer; heterodimer p52-RelB and homodimer p50-p50 are mostly implicated in non-canonical NF- $\kappa$ B activation. NF- $\kappa$ B dimer is sequestered in the cytoplasm by a family of inhibitors, I $\kappa$ B, which is inactivated and further degraded when phosphorylated by a complex IKK in response to TLR activation. In the nucleus, NF- $\kappa$ B promotes gene expression by binding to consensus binding site (Cogswell 1994, Udalova 2002, Martone 2003). Genes regulated by NF- $\kappa$ B are mainly genes implicated in inflammatory response, including different cytokines like IL-1, IL-8, interferon- $\gamma$  or TNF- $\alpha$ . Other responses associated with NF- $\kappa$ B activation are mainly stress response, regulation of adhesion or apoptosis as NF- $\kappa$ B binding domain site is present in the promoter of many genes (Pahl 1999, Gilmore 2006) (Figure 20).

#### *TLRs in epithelial cells and enteroendocrine cells*

Intestinal epithelial cells are continuously in direct contact with microbes present in the lumen and form an active barrier between commensal bacteria and the host. Epithelium prevents internalization of microbes, but also modulate immune response to shape microbiota and participates in the regulation of immune cells by secreting different cytokines (Stadnyk 1994). However, their response must be limited to avoid induction of inflammation whereas they are exposed to a high number of microbes. Moreover, presence in the epithelium of dendritic cells and T cells, that express TLRs at high levels, participate in the sensing of microbial communities and response. Intestinal epithelial cells have been demonstrated to express all TLRs except TLR10 (Abreu 2010, Graves 2014). Localization of TLRs is important as depending on localization on apical membrane or basolateral membrane, they are exposed very different quantities of MAMPs. Consistently, on polarized cells in culture, some TLRs were only stimulated on the apical membrane

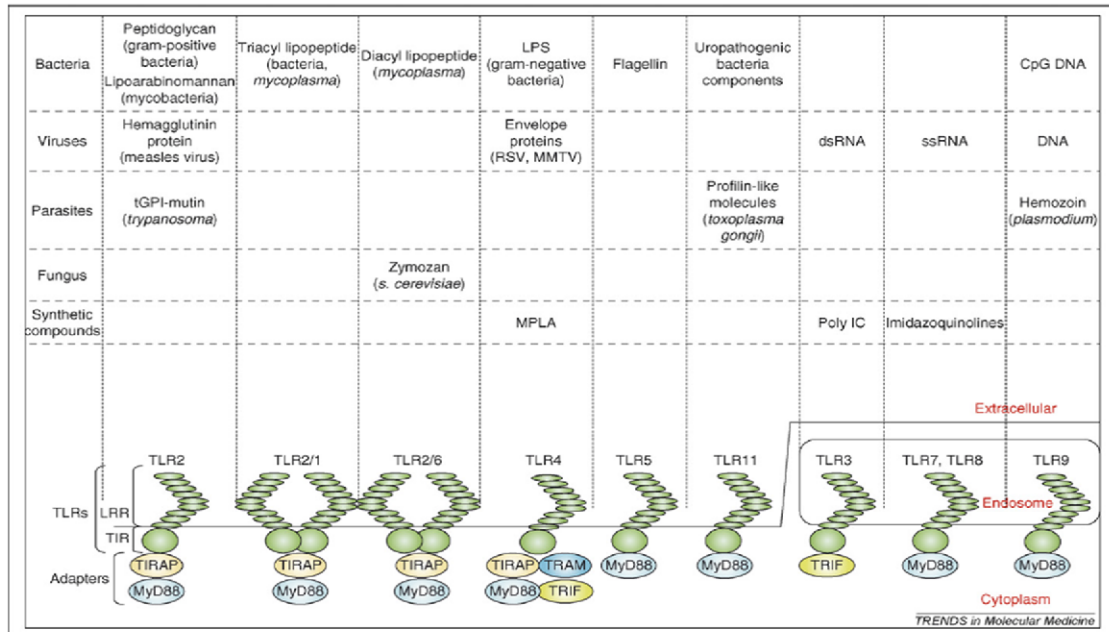


Figure 19 : Patterns recognized by TLRs and their main adaptators (Kaway 2007)

Each TLRs can recognize several microorganisms by binding to specific family of molecule produced by these organisms

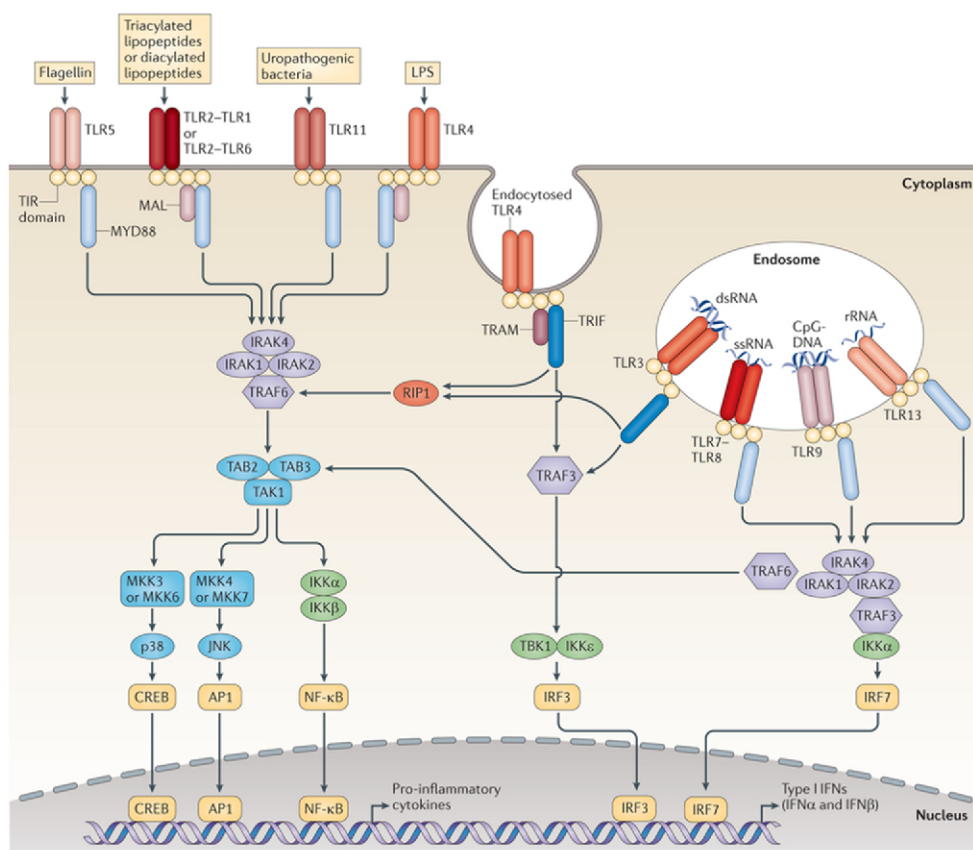


Figure 20 : TLR signaling activating NF-κB pathways (from O'Neill 2013).

Representation of main pathways implicated in response to TLR stimulation. TLR11 and TLR13 are only present in mice.

or on the basolateral membrane (Lee 2006). Moreover, in this same study, whereas TLR9 is present at both the apical and the basolateral membrane, only basolateral activation induced IL-8 secretion whereas apical stimulation decreased response to other TLR stimulation, indicating different roles of TLRs depending on their localization. TLR4 was implicated in response to barrier injury by enhancing cell proliferation (Zheng 2009, Fukata 2006). Activation with TLR2 increases ZO-1 activation in a PKC dependent and Myd88 independent pathway and it upregulates connexin43, therefore strengthening the tight junctions. (Cario 2004, Ey 2009). Expression of TLR3, TLR4, TLR5 and TLR9 in Paneth cells has also been described and has been implicated in degranulation and release of antimicrobial peptides (Rumio 2004, Rumio 2012). Interestingly, agonists of TLR3 and TLR9 induced a rapid degranulation, in less than 3 hours whereas agonists of TLR4 and TLR5 induced degranulation only after 12 hours, indicating potentially an indirect pathway.

In human colon, EC cells stained for 5-HT colocalized with high expression of TLR1, 2, 4 and 6, indicating that these TLRs are much more expressed in enterochromaffin cells than in other intestinal epithelial cells (Bogunovic 2007), and LPS induces 5-HT secretion (Kidd 2009). However, expression in other types of enteroendocrine cells was not assessed and it is therefore not known if EECs in general express TLRs. STC-1 a murine cell line modeling poorly differentiated enteroendocrine cells expressing CCK, GLP-1 and GIP expresses TLR1, 2, 4, 5, 5 and 9, but other TLRs were not tested (Bogunovic 2007, Palazzo 2007). Interestingly, stimulation of TLR4, 5 and 9 induces secretion of CCK, in a Myd88 dependent manner, indicating that enteroendocrine cells may sense microbes through TLRs and respond by increasing gut hormone release. Moreover, LPS induces a rapid weak cytosolic calcium concentration rise, a mechanism needed for induced secretion, but mechanisms implicated in this response remain unknown. Moreover, precise localization of TLRs on polarized EECs is not known, nor if this feature is shared among different types of enteroendocrine cells and between species.

Moreover, colonic enteroendocrine cells, and more particularly L-cells, are exposed to a higher quantity of bacterial compounds, and may therefore have different sensibility to bacterial content compared to proximal enteroendocrine cells. Expression of TLRs in L-cells is not known we hypothesized that it may participate in the regulation of PYY production and secretion. Moreover, L cells are also in contact with many over bacterial products including butyrate which induces different responses in these cells. Using NCI-h176 as a model of L-cells, we tested the functional expression of TLRs in non-treated cells as well as cells incubated with butyrate which may be a better representation of physiological conditions and assessed their stimulation on PYY expression and secretion.



# Model Development





NCI-h716 cells are derived from a caecal adenocarcinoma and have been described as expressing endocrine features when differentiated on extracellular matrix Matrigel (De Bruine 1993). Most studies using this cell line has been done using NCI-h716 coated on Matrigel (Reimer 2001, Jang 2007, Le Nevé 2011), but a few reports showed no differences between cells coated on matrigel or kept in suspension or coated on poly-lysine (Cao 2003, Anini 2003, Liu 2013). We therefore tested effect of matrigel on gene expression in NCI-h176 and could not find any difference concerning *Pyy*, *Glp-1*, differentiation factors or some receptors in cells coated on matrigel or kept in suspension. Treatment with butyrate induced similar responses in both conditions, except for MCT-1 and GPR93. The first did not seem to be upregulated after butyrate treatment in cells coated with matrigel, and effect concerning GPR93 down regulation was weaker in cells coated with matrigel (Figure 21). Moreover, NCI-h716 cells coated on Matrigel secreted similar amount of PYY to uncoated cells when tested twice ( $17.4 \pm 2.3$  pg/mL vs  $15.6 \pm 1.6$  pg/mL respectively). Thus, for practical reasons, we did not coat cells on Matrigel before experiments for the following studies. Interestingly, we could observe that in buffers or medium without serum, cells adhered weakly to plastics and were not washed away when solutions were added or removed carefully. Consequently, in our studies, cells were not coated on Matrigel or Poly-Lysine but just plated in serum free medium.

Our project was to do high throughput screening of bacterial products to assess regulation of *Pyy* expression and PYY secretion, thus, we had to develop models to report easily these activities by measuring an accessible readout like fluorescence or enzymatic activity. Indeed, ELISA or Luminex to assess secretion and RT-qPCR to assess gene expression cannot be used in a high throughput manner.

## Development of a reporter system for *Pyy* gene expression

Human *Pyy* gene structure is complex, and little is known about its promoter or regulatory elements modulating its expression. Indeed, genome annotation reveals that the gene encoding for PYY preproprotein (called preproPYY afterward) is composed of seven exons spanning on more than 50,000 bases and maturated mRNA is 1099 base long (gene id: 5697, NM\_004160.4). This is rather unexpected to encode just a small peptide hormone. Rapid analyses of *Pyy* encoding gene reveals that this important size is common to hominidae and many other primates have a smaller gene, but which is still much longer than rodent one. Interestingly, final structure of the last four exons is conserved in mammals and preproPYY is encoded in the last three exons. Proximal promotor of a gene is often considered to be the 500 to 2000 pair base upstream the first exon. This sequence often includes only limited but essentials sequences implicated in the regulation of the gene. However, many other sequences can be present in the introns and in more distal regions and participate in the regulation of the gene expression. Considering *Pyy* gene, study of the proximal sequence before the first exon may not be sufficient to analyze regulation of its expression. Indeed, this large structure indicates potential regulatory regions in the introns.

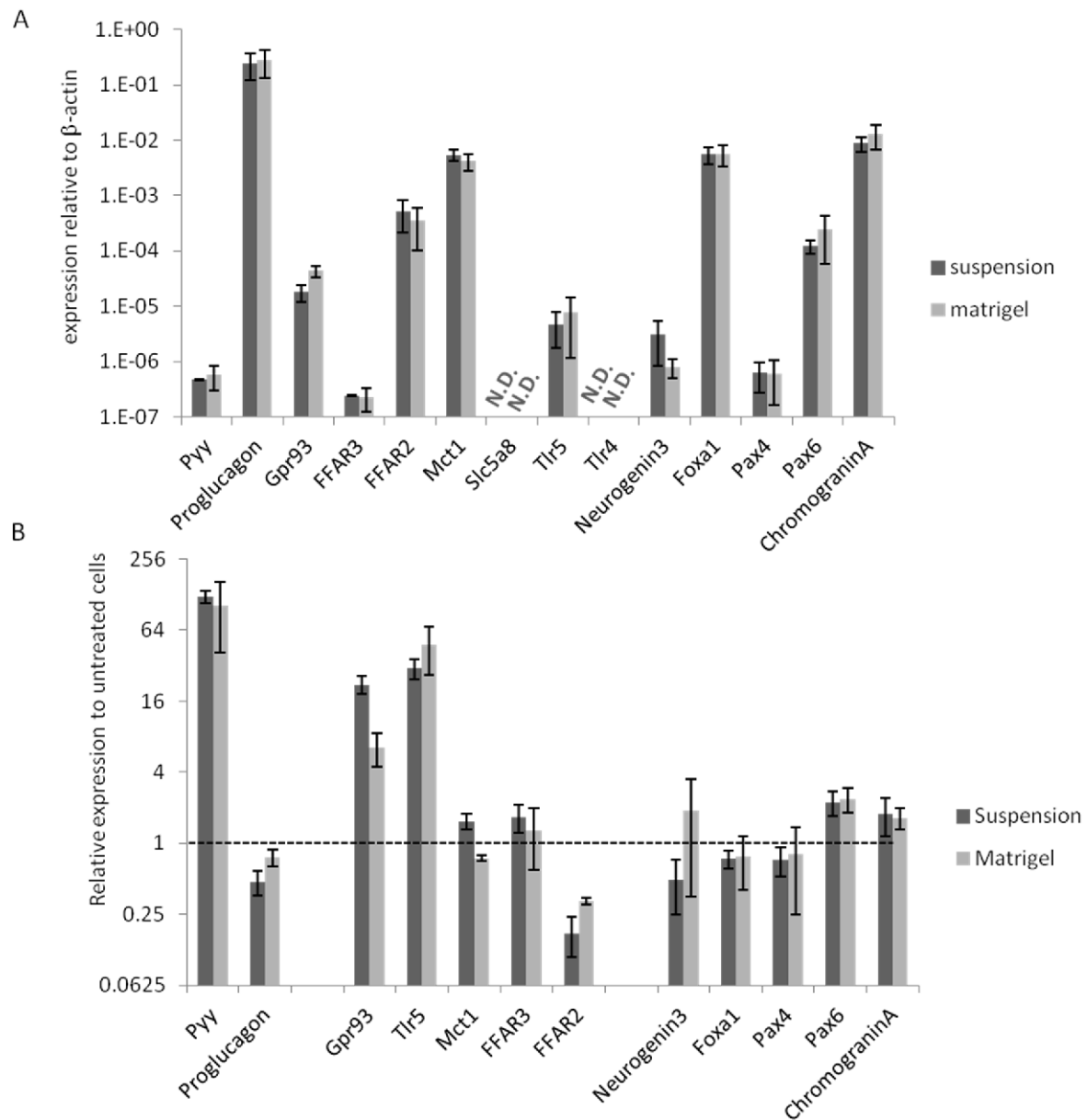


Figure 21 : Matrigel does not affect gene expression in NCI-h716 cells

A: Expression of L-cells markers, SCFA transporters and receptors and specific enteroendocrine differentiation factors in NCI-h716 in suspension (dark grey) or coated on matrigel (light grey) were detected by RT-qPCR. Data are expression relative to beta-actin determined by the  $2^{-\Delta Ct}$  method, on 3 distinct experiments performed in duplicate, represented as means  $\pm$  s.e.m. N.D. : Not Detected

B: Effect of butyrate on gene expression in NCI-h716 in suspension (dark grey) or coated on matrigel (light grey), measured by RT-qPCR. Data are relative expression to untreated cells, determined by the  $2^{-\Delta Ct}$  method, on 3 distinct experiments performed in duplicate, represented as means  $\pm$  s.e.m.

## Classical reporter systems

The reporter system used to measure gene expression is often composed of the promoter region of the gene of interest and a reporter gene which has a detectable activity when expressed allowing easy quantification of its expression. Reporter gene can be an enzyme which activity on a substrate as an easy readout, including luminescence or light absorption (luciferase, secreted alkaline phosphatase), or directly fluorescent protein. However, construction of such a reporter system necessitates to know the expected promoter region of the gene to clone it and integrate it before the reporter gene to regulate its expression similarly to the gene of interest. This system is quite simple and effective for many genes when regulation is not dependent on distal promoters. This system is integrated in the genome to be expressed stably by cell lines because a homogeneous model is required to compare effect of different molecules/conditions on expression of the gene of interest. However one major drawback is that integration in the genome is random and can therefore interact with other regulatory sequences in the genome. To avoid these drawback and have access to whole sequences implicated in regulation of *Pyy* gene, as we could not be sure of the size of the proximal promoter nor the relative importance of other regulatory regions, we wanted to take advantage of recently developed tools to integrate precisely a reporter gene in the sequence of our gene of interest by homologous recombination after DNA double strand break.

## DNA repair to induce mutations or integrate a specific sequence

Rapidly, after a double strand break, DNA can be repaired by two different pathways: non homologous end joining (NHEJ) which consist of DNA repair by itself but is error prone as insertions or deletions can happen during this process, or homologous recombination (HR) which uses a similar (homologous) template of DNA for repairing (*Figure 22*). Both pathways implicate complex molecular cascades to achieve DNA repair, and first steps are exclusive, meaning that only one type of repair can be done for one DNA break. NHEJ requires recognition of DNA break ends and their stabilization by a heterodimer, Ku, which recruits elements of the NHEJ complex (Lieber 2010, Davis 2013) before ligation. However, NHEJ is considered as error prone as it requires to remove some DNA overhangs or integrate DNA elements during the ligation of the two strands and is often used to introduce random mutations in a gene to delete its function (knock-out). The other mechanism necessitates a donor template which can be ever sister chromosome or any other sequence that has important homology to the sequence upstream and downstream of the double strand break (Elliott 1998, Li 2008). In that case, one strand of the donor template is replicated to replace the deleted part in proximity of the double strand break. Therefore, if the donor template has a different sequence close to the break, the different sequence is copied. This mechanisms can therefore be used to insert or delete specifically a mutation in a gene, or even insert a larger fragment at one precise place, including a reporter gene at a precise place if one can induce double strand break at a precise localization.

## DNA double strand break

Two methods have been recently developed to create DNA double strand break at specific locus, based on the same principle: Talens, for transcription activator like effector nucleases and Crispr/Cas9 for Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR Associate Protein 9. Both of them are composed of a domain recognizing specific sequence and a nuclease that induce double strand break alone (Cas9), or in dimer (FokI for the Talen), similarly to the older technology based on zing finger nucleases. The main difference between the two technologies is that a Tale is a

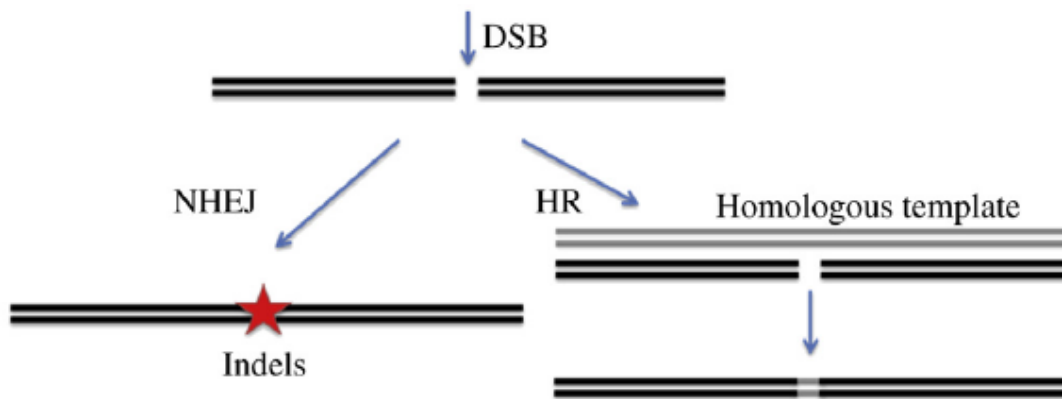


Figure 22 : Mechanisms of DNA repair after a double strand break

Representation of 2 different main pathways for double strand break repair (DSB) in mammalian cells: the error prone non homologous end joining (NHEJ), resulting in insertion or deletion of some nucleotides and the homologous recombination (HR) using a donor template for reparation (Adapted from Wei 2013)

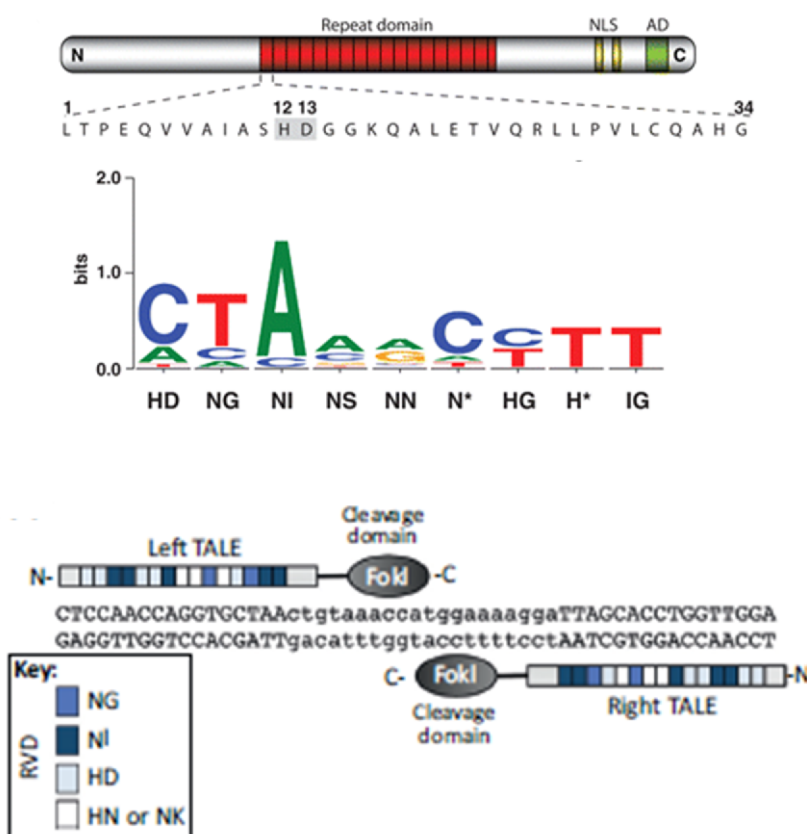


Figure 23 : TALENs induce DNA double strand break at a precise locus

A: representation of a TALE including a central repeat domain and the code corresponding to the amino acids in 12th and 13th position recognizing specific nucleotide (from Boch 2009)

B: Two TALEs associated with FokI nuclease recognizing two DNA sequences separated by a 20 base spacer to induce a double strand break at this locus (From Gaj 2013)

protein including a central domain composed of 33 amino acid long repeats with two amino acids variable in position 12 and 13 and that constitutes the DNA binding domain. Each repeat recognizes a nucleotide with a code that was deciphered recently, and succession of repeats allow binding to specific sequences. Thus, construction of a Talen that recognizes specific sequence by assembling repeats corresponding to the desired sequence is possible (*Figure 23*) (Boch 2009, Cermak 2011, Chen 2013). Activity of the nuclease FokI requires formation of a dimer, therefore a pair a Talen recognizing about 18 base long sequences separated by about 15 bases are required. Crispr is based on DNA recognition by a guide RNA and a transactivating RNA that recruits a Cas9 nuclease. Guide RNA corresponds to the targeted sequence and is followed by a two nucleotides PAM sequence that is dependent on species of origin (*Figure 24*). Crispr Cas9 systems are found in most bacteria and are supposed to represent an adaptive immune system by integrating foreign DNA sequences on a first time and is able to cleave them when found again (Mojica 2000). Crispr/Cas9 is thus easier to construct to target specific sequence as it relies on RNA/DNA construction and not protein construction. Both seems to have similar efficiency, but Crispr/Cas9 is supposed to have more off-targets (Ran 2013, Le Cong 2013).

### Strategy for a fluorescent reporter gene integrated in *Pyy* coding gene

As the Crispr technology was not completely developed when we started this project, and some questions about off-targets remained, we decided to use the Talen technology to introduce a fluorescent protein at the PYY locus in NCI-h716, the cell line best modeling human L-cells. Two Talen pairs designed to induce a double break in the 5' coding part of prePYY were purchased from Cellectis and efficiency was tested by mutation induction after NHEJ. A first strategy we developed was to tag the prePYY by adding a mcherryFP sequence at the end of the supposed signal peptide part and just before the start of the peptidic hormone sequence. Homology matrix was constructed by fusing about 1000 pb upstream and 1000pb downstream the target site with the mcherryFP sequence removing the mcherryFP stop codon, and by including it in a plasmid pCMV-eGFP to target transfected cells or integrated plasmids (*Figure 25*).

After transfection with donor plasmid and plasmids coding for each Talen, we sorted twice by flow cytometry cells transfected cells, using the different fluorescent proteins encoded in the donor plasmid. A first sorting was to select transfected cells *i.e.* cells with a high eGFP fluorescence as transfection efficiency in these cells is about 15-20% using Amaxa. Therefore, we could select only cells that had been transfected. After cell culture for two to four weeks, cells were sorted again to get cells that had integrated the mcherryFP in their genome at the targeted site. These cells should have had a higher fluorescence corresponding to expression of mcherryFP but no fluorescence corresponding to eGFP (*Figure 26*). Indeed, cells that would have integrated the mcherryFP could have done by two ways: integration of the sequence due to homologous recombination just after double strand break down at the targeted locus (the cells we were looking for), and random integration of the whole plasmid. In the latter possibility, as the whole plasmid would have been integrated, including the eGFP sequence, these cells would have also express eGFP whereas the other would not have expressed eGFP. Rate of sorting was very poor and cell survival low after these sorts. However, tested population by microscopy had no higher fluorescence corresponding to mcherryFP than untransfected cells but some had a strong fluorescence corresponding to eGFP, indicating that these cells may have integrated the eGFP sequence also despite the sorting parameters.

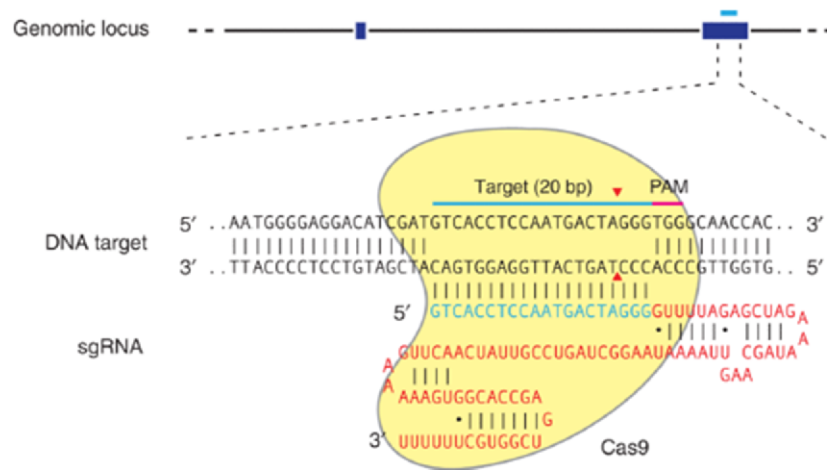
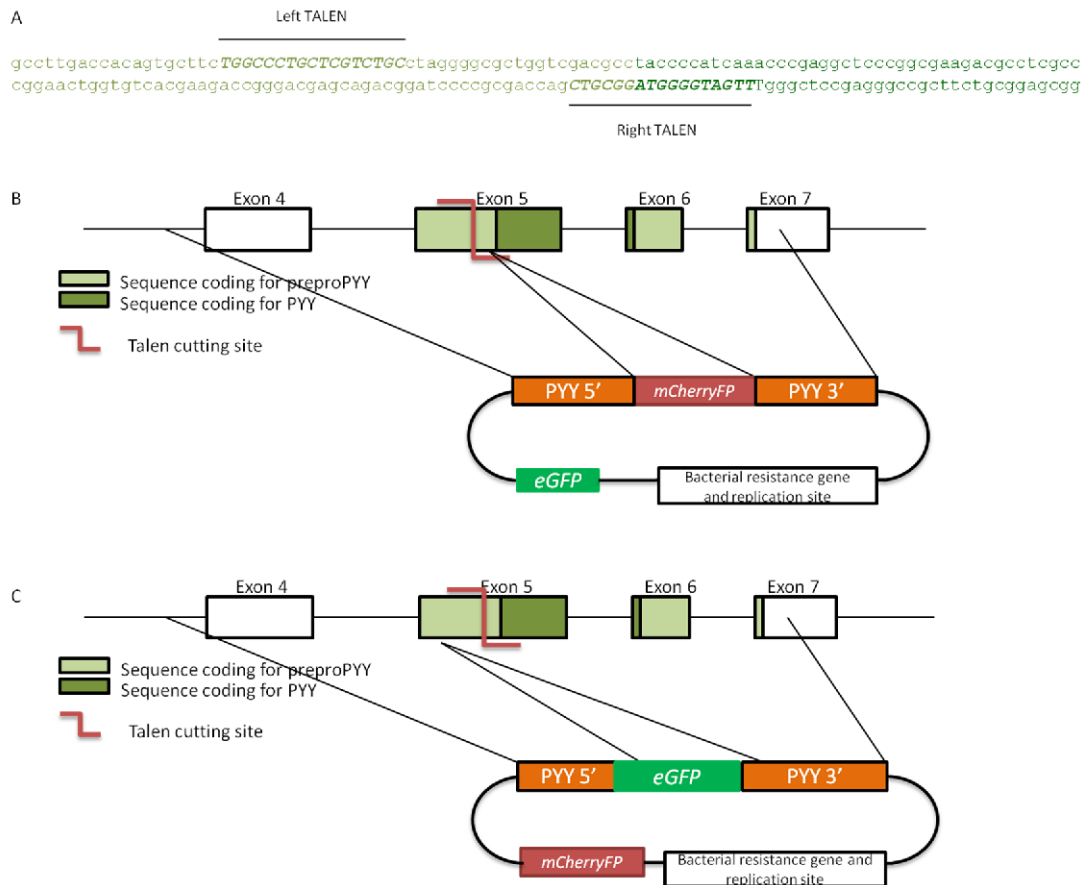


Figure 24: CRISPR/ Cas9 system induces DNA double strand breaks at specific locus  
 Structure of a Crispr/Cas9 complex including a guide RNA (blue) recognizing a target sequence adjacent to a PAM motif (pink), a transactivating RNA (red) and the Cas9 nuclease recruited to induce double strand break. From Ran 2013



**Figure 25 : Strategies to integrate a reporter gene in Pyy gene**

**A** : Sequence targeted by two TALENs to induce a double strand break close to the targeted integration site. Sequences recognized by TALEN are in UPPERCASE. Green: sequence coding for PreproPYY. Dark green: sequence coding for mature PYY.

**B** : donor plasmid with homology sequences upstream and downstream to integrate mcherryFP coding sequence at the end of the signal peptide of PreproPYY

**C** : donor plasmid with homology sequences upstream and downstream to integrate an eGFP coding sequence just after the ATG of prepro PYY.

Green: PreproPYY, dark green: PYY, white non coding Pyy mRNA, orange: homology sequences in the donor plasmid, bright green: sequence coding for eGFP, red: sequence coding for mcherryFP.



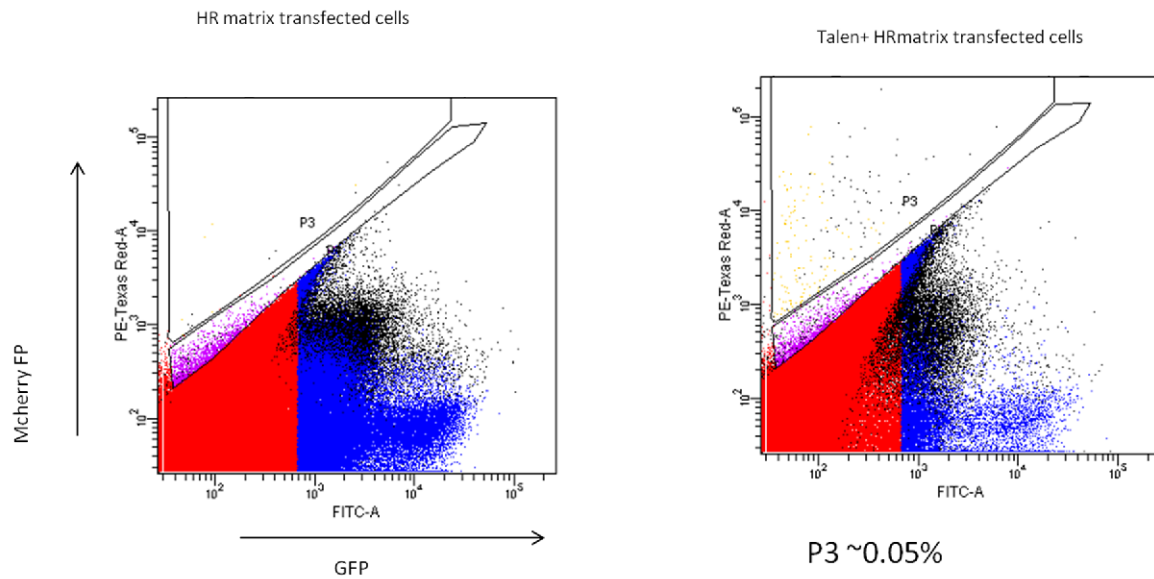


Figure 26: Cells sorted after homologous recombination to introduce a mcherryFP sequence in Pyy locus  
Cells are analyzed depending on their fluorescence and cells having a high intensity of fluorescence corresponding to mcherryFP spectra, but less fluorescence corresponding to eGFP spectra were sorted (P3, yellow dots). Left : control cells transfected only with the donor plasmid. Right cells transfected with TALEN and donor plasmid to induce homologous recombination

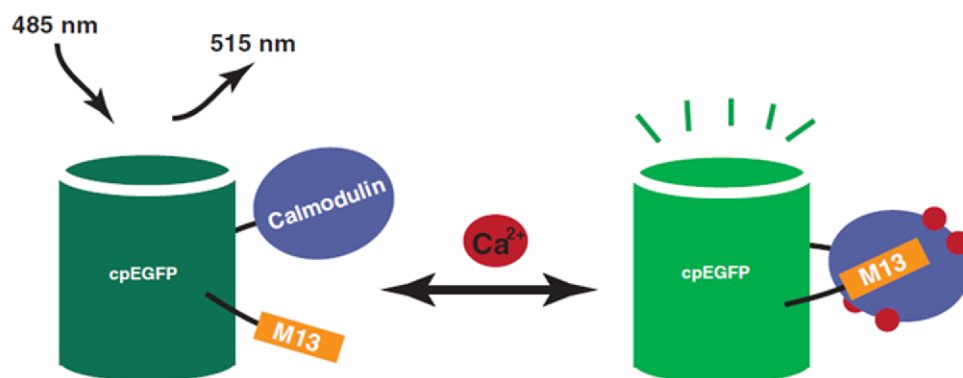


Figure 27: Schematic representation of GCaMP protein  
GCaMP structure has low fluorescence in absence of calcium. Calcium bound to calmodulin induces a conformational change increasing fluorescence of the protein. (Adapted from Tian 2012)

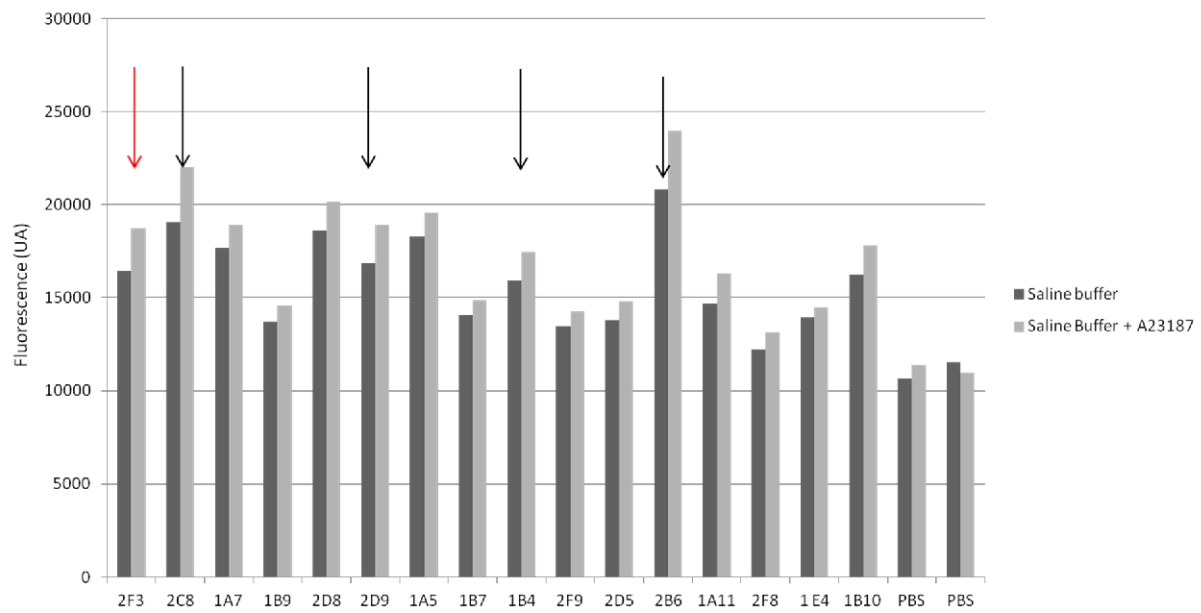
Thus, we supposed that our strategy may not be optimal for fluorescence detection as *Pyy* expression is very low in NCI-h176 cells and mcherryFP is not as bright as eGFP. Moreover, as mcherryFP is much larger than prePYY, potential misfolding or disruption of sorting into secretory granules can happen when mcherryFP is directly integrated in the gene as we did, leading to rapid degradation of the newly synthesized protein. Thus, newly synthesized mcherryFP tagging the prePYY would be misfolded or rapidly degraded, resulting in no functional mcherryFP produced. We therefore decided to use as reporter system an eGFP rather than a mcherryFP and to integrate the sequence at the starting codon of the prePYY and had stop codons just after the eGFP sequence. In this model that is still under construction, eGFP alone would be expressed instead of preproPYY, but its expression would be dependent on all regulations of *Pyy* gene (*Figure 25*).

## Development of reporter systems for secretion

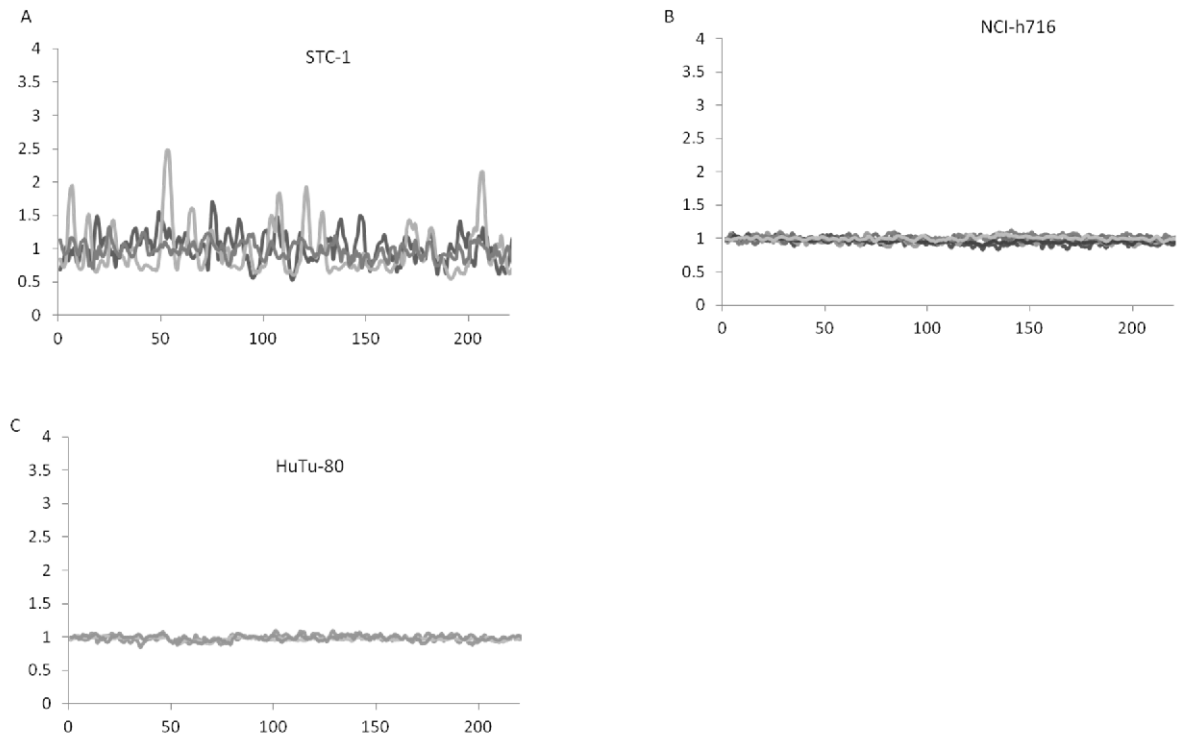
### Calcium sensors

The second model we wanted to develop was to screen for bacterial products responsible for PYY secretion. One possible readout is by measuring calcium fluxes in the cell as a rise of cytosolic calcium concentration is necessary for secretory granule fusion and secretion. Calcium concentration can be assessed by different molecular probes, the most common one being Fura probes (Tsien 1985, Paredes 2008). Recently, genetically encoded calcium sensors have been developed, allowing direct measurement of calcium flux without loading belonging to two main families: the cameleon proteins and the GCaMP. The cameleon protein are composed of two fluorescent proteins of different excitation and emission spectra, most often a CFP and an YFP bound together by a M13 domain and calmodulin a small protein that can bind four calcium cations. Förster Resonance Energy Transfer (FRET) is possible between the two fluorochromes when calmodulin is bound to calcium. Measurement of FRET is therefore a direct indicator of calcium concentration (Miyawaki 1997). The other family is based on similar process, but using a single GFP molecule that have been modified by circular permutation: GFP structure being mainly a barrel of  $\beta$ -strands surrounding the chromophore, fusion of its N and C-terminal parts was possible to introduce in the barrel a calmodulin and M13. This structure has very low fluorescence when not bound to calcium, but conformational changes after binding of calcium to calmodulin induces a conformational change of the protein and consequently a strong increase of fluorescence of the protein (Nakai 2001). This molecule has been improved for better sensitivity to calcium fluxes (Akerboom 2012) (*Figure 27*). We chose to use a GCaMP protein to assess calcium fluxes in NCI-h716 cells as it is based on only one fluorochrome and has properties corresponding to our objectives such as little quenching, high sensitivity, high response/noise ratio, and selected the GCaMP-5G protein for our studies.

We therefore established NCI-h176 cells stably expressing GCaMP-5G and a resistance gene to G418 and could test different clones responding to calcium. Cells were first tested using a calcium ionophore, A23187 permitting entry of calcium from the outside as calcium concentration was fixed to 2mM in a saline buffer with glucose, which is much higher than cytosolic calcium concentration. Increased of fluorescence in these cells was therefore expected. Selection of clones was done using a fluorimeter to test fluorescence variation after addition of ionophore. Control was made in cells in calcium free buffer to control that fluorescence increased was not dependent on drug addition but calcium concentration increase (*Figure 28*). We selected several clones and confirmed their activity by live imaging, enabling better sensitivity by measure fluorescence per cell. Similar models were developed in human HuTu-80 cells and mouse STC-1 cells.



**Figure 28 : Selection of clonal populations of NCI-h716 stably expressing GCaMP-5G**  
Mean fluorescence of clonal population transfected with pCMV GCaMP-5G and selected for G418 resistance was measured in PBS + 2mM  $\text{Ca}^{2+}$  before (dark grey) and after (light grey) ionophore A23187 addition 96 well plates with a fluorimeter (Infinite 2000, Tecan). Arrows indicate population kept for further analysis and red arrow indicates clone based on reliability, signal/noise ratio and further comparison using calcium imaging



**Figure 29 : cell lines have different basal calcium concentration variations**  
Representative curves of relative fluorescence of 5 STC-1 (A), NCI-h716 (B) or HuTu-80 (C) cells stably expressing GCaMP-5G during 4 minutes without any stimulation acquired 1img/s

## Live imaging for calcium imaging

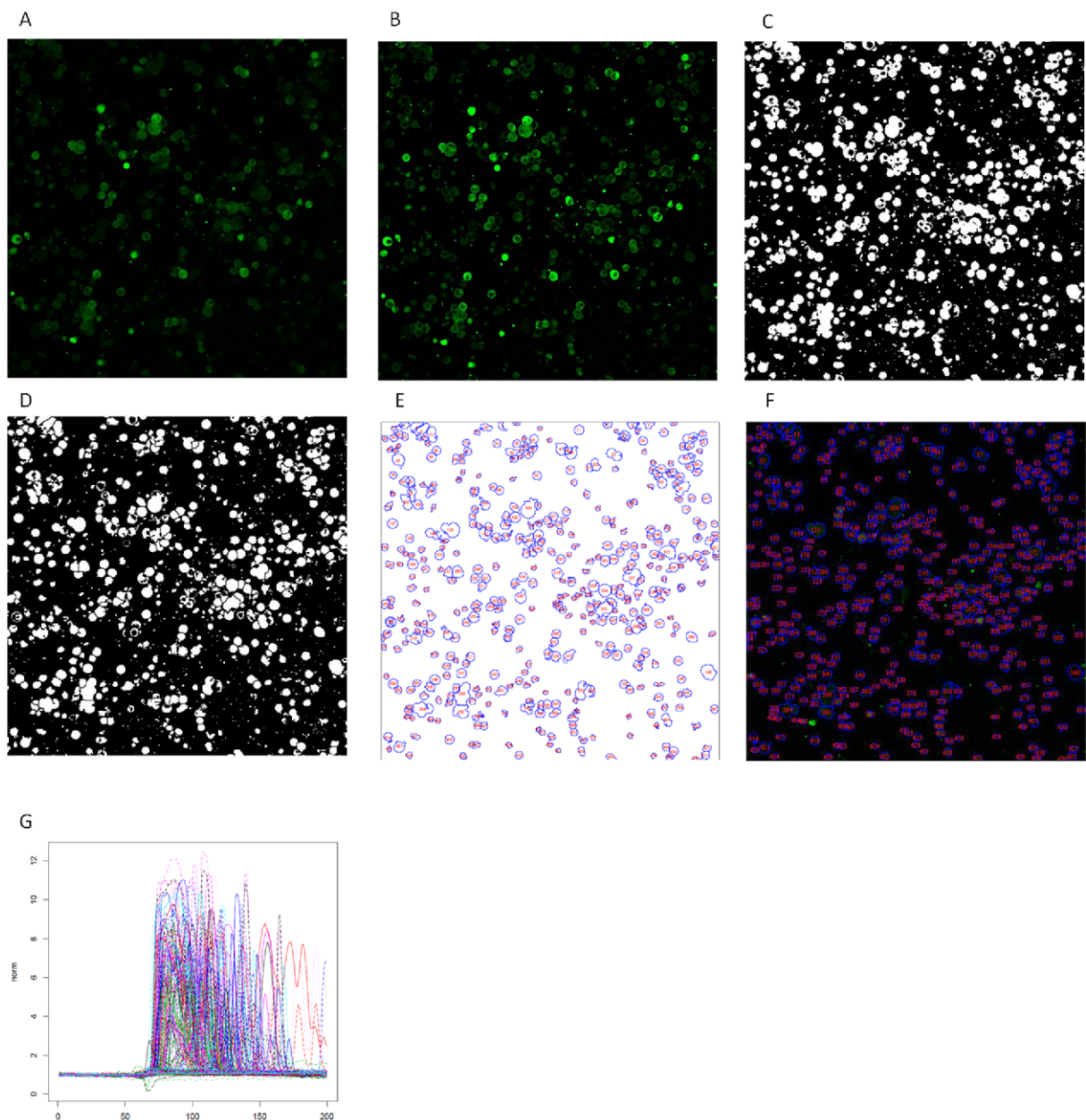
NCI-h716 cells were placed in µclear 96-well plates in 50 or 100µL of saline glucose buffer 2 hours before experiment. This allowed sufficient adhesion of cells for further experiments. Other cell lines were coated the day before experiment for adhesion as they are adherent cells. Using a Zeiss LSM700 confocal laser scanning microscope (MIMA2 platform, INRA), using 20x/0.8 objective (Zeiss 420650-9901) to get a field acquiring between 30 and 100 cells depending on the cell type. This allowed us to follow a sufficient number of cell per test to have more robust results in a single acquisition. Fluorescence was measured during 4 to 5 minutes with one image acquired per second, and no bleaching of fluorescence could be observed. These parameters allowed to capture brief calcium modifications with an acquiring time (~0.7s) sufficient to get a good signal to noise ratio. Of note, whereas NCI-h716 cells and HuTu-80 cells had no modification of  $[Ca^{2+}]_i$  over time when not stimulated, STC-1 had transient intermittent  $[Ca^{2+}]_i$  increase which may reflect induced secretion occurring naturally in these cells (Figure 29) .

Protocol for calcium imaging was developed to ensure robust and reproducible results and avoid side effects. During acquisition, drug was added approximately 50 seconds after start of acquisition. This period was used to establish basal fluorescence level. Drug was delivered in a 2x solution by pipetting with care as mechanical stress due to pipetting induced a short but strong calcium response, since some mechanosensors can modulate calcium signaling in cells and some cells can detach. Indeed, mechanical pipetting of medium alone could induce a calcium response in these cells. The tested drug was added at low concentration to ensure rapid diffusion to the cells and easier pipetting.

Drugs known to induce calcium responses were used to characterize our models and were used to develop image analysis. Calcium ionophore A-23187 was used to calibrate acquisition settings to get the range of signal and avoid saturation with high  $[Ca^{2+}]_i$  but get sufficient signal with close to null calcium concentrations. PMA which activates PKC pathway induced a strong response whereas forskolin stimulated only transiently cells. ATP on these cells did not induce any response.

## Image analysis

Image analysis was performed using Fiji software (Schindelin 2012), and scripts were developed for automatic analysis. Analysis consisted of measuring fluorescence level in each cell at each time point to follow fluorescence variations after drug addition, reflecting  $[Ca^{2+}]_i$  variations. The script developed and used for further analysis is presented in annex 2 with annotations. Figure 30 represents the images obtained after main steps described thereafter. Acquisition corresponded to two or three stacks of images corresponding to different channels acquired, *i.e.* transmitted light, channel to detect GFP fluorescence (corresponding at the channel for excitation and emission of GCaMP) and if necessary channel to detect mCherryFP fluorescence to determine transfected cells in some experiments. An image corresponds to a matrix of values corresponding to pixel intensities representing fluorescence level. First steps consisted of recognition of single cells, *i.e.* determination of pixels belonging to a same cell and distinguishing them from pixels corresponding to the outside of the cell. This allowed us to create masks for single cells. Then, the mask was used to measure average pixel values of a cell in every single image of the GFP stack. Fluorescence was first averaged over time to reduce noise and contrast was increased by applying an unsharpening mask, a transformation that reduces pixel value when its neighbors have greater pixel values and increase pixel value otherwise. Then, a threshold was applied, consisting of separating the pixels in two populations to get a binary image: pixels with a value inferior to the local threshold are considered as negatives (0) and pixels that have a value superior to the threshold are the positive ones(1). Positive pixels correspond to



*Figure 30 : Steps of image analysis developed to measure automatically fluorescence intensity per cell upon time. Images are the intermediary results of successive steps from analysis of an acquisition (corresponding to addition of Epi300 supernatant)*

*A : Stack is reduced to a single image by averaging of for each pixel intensity over time*

*B: Contrast is increased by application of an unsharp mask filter*

*C: Image is binarized by application of a threshold*

*D: Application of a watershed algorithm to discriminate partially adjacent cells*

*E: Automatic detection of intensity borders defining particles when shape corresponds to size and circularity criteria*

*F: Particles are used as masks on initial stack of image to measure mean fluorescence for each particle on each image, determining, intensity fluorescence per cell over time*

*G: Representation of normalized fluorescence to basal fluorescence for each cell recognized in the acquisition.*

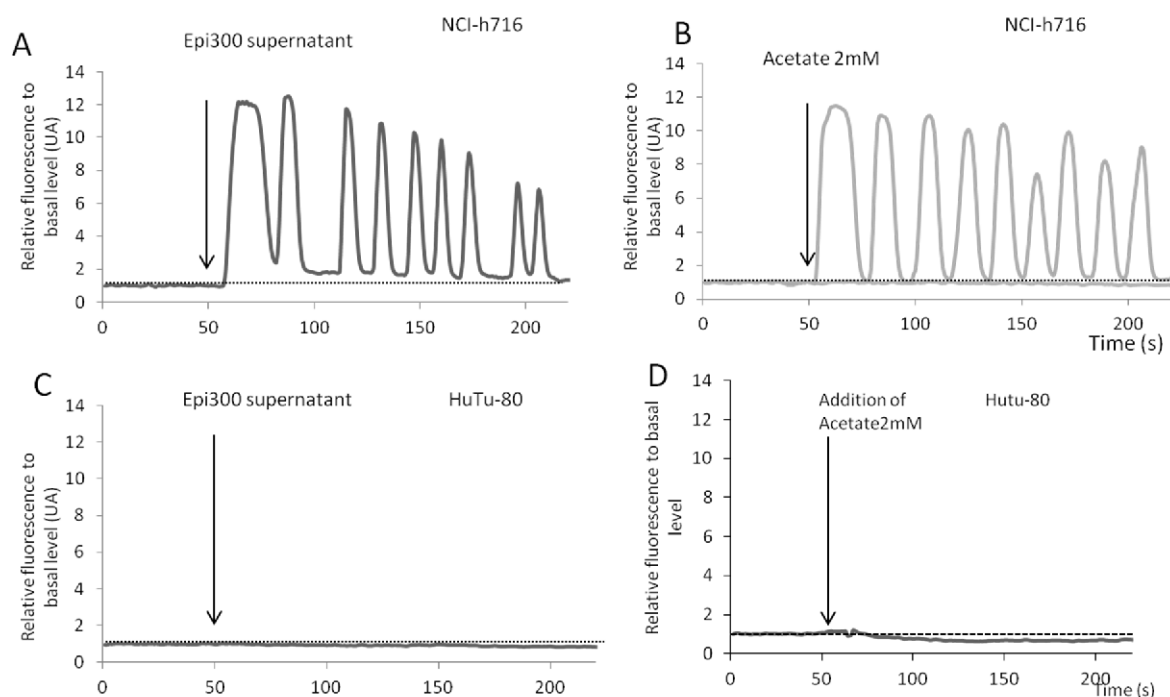
pixels with a higher intensity than the threshold, so they belong to a fluorescent structure whereas the other belongs to the background. If cells are sparse enough, grouped positive pixels will belong to a single cell. Otherwise, a watershed algorithm can be used to delimit different structures based on intensity but it remains imperfect when cells are too dense and superposing cells cannot be deciphered. Thus, acquiring conditions were important to facilitate automatic cell detection. Finally, a last algorithm was used to detect positive groups of pixels surrounded by negative pixels creating a particle for each structure. Size and circularity of particles can be chosen for the automatic detection to include only particles that are the size and the shape of cells. Figure represents images after different steps of analysis

Fluorescence intensity per cell was then measured using the particles as masks for each time point, and fluorescence intensity per cell over time was then recorded for further analysis using R software. Analysis consisted of creating normalized curves per cell to get a visual analyze of the responses, and a curve analysis by determining maxima, minima, means and oscillations characteristics. Script developed for this analysis is presented in annex 3 commented in French. As basal fluorescence was not similar between cells due to different cell size and GCaMP expression level, normalization was required to compare cells together. To do that, we considered that basal  $[Ca^{2+}]_i$  was similar between cells and fluorescence intensity was linear with  $[Ca^{2+}]_i$ . Basal fluorescence corresponded to average fluorescence intensity during the forty first seconds. Moreover, to smooth the curves to limit noise effects, each time point was averaged with half weight with the two proximal points and then normalized to basal fluorescence, giving the normalized fluorescence intensity in single cells which was graphically presented and most often used as a result. However, to get more information to compare different responses and have quantified measures, curve analysis was performed. Maxima, minima and mean of normalized fluorescence were measured per cell, as well as characterization of oscillations. We defined local maxima a  $t$  point which intensity is maximum on  $[t-4s ; t+4s]$  and is at least 110% of mean intensity during this period. This was done to avoid relative maxima corresponding to noise as we tested that before drug addition, values did not vary more than by 10%. Peak duration was calculated as time while intensity was superior to local maximal intensity minus one (corresponding to the background) divided by 2. We considered the response was oscillatory when three separated maxima could be found. For oscillatory responses, periodicity was measured by measuring mean time interval between two maxima (period) and standard deviation of these intervals. Data for each cell of an experiment was recorder. Moreover, for each experiment, means and deviations were measured for each parameter described before, as well as the proportion of responding cells, *i.e.* the proportion of cells that have mean higher than 1.1 and the proportion of oscillating cells. These values were exported and could be compared between experiments.

These measures were reproducible from one experiment to another, and thus validated our model for further experiments (

Table 2). Our aim was to use a functional metagenomics approach to screen for bacterial genes that may regulate PYY secretion using calcium signaling as a reporter system. We therefore tested the control used in this approach, supernatant of *E.coli* Epi300 containing an empty fosmid. Addition of *E.coli* supernatant on NCI-h716 GCaMP cells induced a strong oscillatory response whereas addition of growth medium alone (LB + chloramphenicol) had no effect. We tested different *E.coli* metagenomic clones that were positive for different activities characterized on NF- $\kappa$ B reporter system to test for potential different calcium activation compared to the *E.coli* control tested. No difference could be found between different clones (Figure 31). We tested different bacterial components that could have effect like LPS or Flagellin, and as presented in the second paper, none of these molecules had an effect on calcium fluxes. However, comparison between response to stimulation by *E.coli* supernatant or by

acetate revealed that both responses were quite similar regarding response amplitude and oscillation periods, Moreover, on HuTu-80 cells that do not



**Figure 31: Cells respond similarly to Epi300 supernatant and acetate**

Calcium response of NCI-h176 (A, B) or HuTu-80 (C, D) GCaMP-5G cells to addition of Epi300 supernatant (A, C) or Acetate 2mM (B, D) Curve represents relative fluorecence to mean fluorecence before drug addition in one representative cell of 50-100 cells acquired per experiment, repeated at least three times. Drug addition is indicated by a vertical arrow

element tested	Epi300 control supernatant 1/10			Epi300 with activities on NF-kB 1/10			Acetate 2mM			saline solution	
replicate	1	2	3	1	2	3	1	2	3	1	2
min	0.97	0.90	0.95	0.94	0.91	0.96	0.85	0.83	0.85	0.92	0.94
max	10.3	10.2	9.5	11.1	11.3	10.4	11.1	10.3	11.5	1.1	1.1
mean	2.8	2.9	3.1	3.0	2.7	2.6	3.1	2.9	3.5	1.0	1.0
oscillating cells (%)	23.0	0.3	33.0	34.0	26.0	41.0	56.0	60.0	60.0	0.0	0.0
period	16.9	16.3	18.5	16.1	17.9	15.8	17.0	17.8	15.6	0.0	0.0

**Table 2: Metagenomic clones and have similar effect to acetate**

Data from separate acquisition where analyzed measuring main parameters per cell, and data is the mean on responding cells in one acquisition, three seprate acquisitions per condition are presented to show the relative steadiness of results.



respond to acetate, *E.coli* seemed to produce no effect (n=1). As *E.coli* produces acetate even in aerobic culture when carbon is in excess, we hypothesized that response induced by *E.coli* was acetate dependent. This strong effect of *E.coli* by itself on calcium signaling limited potential use of this model to screen for new responses, as they might be concealed by the response to acetate.

As we could show that calcium response to acetate is dependent on FFAR2, a model depleted for expression of FFAR2 has been considered. Two approaches are developed in the laboratory, one using shRNA to decrease expression and the other using Crispr/Cas9 to induce insertions/deletions by NHEJ in the FFAR2 locus. Depletion of FFAR2 in these cells will help us to determine the precise function of this receptor and confirm the data presented. Moreover, FFAR2 being potentially implicated in the calcium response of NCI-h716 to the control metagenomic clone Epi300 that are producers of acetate, cells depleted for its expression may no longer respond to it, and may therefore be further used for functional genomic screening.

## Material and methods:

Cell Culture, RNA extraction and calcium imaging techniques were the same as reported as in the next papers.

### *Cell coating, cell sorting and transfection*

For Matrigel coating assays, 50 $\mu$ L of 4°C Matrigel matrix (BD biosciences 354234) were deposited in 24 well plates to cover the whole surface and incubated for 2 hours at 37°C for coating. Coating solution was rinsed twice with sterile PBS before 5x10<sup>5</sup> NCI-h716 cells were seeded in these plates and allowed to differentiate for 48 hours before cells were used for tests. For RNA extraction, lysis buffer was directly added on PBS-rinsed coated cells.

Cells were sorted using a FACS Aria III (BD biosciences) at Metagenopolis (INRA, Jouy-en-Josas, France) platform. Cells were kept in a PBS EDTA 0.5mM solution during sorting, and parameters of sorting were defined depending on fluorescent levels of control cells. Cells were either sorted at one cell/ well in 96 well plates or positive cells pooled in a single tube, that was then centrifugated and cells were put in culture in fresh medium. For single sorted cells, medium from cultured cells was added to enhance their growth.

NCI-h716 cells were transfected by electroporation using an Amaxa Nucleofector (Lonza). Transfection was done using 2x10<sup>6</sup> cells with 2 $\mu$ g plasmid DNA in 100 $\mu$ L Tyrode solution (137mM NaCl, 2.7mM KCl, 1.05mM MgCl<sub>2</sub>, 1.8mM CaCl<sub>2</sub>, 0.4mM NaH<sub>2</sub>PO<sub>4</sub>, 11.9mM NaHCO<sub>3</sub>, 5.6mM glucose, pH adjusted at 7.3. Transfection efficiency was between 20% and 30%. For multiple transfections to introduce the two TALEN coding plasmids and the homology matrix containing plasmid, plasmid DNA quantities were 1.2 $\mu$ g for two TALEN plasmids and 1.6  $\mu$ g for homology matrix plasmid.

### *Plasmid construction*

Genomic region around cutting site was amplified from genomic DNA by PCR and mcherryFP coding sequence was amplified from pCMV mcherryFP-N1 plasmid, a kind gift from A. Echard (Institut Pasteur, Paris, France) and Sall cutting site was added on both ends of mcherryFP plasmid as a Sall cutting site is present in the *Pyy* gene sequence at the site where mcherryFP coding sequence was decided to be integrated. PCR products were digested by Sall (Biolabs) for 1 hour at 37°C and ligated using a T4 ligase (Promega) for 4 hours at room temperature. After 1% agarose gel migration, product of ligation at the expected size (~2600 pb) was extracted (Qia Quick gel extraction kit, Qiagen) and integrated in a pGemT-easy vector (Promega) by ligation. After transformation in DH10B electro-competent cells by electroporation, clones were selected for integration of the PYY-mcherryFP construct with integration of the mcherryFP coding sequence in the same frame as PYY sequence and checked by sequence analysis. This construction was then integrated in a second vector coding also for an eGFP under a strong promoter (pCMV eGFP N1).





# Results



# Short Chain Fatty Acids differently regulate Peptide YY expression and secretion in human enteroendocrine L-cells

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**Keywords :** L-cells, Human gut, SCFA, PYY, GLP-1

*Abbreviations used in this paper:* 1-MCPC: 1-methylcyclopropane carboxylate; 2APB: 2-aminoethyl diphenylborinate; cytosolic calcium concentration: [Ca<sup>2+</sup>]; EEC: enteroendocrine cells; FFAR: free fatty acid receptor; GLP-1 glucagon-like peptide-1; GPCR : G-protein coupled receptor; K/HDAC: Lysine/Histone Deacetylase; SCFAs: short chain fatty acids; PYY: peptide YY; TSA: Trichostatin A

## Abstract

*Background and aim:* Short chain fatty acids (SCFAs) produced during fermentation of fibers by gut microbiota have many beneficial effects, and have been reported to increase plasma levels of Peptide YY. However, the precise mechanisms regulating this effect is unknown. We investigated the precise effects of SCFAs on PYY expression and secretion in human L-cells.

*Methodology:* Taking advantage of NCI-h716 and HuTu-80 cell lines as models of human enteroendocrine L-cells, we assessed the effect of the main colonic SCFAs (acetate, propionate and butyrate) on Pyy expression, calcium fluxes and PYY secretion.

*Results:* SCFAs increased the expression of Pyy differently, butyrate and propionate being the most potent (with respective folds of 120 and 40). Increased expression was induced by stimulation of SCFAs receptors FFAR2 and FFAR3 and more importantly by a mechanism involving inhibition of Lysine/Histone deacetylases. SCFAs also induced calcium signaling that were associated with secretory responses. Moreover, butyrate decreased regulated secretion by decreasing cytosolic calcium concentration through activation of FFAR2, FFAR3 and GPR109a as well as by decreasing expression of *Ffar2*. FFAR2 was the receptor implicated in the secretory response to SCFAs by activating Gαq/PLC/IP3 pathway. Altogether, butyrate strongly increased PYY production but decreased induced secretion whereas acetate had mainly a short term secretory response.

*Conclusions:* SCFAs are important regulators of human L-cells by modulating gut hormone production and secretion and participate in the regulation of release of PYY with long term and short term effects depending of their chain length.

## Introduction

The intestine harbors the highest density of micro-organisms of the human body, referred to as the gut microbiota<sup>1</sup>. Gut microbiota has important functions for the host such as fiber fermentation, vitamin and amino acid production, maturation of the immune system, defense against pathogens<sup>2-4</sup>. In addition, gut is one of the largest endocrine organs, secreting more than 20 different hormones that participate in the regulation of important physiological functions, including insulin secretion, intestinal absorption and motility, gastric enzyme secretion, food intake<sup>5-7</sup>. Hormones are secreted in response to food intake for most of them by a family of cells representing less than 1% of intestinal epithelial cells, namely enteroendocrine cells (EECs). Depending on their localization and expression of differentiation factors<sup>8-10</sup>, EECs secrete a specific pattern of hormones in response to the composition of luminal content or peripheral signaling. EECs are able to sense luminal content through expression of a great variety of receptors and transporters<sup>11-15</sup> including GPCRs recognizing fatty acids<sup>16,17</sup>, protein hydrolysates<sup>18</sup>, secondary bile acids<sup>11,19</sup>; taste receptors<sup>20-24</sup>.

Among EECs, colonic L-cells play a central role in regulation of food intake and insulin secretion as they secrete glucagon-like peptide-1 and -2 (GLP-1 and GLP-2), peptide YY (PYY), and Insulin-like Peptide 5<sup>25,26</sup>. GLP-1 has been largely studied for its incretin properties, and analogs of GLP-1 have been used as treatment for type II diabetes<sup>27,28</sup>. PYY belongs to the NPY family of peptides and has been implicated in the regulation of gut motility and electrolyte absorption, gastric emptying and food intake<sup>29-34</sup>. Moreover, it has been shown that PYY plasma levels are decreased in obese patients and increased after bariatric surgery during weight loss<sup>35,36</sup>. Therefore, an increase of PYY plasma levels can be seen as a good pharmacological strategy to reduce food intake in obese patients<sup>37</sup>.

L cells localization follow a proximo-distal gradient and PYY is mostly expressed in the colon, where bacterial density is the most important. Therefore, L-cells may not only sense directly nutrients after food intake but rather bacterial and nutrient degradation products, such as peptones<sup>38</sup>, secondary bile acids<sup>20</sup> or fatty acids<sup>39</sup>. Indirect sensing of nutrients in duodenum through vagous nerve signaling has also been shown implicating of cholecystokinin (CCK) secretion from I-cells in the duodenum<sup>40-42</sup>. Among fatty acids, short chain fatty acids (SCFAs) are major bacterial metabolites. Acetate, propionate and butyrate are the most abundant ones, with concentration of about 60mM, 20mM and 20mM respectively, in the colon lumen<sup>43,44</sup>. SCFAs are end-products of fiber fermentation by bacteria and are used by host cells as energy substrate and for lipogenesis. Different studies have shown that diets enriched in fibers fed for several weeks increased colonic SCFAs by bacterial fermentation and induced an increase of fasting PYY plasma levels and response to food intake<sup>45-47</sup>, but effects were different depending on type of fiber and therefore SCFAs concentration and proportions in the colon. Different mechanisms can be hypothesized to explain these results such as direct secretion of PYY, inhibition of PYY degradation, increased PYY production, by modulation of expression of *Pyy* gene or post transduction modulation, or increased numbers of L-cells<sup>48,49</sup>.

Effects of SCFAs on L-cells are multiple as they can serve as energetic substrate, inhibit Lysine/Histone Deacetylases (K/HDAC)<sup>50</sup> and are recognized by specific G-coupled receptors, Free Fatty Acid Receptor 2 and 3 (FFAR2 and FFAR3, respectively named GPR43 and GPR41) and GPR109a<sup>51-55</sup>. It has been shown that the two first receptors are expressed in L-cells, but at different levels in humans, FFAR2 being much more expressed than FFAR3<sup>56-59</sup>. The affinity for SCFAs differs between receptors and species<sup>52,60</sup>. In humans, FFAR2 recognizes with equally high affinity acetate, propionate and butyrate whereas FFAR3 recognizes with equal affinity as FFAR2 butyrate and propionate but has a much lower

affinity for acetate. GPR109a, which mainly recognize niacin, recognize butyrate with a much lower affinity than FFAR2 and FFAR3<sup>54</sup>. FFAR2, FFAR3 and GPR109a can activate G $\alpha$ i/o signaling inducing inhibition of adenylyl cyclase and thus decreased concentration of cytosolic cAMP<sup>52,53</sup>. FFAR2 can also induce G $\alpha$ q signaling, activating Phospholipase C (PLC) leading to hydrolysis of phosphatidylinositol(4,5)diphosphate into diacylglycerol and inositol triphosphate (IP3). Diacylglycerol activates protein kinase C, regulating many cell functions, whereas IP3 activates a specific receptor on sarcoplasmic reticulum releasing calcium into the cytosol.

Direct administration of SCFAs in the colon increased PYY plasma levels, and *in vitro* administration on primary colonic crypts also increased released PYY concentration suggesting a direct effect of SCFAs on secretion<sup>61–64</sup>, which may be mediated by one of the receptors to SCFAs. However, several studies showed different responses<sup>46,64–66</sup> and could not allow to decipher the precise mechanisms implicated in PYY increased levels. Moreover, differences in receptor affinities between human and rodent genes limited the conclusion from rodent models. SCFAs also increased the number of L-cells and impacted on the expression of differentiation factor in the colon of rats or in organoids, but at different extent between human and mouse organoids. These results indicate that increased PYY plasma levels after long term fiber rich diet could also result from increased number of L-cells and therefore increased production of PYY<sup>48,67</sup>. However, precise mechanisms and pathways implicated in the response of L-cells to SCFAs resulting in increased PYY circulating levels remain unknown, nor direct effect of SCFAs on PYY production.

In this study, we used two human cell lines expressing PYY and modeling L-cells to assess *in vitro* the effects of SCFAs on *Pyy* gene expression and PYY secretion and to decipher the precise mechanisms implicated in human L-cell response to SCFAs.

## Materials and Methods

### Cell culture and reagents

NCI-h716 cells (a kind gift from C. Roche, Lyon, France) were maintained in suspension in RPMI-160 (Gibco, Life Technologies), supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 50IU/mL penicillin and 50µg/mL streptomycin in a humidified incubator at 37°C, 5% of CO<sub>2</sub>. HuTu80 (obtained from ATCC, HTB40) were maintained in EMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum, 2mM L-Glutamine and 50IU/mL penicillin and 50µg/mL streptomycin in a humidified incubator at 37°C with 5% of CO<sub>2</sub>. Drugs and reagents were from Sigma-Aldrich. SCFAs, tiglic acid, 1-methylcyclopropane carboxylate (1-MCPC), niacin were solubilized in water and pH adjusted between 7.2 and 7.4. U-73122, PMA and 2-aminoethyl diphenylborinate (2-APB) were solubilized in DMSO, final DMSO concentration was 0.1% at maximum and did not have detectable effect on cells. Pertussis toxin was kept in 50% glycerol solution, final glycerol concentration was 0.05% and had no detectable effect on cells.

### Plasmid construction and transfection

Cloning of FFAR2 was performed by PCR amplification from NCI-h716 genomic DNA and integrated after EcoRI and XhoI digestion into a pCMV-eGFP-N1 vector (a kind gift from A. Echard, Institut Pasteur). Oligonucleotides used for amplification of FFAR2 were 5'-*aaaactcgagatgctgccggactggaa*-3' and 5'-*aaaagaattctactctgtagtgaagtccga*-3'. HuTu-80 cells were seeded at 2x10<sup>5</sup> cells per well in 6 well plates 24h before transfection. For transfection, medium was removed and replaced with Optimem (Gibco, Life Technologies) and 2µg of plasmid DNA mixed with 4 µL lipofectamine 2000 (Invitrogen) in 50 µL Optimem were added following manufacturer's instructions. Medium was replaced 6h after transfections, and experiments were performed 24 to 48h after transfection.

### Immunostaining

PYY rabbit polyclonal antibody (11431, used at 1/40) and monoclonal antibody against Chromogranin A (LK2H10, used at 1/20) were purchased from Progen Biotechnik (Germany). Fluorescent secondary antibodies, goat anti mouse IgG coupled to AlexaFluor 488 (1/5000) and goat anti rabbit coupled to AlexaFluor 565 (1/2000) were purchased from Life Technologies. 8x10<sup>4</sup> NCI-h716 cells were coated on cover slips in serum free media 4h before fixation and 2x10<sup>4</sup> HuTu-80 cells were coated on coverslips 24h before fixation. Immunostaining was performed after 4% PFA cell fixation and 1% saponin permeabilization. Primary antibodies were incubated for 1h and secondary antibodies for 45min in saponin bufer (PBS with 5% donkey serum and 1% saponin solution) at room temperature. Slides were mounted with Mowiol. Images were acquired on a Zeiss LSM700 confocal laser scanning microscope (MIMA2 platform, INRA) and images analyzed using Fiji software.

### RNA extraction and qPCR

NCI-h716 (1x10<sup>6</sup> in twelve well plates) or HuTu80 (5x10<sup>5</sup> in six well plates) cells were seeded 48 hours before lysis. Drugs were added 24h before RNA extraction if not indicated otherwise. RNA was extracted using a Qiagen RNeasy minikit with a DNase treatment (Qiagen). 2µg of RNA were used for reverse transcription using High capacity cDNA Reverse transcription kit (Applied Biosystems). qPCRs were performed on a AbiPrism 7000 system with Taqman gene expression assay probes (Supp Table



1) and Taqman gene expression master mix (Life Tech). Data was analyzed using 7000 System SDS software (Applied Biosystems) and Student unpaired t-test analysis was used for statistical analysis.

### Calcium Imaging

Clonal cell lines stably expressing GCaMP-5G (a gift from D. Kim and L. Looger (Addgene plasmid #31788)) were established with resistance to G418 (500 $\mu$ g/mL). HuTu-80 GCaMP-5G cells were seeded 48h before experiments in a 96wells plate with  $\mu$ clear bottom for microscopy (Greiner BioOne). EMEM was removed one hour before experiments and replaced by saline glucose buffer (NaCl 140mM, KCl 5mM, MgCl<sub>2</sub> 2mM, CaCl<sub>2</sub> 2mM, Hepes 10mM, sucrose 6mM and glucose 10mM, pH adjusted to 7.35). NCI-h716 GCaMP-5G cells were coated on the morning before experiments in saline buffer. Same buffer with CaCl<sub>2</sub> replaced by 2mM MgCl<sub>2</sub> and 5mM EGTA was used as a calcium-free saline buffer. Cells were observed on a Zeiss LSM700 confocal laser scanning microscope (MIMA2 platform, INRA) at 25°C and filmed for 3 minutes at 1 image per second. 30 to 100 cells were acquired in a same field. Reagents were added volume/volume 50s after beginning of acquisition. Image analysis and cell fluorescence was quantified using Fiji software<sup>68</sup>, data analysis was performed using R software.

### Elisa assay

For secretion studies, 4x10<sup>5</sup> NCI-h716 cells per condition were centrifuged and rinsed twice with saline buffer. Cells were incubated 2h in 200 $\mu$ L of saline buffer with DPP-IV inhibitor (Merck Millipore) at 37°C. Supernatant was centrifuged once and stored at -80°C until used for ELISA. PYY concentration in the supernatant was measured using PYY Elisa kit EZHPYYT66K (Merck Millipore) using manufacturers' instructions, except that 40 $\mu$ L of supernatant was used instead of 20 $\mu$ L. Measurements were performed with a microplate reader (Infinite 2000, Tecan). Data was analyzed using R and statistical analysis was assayed by one-way ANOVA.

## Results

### NCI-h716 and Hutu-80 cell lines express markers of human L-cells

NCI-h716 cell line, derived from a colonic adenocarcinoma, has previously been characterized to express and secrete GLP-1<sup>69</sup>. We confirmed expression of specific L-cells early and late differentiation factors, *Neurogenin3*, *FoxA1*, *Pax4* and *Pax6* and specific marker *ChromograninA* using qRT-PCR (Fig 1A) and showed expression of *Pyy* at low levels. Similarly to colonic L-cells, they expressed several transporters and receptors for SCFAs, the carbohydrate transporter *MCT-1*, receptors *FFAR2*, *FFAR3* and *GPR109a*. Notably, *FFAR2* was much more expressed than the two others, in accordance with literature. SCFAs transporter *SLC5A8* expression was not detected in our cells. We also monitored the expression of these factors in another cell line described to express and secrete GLP-1 as well as CCK, namely HuTu-80, derived from a duodenum adenocarcinoma. These cells have a different pattern of expression of differentiation factors, with a higher expression of *Pax6* and a lower expression of *Pax4*, but also expressed *Pyy* (Fig 1A). SCFAs receptor gene expression did not differ from NCI-h716 cells, except *FFAR2* which was much less expressed in HuTu-80 cells. We therefore focused our study on NCI-h716 cells as models of human L-cells and used HuTu-80 cells to confirm our main results. The immunostaining confirmed that both cell lines express chromogranin A and PYY at the protein level (Fig1B).

### SCFAs modulate *Pyy* gene expression in L-cells.

SCFAs have been associated with increased PYY plasma levels, but effects on *Pyy* expression have not been monitored so far. Addition of SCFAs to culture media induced an increase of *Pyy* gene expression in NCI-h176 cells, but the effect was dependent on chain length (Fig 2A). Indeed, acetate induced a two fold increase of *Pyy* expression compared to untreated cells, whereas propionate and butyrate induced a much higher gene expression, with a fold of 50 and 120, respectively. We further characterized that butyrate effect was time and dose dependent (Fig2B), with no saturating effect in the range of concentration tested. A low, but statistically significant increase was observed as soon as one hour after addition of butyrate, and increased importantly until 48 hours. At a low concentration of 0.1 mM, *Pyy* gene expression was increased by a fold of 6, and dose dependently increased to reach 420 fold for a 5 mM concentration after 24h incubation (Fig2B). Butyrate is known to have inhibitory effects on cell proliferation. We therefore set up our experiments at concentration below 5mM and for no longer than 48h to avoid this phenomenon (data not shown). Using Hutu-80 cell lines, we reproduced the high *Pyy* overexpression induced by butyrate, but at a lower extent with an increase fold of about 15. Propionate had a much lower effect, increasing *Pyy* expression by a fold of 2. Moreover, acetate did not have any effect on *Pyy* expression (Fig 2A). Hutu-80 cells were therefore much less sensitive to SCFAs compared to NCI-h716 cells, which can be explained by differences in *FFAR* receptors expression (Fig 1A) and tissue origin of the cells, but still reproduced partially SCFAs effects on *Pyy* expression.

It was shown that SCFAs can induce an increase of the number of L-cells in human and rodent organoid models<sup>48</sup>, indicating an effect on cell differentiation. Thus, we analyzed the impact of butyrate on the expression of differentiation factors to decipher if the increased expression of *Pyy* was dependent on cell differentiation. Only slight modifications of expression of these factors were detected, with a

decrease of expression of early differentiation factors Neurogenin3 and Foxa1 and an increase of Pax6 expression (Fig 3A). Thus, butyrate action may not be a consequence of modification of expression of differentiation factors in NCI-h716 cells. Interestingly, butyrate induced a stronger modification of differentiation factors expression in HuTu-80 cells (Suppl Fig 1 A), with a high increase of Neurogenin3 and Pax4 expression, but this could not be associated with increased *Pyy* gene expression. We could therefore hypothesize that butyrate effect on *Pyy* expression was not mediated by cell differentiation but depended on other signaling pathways.

#### HDAC inhibition strongly increases *Pyy* expression

Two main mechanisms have been implicated in response of human cells to SCFAs: activation of specific GPCRs and inhibition of K/HDAC. We used agonists for SCFAs main receptors and Trichostatin A (TSA), which mimic butyrate and propionate K/HDAC inhibitory activity on NCI-h176 cells. We showed that FFAR2 and FFAR3 activation by respectively tiglic acid and 1-methylcyclopropane carboxylate (1-MCPC), but not GPR109a activation by niacin, induced a slight increase of *Pyy* expression similar to acetate (Fig 3B). We highlighted the importance of FFAR2 in the response to SCFAs using HuTu-80 cells as HuTu-80 cells do not respond to acetate but express very low levels of *Ffar2*. In HuTu-80 cells transiently overexpressing FFAR2 but not in those expressing a control vector, *Pyy* expression was increased in response to acetate, tiglic acid but not 1-MCPC (Suppl fig1B). Finally, PMA, a known activator of Protein Kinase C, downstream of  $G_{\alpha q}$ , a small G-protein implicated in the response of some GPCRs including FFAR2, also induced a 7 fold increase of *Pyy* expression (Fig 3B). Moreover, we showed that TSA induced a strong *Pyy* overexpression similar to propionate and butyrate (Fig 3C), indicating that inhibition of K/HDAC induced a strong over expression of *Pyy*. Altogether, these results indicated that both mechanisms were implicated in *Pyy* increased expression but at different extent: activation of FFAR2 or 3 by SCFAs induced a low increase of *Pyy* mRNA levels, which may be the main pathway implicated in L-cells response to acetate, whereas K/HDAC inhibition induced a much stronger increase of *Pyy*, mRNA levels which seems the main pathway implicated in butyrate and propionate effect.

#### SCFAs differently modulate calcium flux in L-cells

SCFAs are predicted to induce secretion in these cells<sup>61–64</sup>. We assessed secretion indirectly using a reporter system for cytosolic  $Ca^{2+}$  concentration, as a rise of cytosolic calcium concentration  $[Ca^{2+}]_i$  is necessary for membrane fusion and secretion. Clonal population of NCI-h716 cells and HuTu-80 cells stably expressing GCaMP-5G, a protein which fluorescence is dependent on cytosolic calcium concentration, were selected for their response to known modulators of cytosolic concentration. Addition of acetate on these NCI-h716-GCaMP-5G cells induced a strong oscillatory response similar to secretion patterns observed in other secreting cells (fig 4A). At higher dose, acetate and propionate induced a strong calcium rise, with limited oscillations (Supp Fig 2A and 2B). On the contrary, butyrate induced a smaller increase of  $[Ca^{2+}]_i$  which may signal for secretion but also induced a  $[Ca^{2+}]_i$  decrease or hyperpolarization, meaning a potential inhibition of secretion (fig 4C). Propionate induced an intermediate response, closer to acetate (fig 4B). Using higher doses of butyrate resulted in a stronger  $[Ca^{2+}]_i$  decrease (fig 4D). Thus, butyrate induced two different effects on  $Ca^{2+}$  flux: a rapid, pulsatory rise, which may signal for secretion and a long-term decrease of  $[Ca^{2+}]_i$  which may inhibit secretion.

Similarly to regulation of *Pyy* expression, chain length determined the response as butyrate induced a higher  $[Ca^{2+}]_i$  decrease and acetate a higher pulsatory  $Ca^{2+}$  rise. SCFAs mixes, in similar proportion as found in the colon induced intermediary phenotypes and butyrate had the strongest effect at higher doses (Supp fig 2C and 2D).

FFAR2 mediates SCFAs induced calcium concentration rise through a  $G\alpha_q$  activation

Activation of FFAR2 by tiglic acid resulted in a rise in  $[Ca^{2+}]_i$  followed by a diminution of the basal  $[Ca^{2+}]_i$  associated with (fig 5A), whereas activation of FFAR3 and GPR109 by 1-MCPC and niacin respectively only resulted in a decrease of  $[Ca^{2+}]_i$  (Fig 5B and 5C). This suggested that FFAR2 activation induced secretion in response to SCFAs, and that activation of other SCFAs receptors reduced basal calcium level. It is noteworthy that none of the agonists tested could totally reproduce acetate-induced strong oscillatory response whereas tiglic acid and butyrate had very similar effects. To further document the role of FFAR2 in response to acetate and butyrate, we transiently overexpressed FFAR2 in HuTu-80 cells, as these cells express very low levels of FFAR2. In control HuTu-80, acetate and butyrate only induced a low  $[Ca^{2+}]_i$  decrease (Fig 5D and 5E). In cells transfected with pCMV-FFAR2, acetate and butyrate both induced a strong rise in  $[Ca^{2+}]_i$  (fig 5F and 5G), with acetate inducing a higher  $[Ca^{2+}]_i$  increase underlining the role of FFAR2 in SCFAs effects on calcium fluxes.

FFAR2 is coupled to both  $G\alpha_q$  and  $G\alpha_i/o$  whereas FFAR3 and GPR109a are only coupled to  $G\alpha_i/o$ . As  $G\alpha_q$  pathway induces rise in  $[Ca^{2+}]_i$  through PLC/IP3 activation whereas  $G\alpha_i/o$  decreases cAMP concentration also implicated in calcium response, we hypothesized that activation of  $G\alpha_q$  pathway may induce secretion whereas activation of  $G\alpha_i/o$  pathway may be responsible for the calcium hyperpolarization. Indeed, activation of PKC, acting downstream of  $G\alpha_q$ /PLC pathway, by PMA induces a strong calcium concentration increase (supp Fig 2E). To study the implication of  $G\alpha_q$ /PLC/IP3 pathways, we took advantage of U-73122 that inhibits PLC and 2-APB that is an antagonist of IP3 receptor. Both drugs completely abolished acetate and butyrate induced rise in  $[Ca^{2+}]_i$  but did not modified butyrate mediated  $Ca^{2+}$  hyperpolarization (Fig 6A-C). Moreover, inhibition of  $G\alpha_i/o$  pathway using Pertussis Toxin (Ptx) did not prevent  $[Ca^{2+}]_i$  increase, but decreased calcium hyperpolarization induced by SCFAs (Fig 6D). Consistently with results on receptors activation, we demonstrated that SCFAs-induced  $[Ca^{2+}]_i$  increase was  $G\alpha_q$ /PLC/IP3 dependent whereas cell hyperpolarization was  $G\alpha_i/o$  dependent (fig 5H-I).

SCFAs increase secreted PYY quantity by regulation of its secretion and its expression

To confirm our previous results at the protein level, we measured the amount of PYY secreted by NCI-h716 cells during two hours. Acetate, propionate and butyrate increased PYY secretion by a fold of 1.5 compared to control cells (Fig 7A-right). We could not detect difference between acetate, propionate and butyrate induced secretion, on the contrary to results found using calcium imaging. This may be due to limit of sensitivity of ELISA in these experiments, as NC-h716 cells secreted levels of PYY close to the lower sensibility level of the ELISA test.

We could also confirm effect of butyrate on *Pyy* induction at the protein level by the increase of secreted PYY during two hours saline glucose buffer by a fold of 4 in cells that were previously incubated with butyrate compared to untreated cells. However, following 24 hours butyrate incubation prior to measurement of secretion during 2h in saline glucose buffer, acetate did not induce any increase of secretion and only propionate induced a statistically significant increase of PYY

secretion. Surprisingly, butyrate addition even decreased PYY secretion, indicating that butyrate may decrease cell sensitivity to SCFAs and even partially inhibit secretion. We confirmed this decreased sensitivity of NCI-h716 cells to SCFAs after incubation with butyrate for 24h by calcium imaging as acetate induced a much lower response in butyrate-treated cells than in untreated cells (Fig 7B).

Two mechanisms could be implicated in the reduced response to SCFA induced by incubating the cells with butyrate for 24h: an inhibitory effect of butyrate on calcium fluxes associated with induced calcium hyperpolarization and decreased expression of receptors for SCFAs. When cells were exposed to acetate and 50 seconds later another dose of acetate or saline was added,  $[Ca^{2+}]_i$  oscillation were still present (Fig 7C). However, when butyrate was applied 50s after acetate it resulted in a stop in  $[Ca^{2+}]_i$  oscillation. This indicated a direct effect of butyrate inhibiting  $Ca^{2+}$  flux. Moreover when SCFAs were added together, response was decreased compared to treatment with propionate or acetate alone (Supp Fig 2C and 2D). Another mechanism implicated in long-term effect of butyrate was through the regulation of GPCR expression in the cells. By RT-qPCR, we observed that incubating NCI-h716 with butyrate for 24h reduced *Ffar2* expression and to a lower extent *Gpr109a*, but not *Ffar3* expression (Fig7D). This may also explain the long-term effect of butyrate on  $[Ca^{2+}]_i$  response to acetate and on PYY secretion after butyrate treatment (Fig 7B and A, respectively).

## Discussion

These results enabled us to demonstrate the important and complex effects of SCFAs on both PYY gene expression and secretion, with different pathways implicated, and to decipher at least in part, the mechanisms. Using two cellular models for enteroendocrine cells that express *Pyy*, we could demonstrate in human L-cells that butyrate and propionate highly increased *Pyy* gene expression, and that this effect involved K/HDAC inhibition. Indeed, taking advantage of another K/HDAC inhibitor, TSA, we could reproduce the strong increase of *Pyy* expression obtained with propionate and butyrate, indicating that effect of butyrate and propionate were through K/HDAC inhibition. These observations are consistent with known effect of butyrate and propionate on the expression of other genes<sup>70–72</sup>.

Moreover, we demonstrated that activation of the SCFAs receptors FFAR2 and FFAR3, but not GPR109a also increased *Pyy* expression but effect was much weaker than the inhibition of K/HDAC. Increased expression of *Pyy* consequently increased PYY production and PYY secretion as cells incubated for 24h with butyrate had increased PYY secretion. Relative importance of FFAR2 and FFAR3 are not known as both agonists increased expression in NCI-h716 cells but none had effect in HuTu-80 cells. We hypothesized that FFAR2 may be more important as FFAR3 is similarly expressed in both cell lines whereas FFAR2 is much less expressed in HuTu-80 cells than in NCI-h716 cells. Moreover, over-expression of FFAR2 in HuTu-80 cells enabled us to reproduce the increase of *Pyy* expression by a fold of 2 observed in NCI-h716 cells.

Interestingly, we did not observe increased expression of *proglucagon*, the gene coding for the precursor of GLP-1 and GLP-2, by SCFAs stimulation but a low decrease of expression mediated by butyrate, similarly to what was observed in these cells<sup>73</sup>. Considering the pattern of expression of PYY and GLP-1 in human gut and SCFA concentration in the gut, butyrate may be an important regulator only of PYY production, indicating why this hormone is mostly expressed in the colon. PYY may therefore have a unique function in the crosstalk between the microbiota and the host.

SCFAs also had important effects on PYY secretion by modulating calcium signaling in the cells, and we reported two distinct effects. Firstly, all SCFAs induced a rise in calcium concentration resulting in PYY secretion by stimulating FFAR2 and consequently activating  $G\alpha_q$ /PLC/IP3 pathway, releasing calcium from the sarcoplasmic reticulum. Secondly, butyrate and propionate, and acetate at a lesser extent and when used at higher concentrations, decreased  $[Ca^{2+}]_i$ . We could associate this cell hyperpolarization to an inhibition of secretion as cells incubated for a long time with butyrate secreted less PYY when secretion was tested with butyrate than in saline buffer, and their calcium response to acetate was strongly decreased. Interestingly, activation of FFAR2 by its agonist tiglic acid did not reproduce the same calcium response as acetate, which may be explained by the presence of another unknown receptor to acetate in human cells or different signaling after the same receptor activation. Indeed, FFAR2 stimulation induced different responses between NCI-h716 cells and HuTu-80 cells over-expressing FFAR2, highlighting the importance of signaling pathways downstream of receptors in the observed response. Altogether, the expression of three distinct receptors with different affinities for each SCFA enables a finely tuned sensing of total SCFA concentration and their relative concentrations. Modulation of expression of receptors or variation of affinity for SCFAs may therefore induce different responses to SCFAs mixes. Human and mice FFAR2 and FFAR3 have different affinities and order of potency to SCFAs<sup>60</sup>; we therefore hypothesized that mouse L-cells will have different responses to human L-cells, which may explain differences found in L-cell responses to stimulation by SCFAs in human or mouse models<sup>48,63,74</sup>.

Precise localization of FFAR2 and FFAR3 are not known. However, depending on their localization, these receptors are not exposed to similar concentrations. Indeed, SCFA concentrations are about 3 orders of magnitude higher in the colon lumen than in the lamina propria, supposed to be similar to concentrations in the portal vein. Therefore, apical receptors would be exposed to concentration much higher than their affinity, whereas basolateral receptors would be exposed to concentrations in the range of receptor affinity, except FFAR3, which has much lower affinity for acetate. Depending on their localization, these receptors would thus be continuously activated or their activation would depend on variations of local concentrations, sensing concentration variation. GPR109a has been shown localized at the apical membrane<sup>54</sup> and sense variations of butyrate concentration as its affinity for butyrate is in the range of luminal butyrate concentrations. Stimulation of this receptor seems important in the inhibitory effect of secretion mediated by butyrate. Indeed, this effect was mainly found when cells were stimulated by butyrate but not the other SCFAs, and GPR109a is the only receptor with different affinities to butyrate and propionate among the three receptors tested. Therefore variations of butyrate concentration in the colon may be important to regulate secretion as high concentrations inhibit it. Moreover, increased expression of *Pyy* by butyrate and propionate is not dependent on receptor stimulation but on their absorption from the lumen, mainly mediated by MCT-1, and may therefore represent another way to sense SCFA luminal concentration, as at the concentrations tested on our cells, *Pyy* increased expression was concentration dependent, and saturating effect was not reached. Altogether, we described three main effects of butyrate impacting PYY plasma levels on the long term effects: increased production of PYY, inhibition of secretion by cell hyperpolarization and decreased expression of *Ffar2*, decreasing SCFA induced secretion. Altogether, these results indicated an important function of butyrate by increasing the quantity of stored PYY that may be released upon stimulation strong enough to overcome cell hyperpolarization or associated with a decrease of butyrate concentration. Increased stored PYY is important to increase response to cell stimulation, mainly in response to food intake.

Regulation of SCFAs concentration and mainly butyrate and propionate are therefore important to regulate PYY production and secretion. However, little is known about precise SCFAs concentration in the close neighborhood of L-cells and their variations in time. Indeed, epithelial cells absorb actively SCFAs, and therefore decrease local SCFA concentration. Colonic SCFA concentrations are dependent on microbiota fermentation activity and quantity and quality of fibers incoming. Only long term effects have been reported on variation of their concentration, mainly due to enrichment with fibers of the diet, but short term variations after food intake are not known and may not be directly associated with food intake but colonic motility may be an important parameter by modifying local environment.

Our results deciphered precisely the part of the mechanisms implicated in the increase of PYY basal plasma levels and after stimulation, an important parameter to regulate food intake<sup>45,47,75</sup> in response to fiber-rich diets through increased production and modulation of its secretion by SCFA. A third mechanism described in the literature is the effect of SCFAs on number of L-cells<sup>48,49,67</sup>. This effect could not be assessed in our in vitro model, since we did not use stem cells.

Altogether, these results highlighted the beneficial effects of increasing SCFAs, mainly butyrate and propionate, concentrations in the colon as they increase PYY plasma levels and PYY responses. Obese individuals have lower PYY plasma levels, and increasing these levels by intravenous administration decreased food intake<sup>37,76</sup>. Moreover, obesity is associated with a modification of diet, consuming less fiber and more lipid and carbohydrate, potentially resulting in a modification of microbiota with decreased proportion of genes implicated in fibers and butyrate production<sup>77-79</sup>. Our study therefore described potential mechanisms implicated in the decreased PYY basal plasma levels and the decreased response to food intake, and reinforces the conclusions of beneficial effects of fiber-rich

diets regarding PYY by increasing production of SCFAs, and more particularly butyrate and propionate. However, SCFAs have many effects on the host, and comprehensive effects of SCFAs effects on the host on short and long term must be determined.

In this study, we described how microbiota by producing SCFAs impacts the host by modulating PYY plasma levels. PYY functions also modulate the microbiota by regulating food intake and controlling gut motility and intestinal transit. Indeed, food intake and intestinal transit directly affect foodstuff incoming in the colon, and therefore may shape the microbiota and its functions. Moreover, the colonic motility also have a mechanic effect on microbiota by changing local environment and sweeping away some luminal content and direct relationship between modulation of the intestinal transit and the microbiota could be described<sup>80,81</sup>. SCFAs and PYY are therefore important signaling molecules participating in the cross talk between the microbiota and the host among other molecules. Furthermore, targeting PYY in pathologies associated with eating behavior may be an interesting strategy. Fiber-rich diets have proven relative efficiency effects on the long term regarding obesity as fiber-rich diets were not consumed on the long term, but other strategies to increase SCFAs concentration may be considered.

Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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## Figure legends

### Figure1 : NCI-h716 and HuTu-80 express markers of L-cells and receptors and transporters of SCFAs

(A): Expression of L-cells markers, SCFA transporters and receptors and specific enteroendocrine differentiation factors in NCI-h716 (dark grey) and HuTu-80 (light grey) were detected by RT-qPCR. Data are expression relative to beta-actin determined by the  $2^{-\Delta Ct}$  method, on at least 4 distinct experiments performed in duplicate, represented as means  $\pm$  s.e.m. **N.D.** : Not Detected. (B): Representative immunostaining of PYY (red) and Chromogranin A (green) in NCI-h716 (up) and HuTu-80 (down) cells. right corner is control with primary antibody omitted, using same acquisition parameters.

### Figure 2: SCFAs differently regulate *Pyy* expression

(A): Effect of SCFAs (2mM, 24h) on *Pyy* expression (left) and proglucagon expression (right) in NCI-h716 (dark gray) and HuTu-80 (light gray). (B): Time and dose response to butyrate of *Pyy* expression in NCI-h716 cells. Relative expression of *Pyy* is determined by the  $2^{-\Delta\Delta Ct}$  method using beta-actin as control gene. Data are means  $\pm$  sem of at least three distinct experiments. (\*\*\*:  $P < .001$  ; \*\*:  $P < .01$  ; \*:  $P < .05$ ).

### Figure 3: HDAC inhibition increases *Pyy* expression much more importantly than FFAR activation

Relative expression to untreated cells expression of differentiation factors after treatment with butyrate 2mM in NCI-h716 cells. (B) : Effect of FFAR2, FFAR3 and GPR109a agonists (respectively tiglic acid, 1-MCPC and niacin) on *Pyy* gene expression compared to acetate (2mM) and PMA (200 nM). (C) : Effect of TSA (500 nM) on *Pyy* gene expression compared to propionate (2 mM) and butyrate (2 mM) in NCI-h716 cells. Relative expression to untreated cells of *Pyy* is determined by the  $2^{-\Delta\Delta Ct}$  method using beta-actin as control gene. Data are means  $\pm$  sem of at least three distinct experiments. (\*\*\*:  $P < .001$  ; \*\*:  $P < .01$  ; \*:  $P < .05$ ).

### Figure 4: SCFAs induce different calcium response in NCI-h716

Calcium response of NCI-h176 GCaMP-5G cells to addition of acetate 2mM (A), propionate 2mM (B) and butyrate 2mM (C left) and 20mM (C right). Curve represents relative fluorescence to mean fluorescence before drug addition in one representative cell of 50-100 cells acquired per experiment, repeated at least three times. Drug addition is indicated by a vertical arrow.

### Figure 5: Implication of FFAR2, FFAR3 and GPR109a in calcium responses

A-C: Calcium response of NCI-h716 GCaMP-5G cells to addition of FFAR2 agonist tiglic acid (A), FFAR3 agonist 1-MCPC (B) and GPR109a agonist niacin (C).

D-G: Calcium response of HuTu-80 GCaMP-5G cells either transfected with plasmid coding for mcherry (D, E) or for FFAR2 (F, G) to addition of acetate 2mM (D, F) and butyrate 2mM (E, G). Inserted panel (D,E): scale enlargement. Curve represents relative fluorescence to mean fluorescence before drug

addition in one representative cell of 25-50 cells acquired per experiment, repeated at least three times. Drug addition is indicated by a vertical arrow.

**Figure 6:** Implication of  $G\alpha_q$  and  $G\alpha_i/o$  in calcium responses to SCFAs

Calcium response of NCI-h716 GCaMP-5G cells treated with DMSO 0.05% (A) PLC inhibitor U73122 (B), IP3 receptor inhibitor 2-APB (C) and  $G\alpha_i/o$  inhibitor pertussis toxin (D) to addition of acetate 2mM (left) and butyrate 20mM (right).

**Figure 7:** SCFAs increase PYY secretion, but negatively regulate response to SCFAs

(A): Relative secretion of PYY by NCI-h716 preincubated for 24h with butyrate (left) or not in response (right) to different SCFAs (2 mM) to non treated. Secreted PYY was measured by ELISA in 40  $\mu$ L supernatant of  $4 \times 10^6$  cells/mL in glucose saline buffer during 2h in duplicate. Mean basal secretion was  $15.6 \pm 1.6$  pg/mL. Data are means  $\pm$  sem of four distinct experiments. (B): Calcium response of NCI-h716 GCaMP-5G cells untreated (left) or treated for 24h with 2mM butyrate (right) to addition of acetate 2mM. (C): Calcium response of NCI-h716 GCaMP-5G cells to successive addition separated by 50s of 2mM acetate two times (final acetate concentration: 4mM) (left); 2mM acetate addition and saline solution (final acetate concentration: 2mM) (middle) and 2mM acetate then 2mM butyrate (final acetate and butyrate concentration: 2mM) (right). Curve represents relative fluorescence to mean fluorescence before drug addition in one representative cell of 50-100 cells acquired per experiment, repeated at least three times. Drug addition is indicated by a vertical arrow. (D) Relative expression of SCFA receptors and transporter in NCI-h716 cells treated for 24h with butyrate 2mM to untreated cells determined by the  $2^{-\Delta\Delta Ct}$  method using beta-actin as control gene, Data are means  $\pm$  sem of at least three distinct experiments. (\*\*\*:  $P < .001$  ; \*\*:  $P < .01$  ; \*:  $P < .05$ ).

**Figure 8:** Schematic representation of L-cell response to SCFAs

Representation of a L-cell and main pathways implicated in response to SCFAs implicated in regulation of *Pyy* expression (up) and PYY secretion (down), including transport of butyrate and propionate inducing inhibition of HDAC and known receptors to SCFAs : FFAR2, FFAR3 and GPR109a Drugs inhibiting pathways implicated in the responses are indicated.

**Supplementary Figure 1:** acetate requires FFAR2 expression to increase *Pyy* expression

(A): Expression of specific enteroendocrine differentiation factors in NCI-h716 and HuTu-80 treated with butyrate 2mM for 24h or not as detected by RT-qPCR. Data are expression relative to beta-actin determined by the  $2^{-\Delta Ct}$  method, on at least 4 distinct experiments, represented as mean  $\pm$  s.e.m. (\*\*:  $P < .01$  ; \*:  $P < .05$  ; . :  $P < .1$  ; N.S. :  $p > 0.1$ ). (B) : Relative expression of *Pyy* in treated Hutu-80 cells transfected with pCMV-FFAR2 or pCMV-GFP (control) of *Pyy* relative to non-treated control transfected HuTu-80 cells determined by the  $2^{-\Delta\Delta Ct}$  method using beta-actin as control gene. Data is mean  $\pm$  sem on two different experiments.

**Supplementary Figure 2:** Calcium response to SCFAs mixes and PMA

Calcium response of NCI-h716 GCaMP-5G cells in response to acetate 20mM (A), propionate 20mM (B), SCFAs mixes acetate : propionate : butyrate 3:1:1 mM (C) and acetate : propionate : butyrate 30:10:10 mM (D) and PMA 200nM (E). Curve represents relative fluorescence to mean fluorescence before drug addition in one representative cell of 50-100 cells acquired per experiment, repeated at least three times. Drug addition is indicated by a vertical arrow.

**Supplementary Table 1:** list of Taqman assays used for gene expression

**Figure 1**

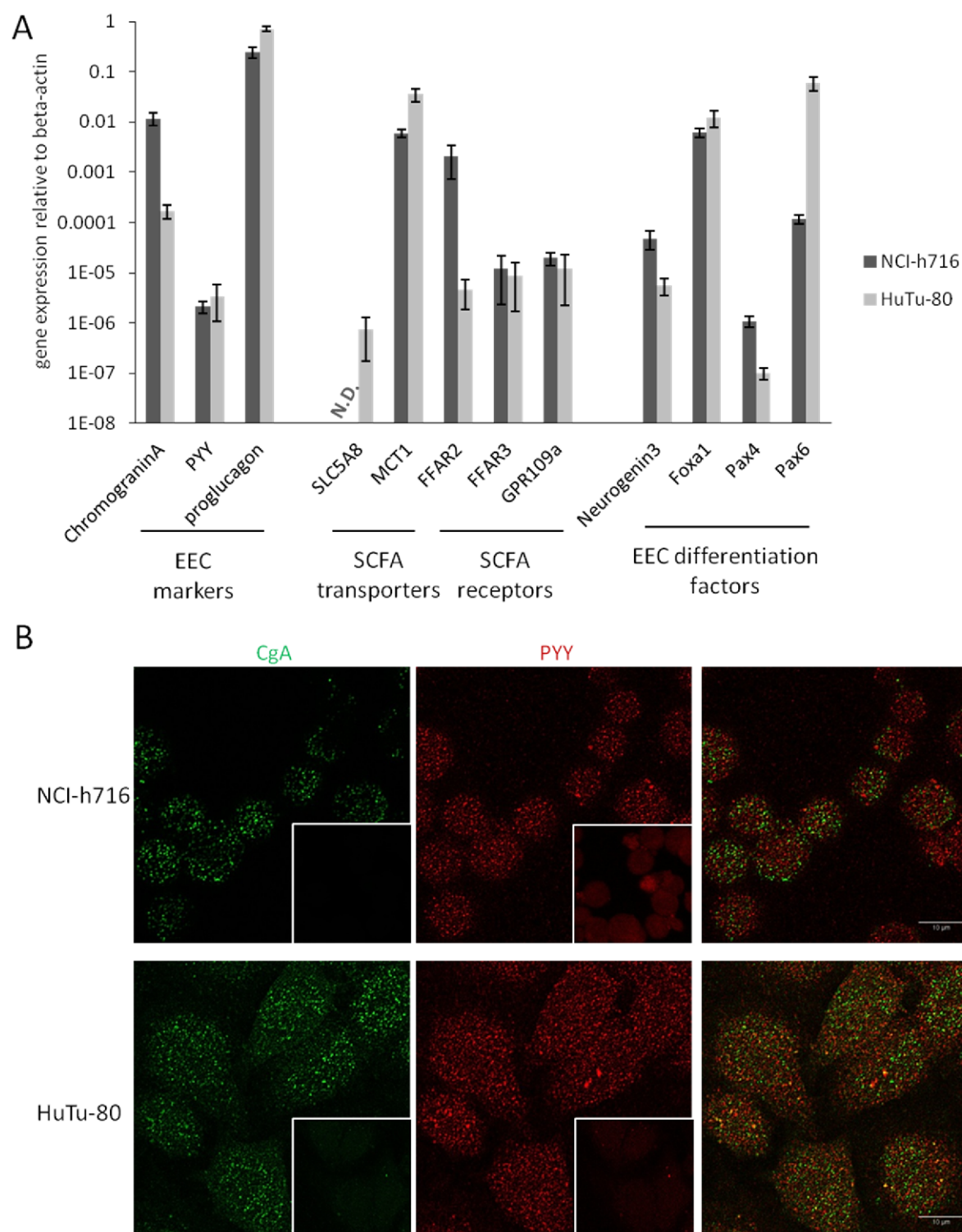
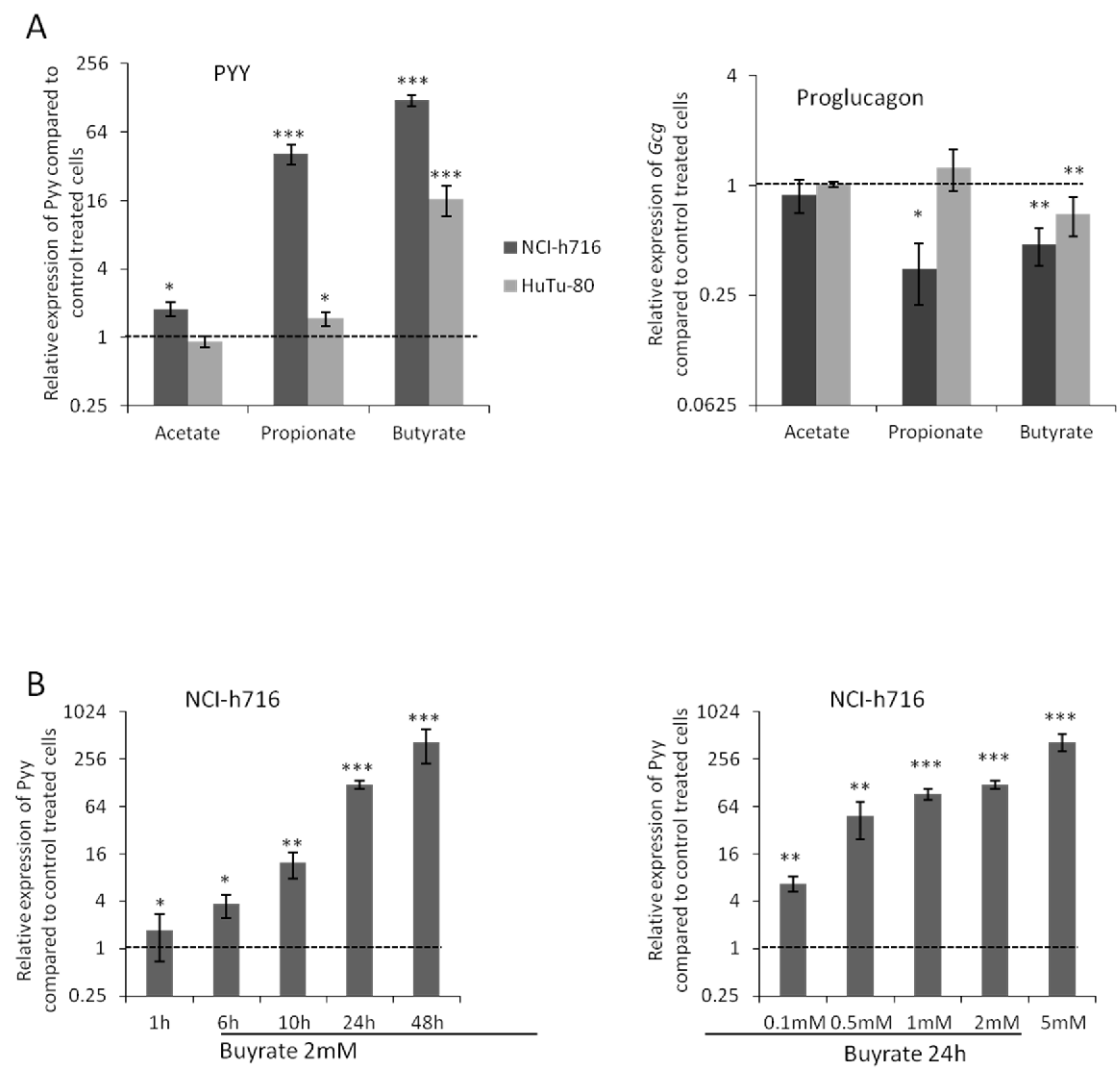
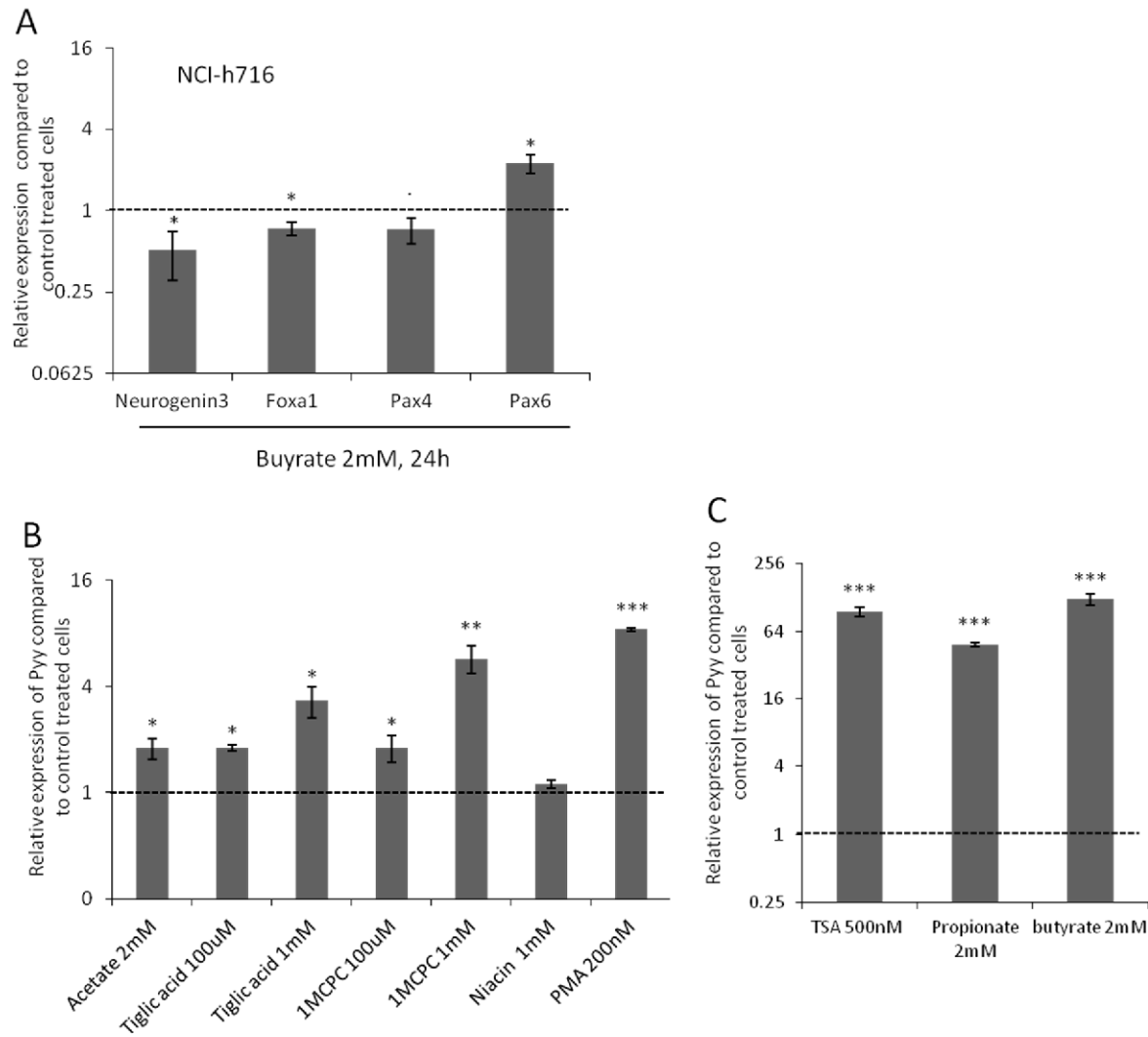


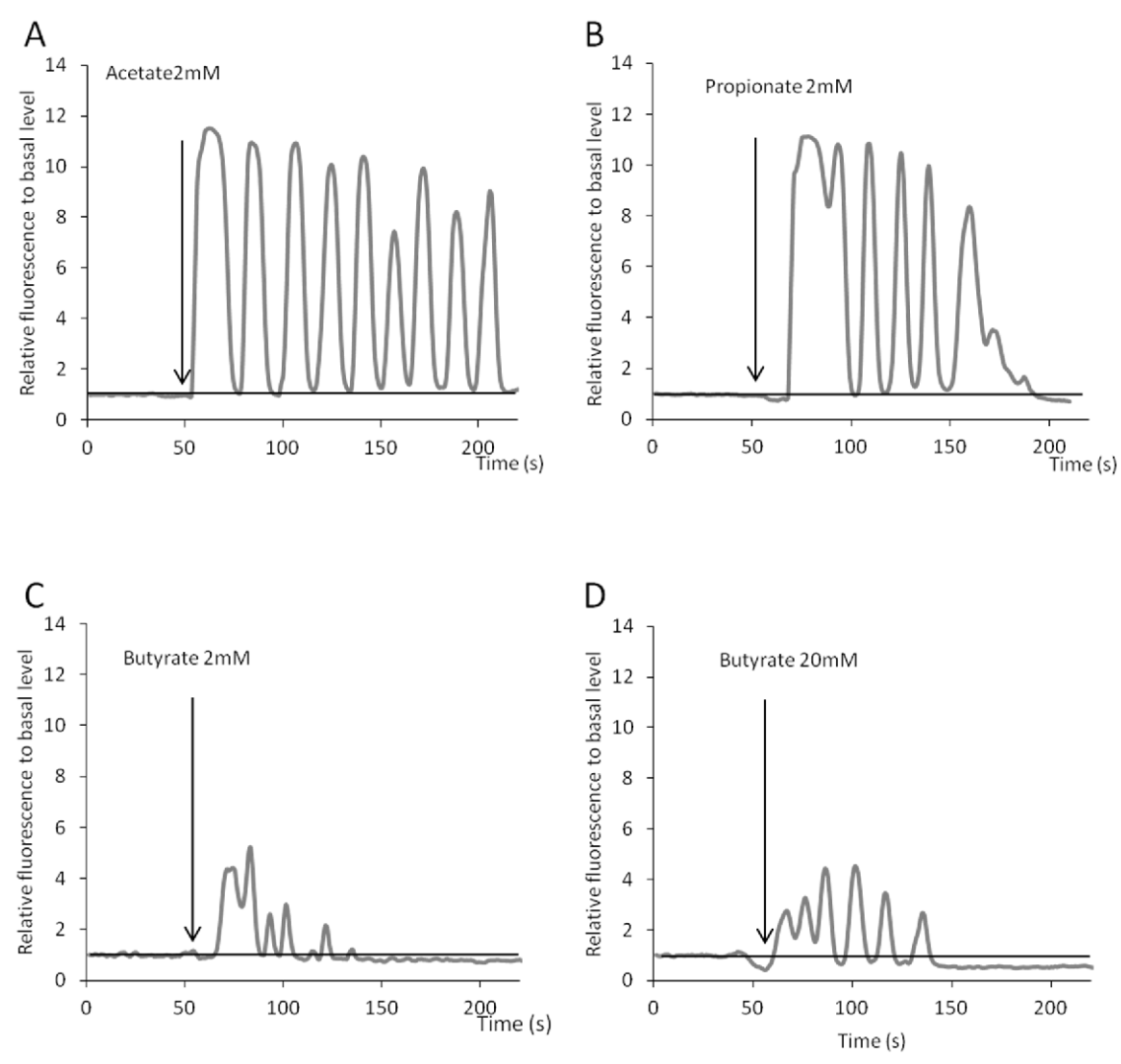
Figure 2



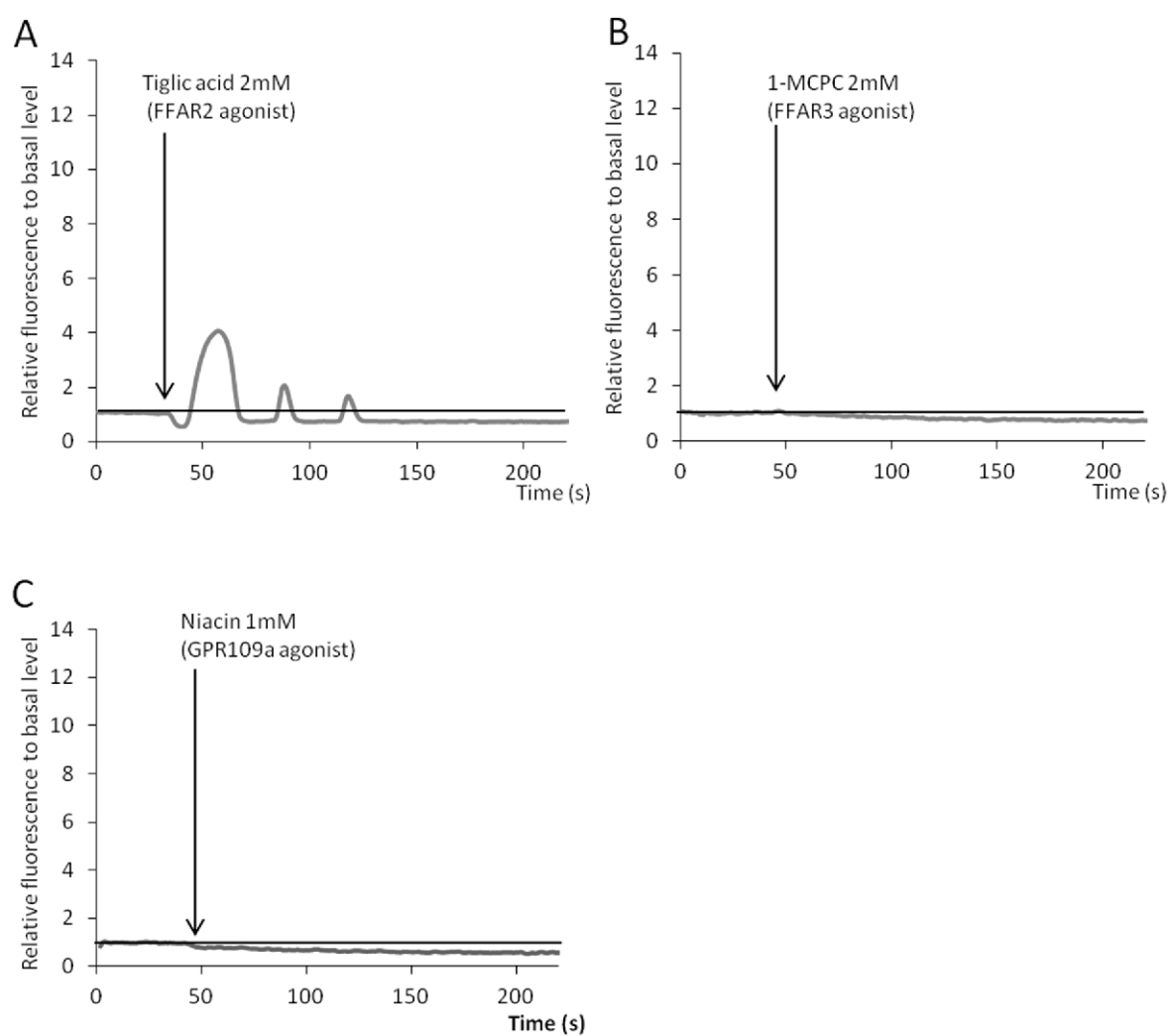
**Figure 3**



**Figure 4**



**Figure 5**





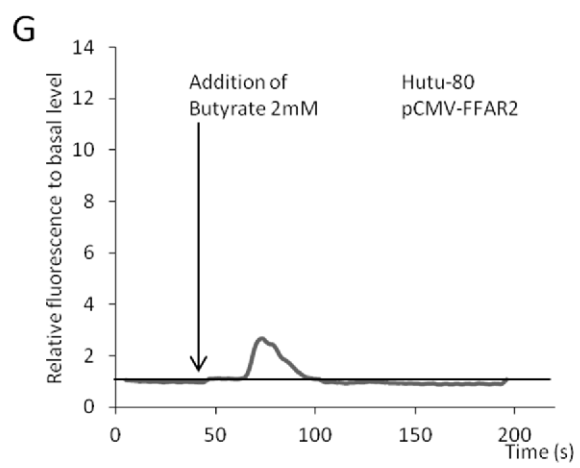
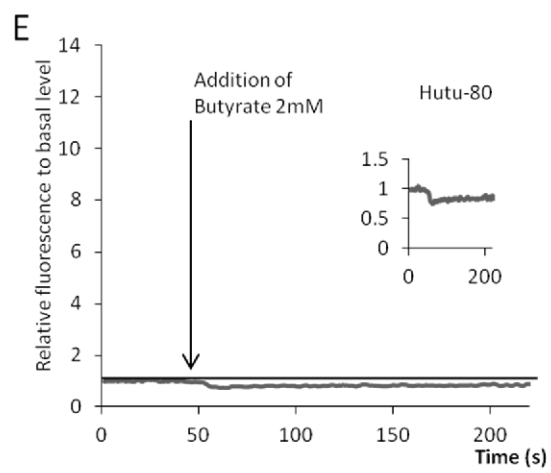
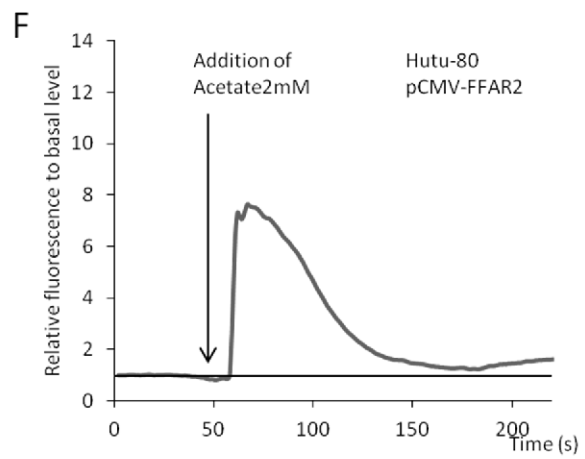
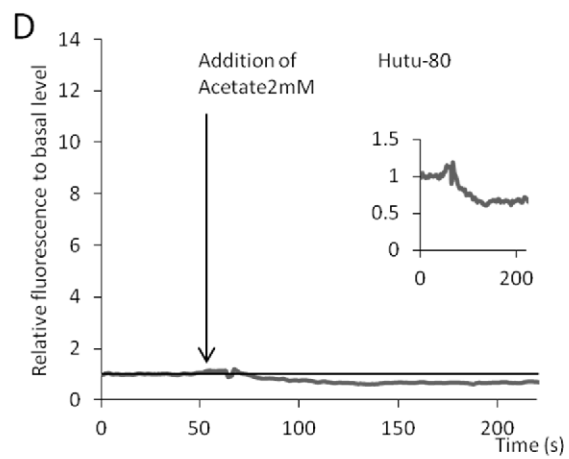
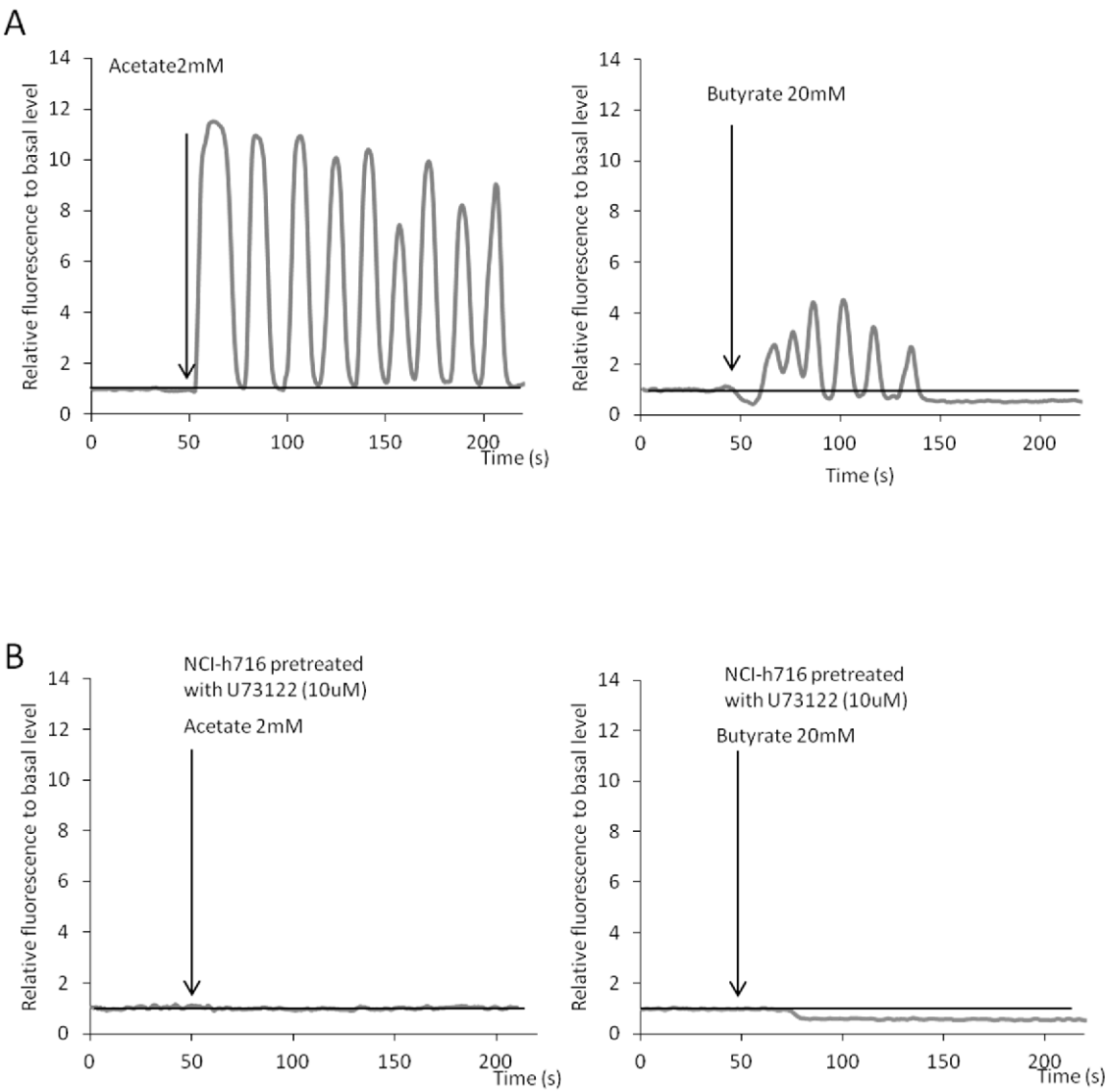


Figure6



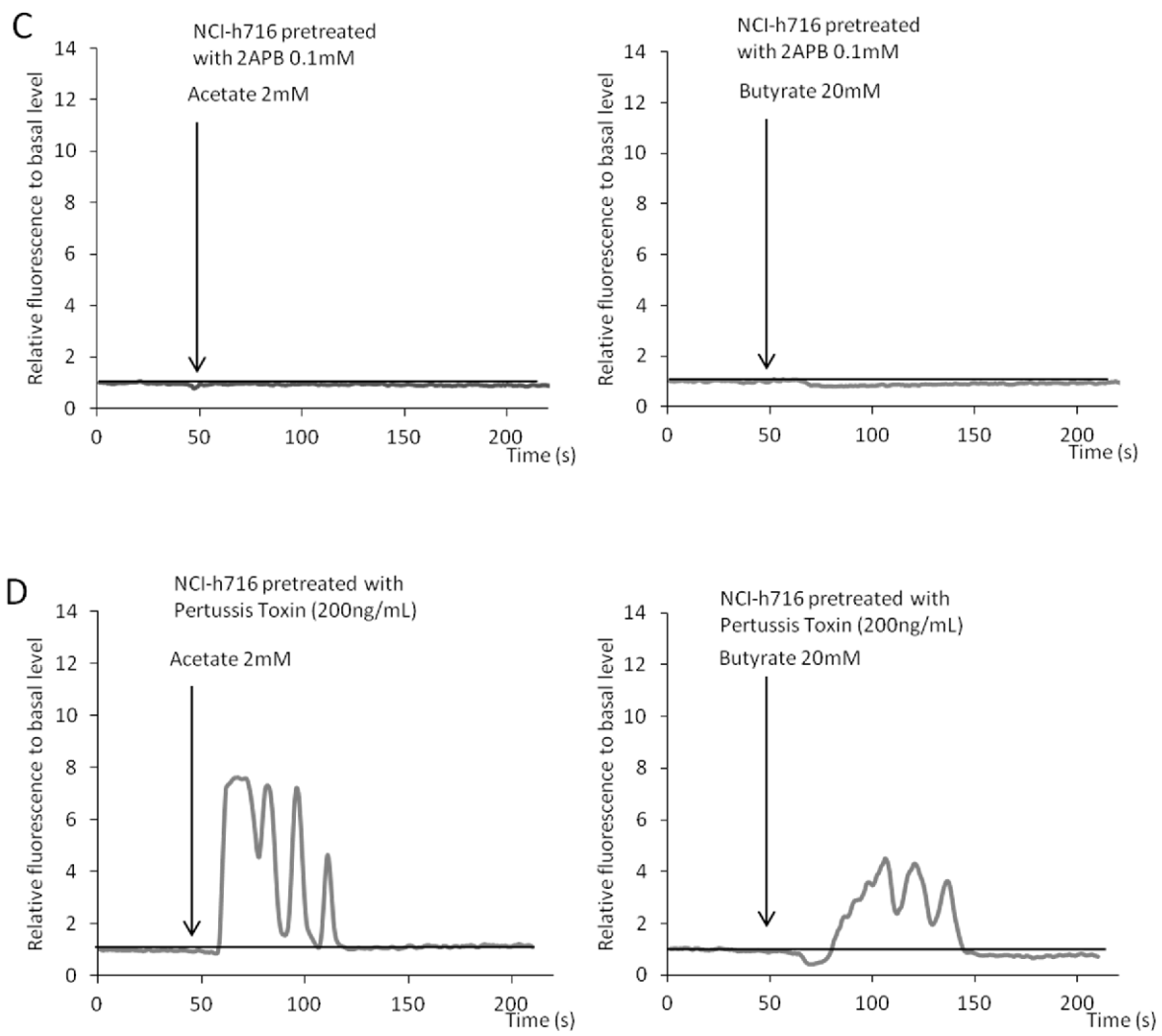
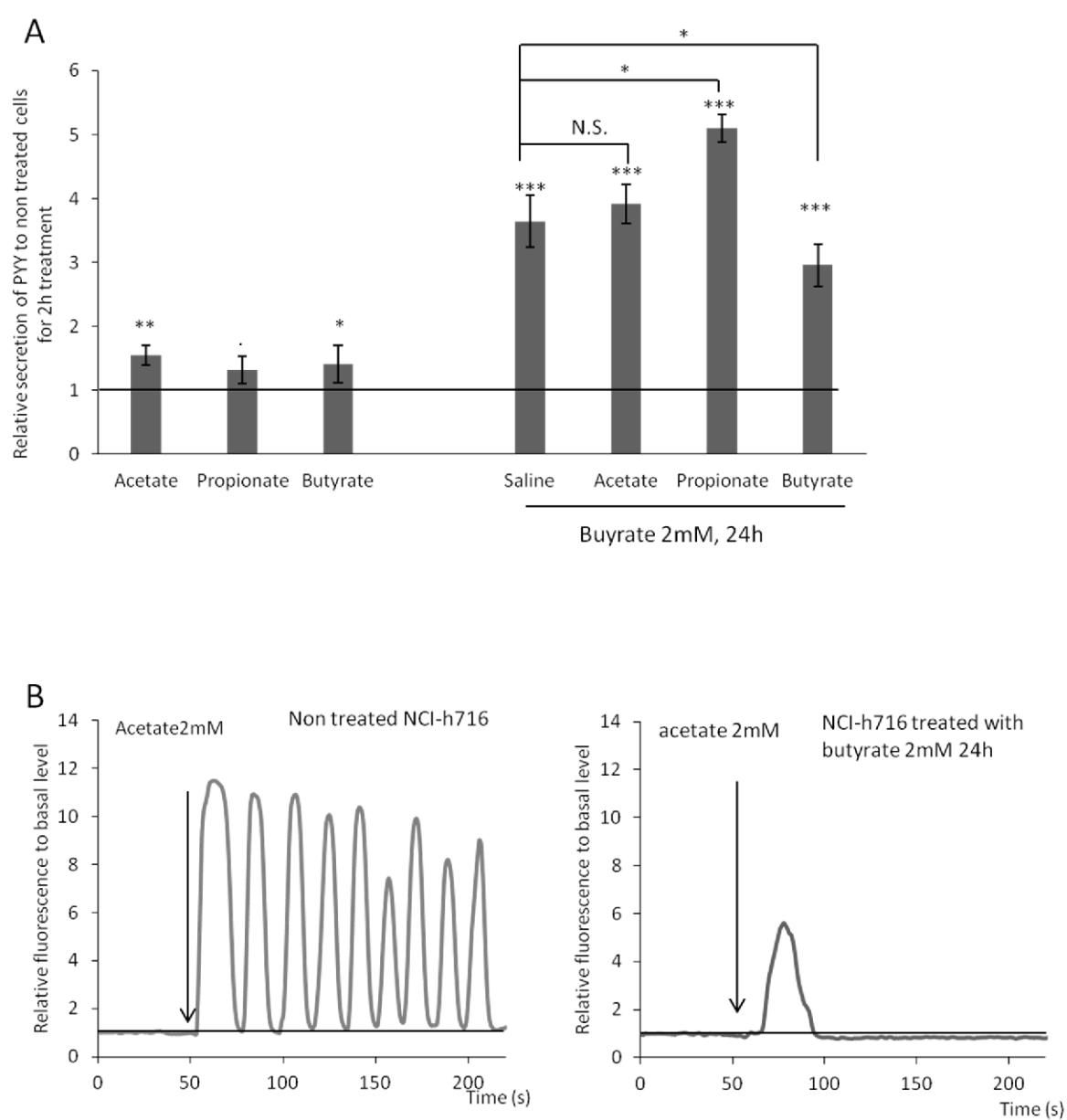
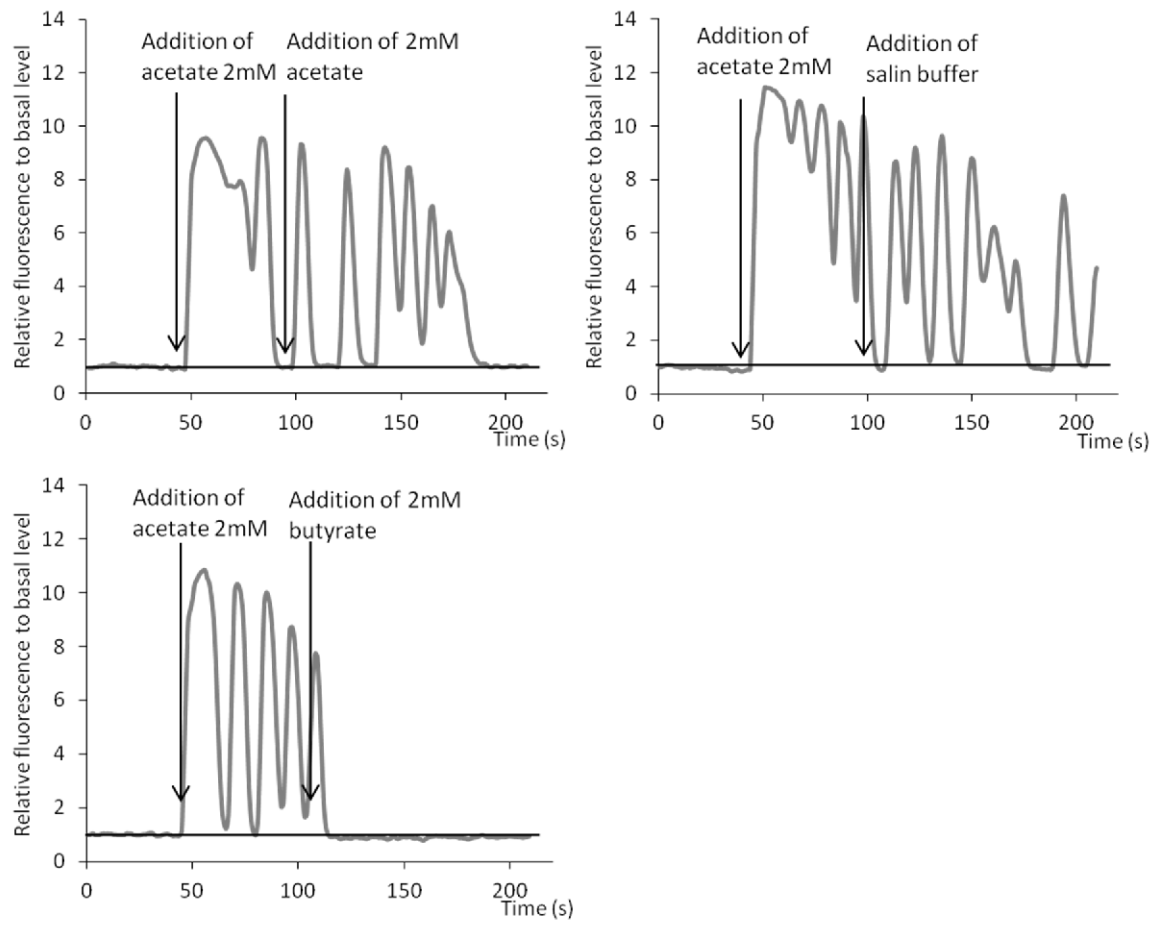


Figure 7



C



D

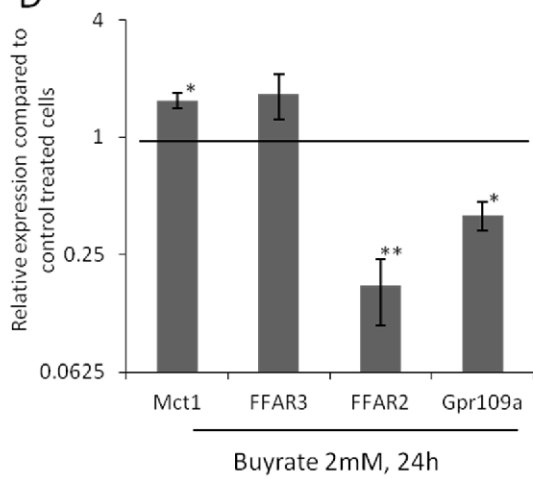
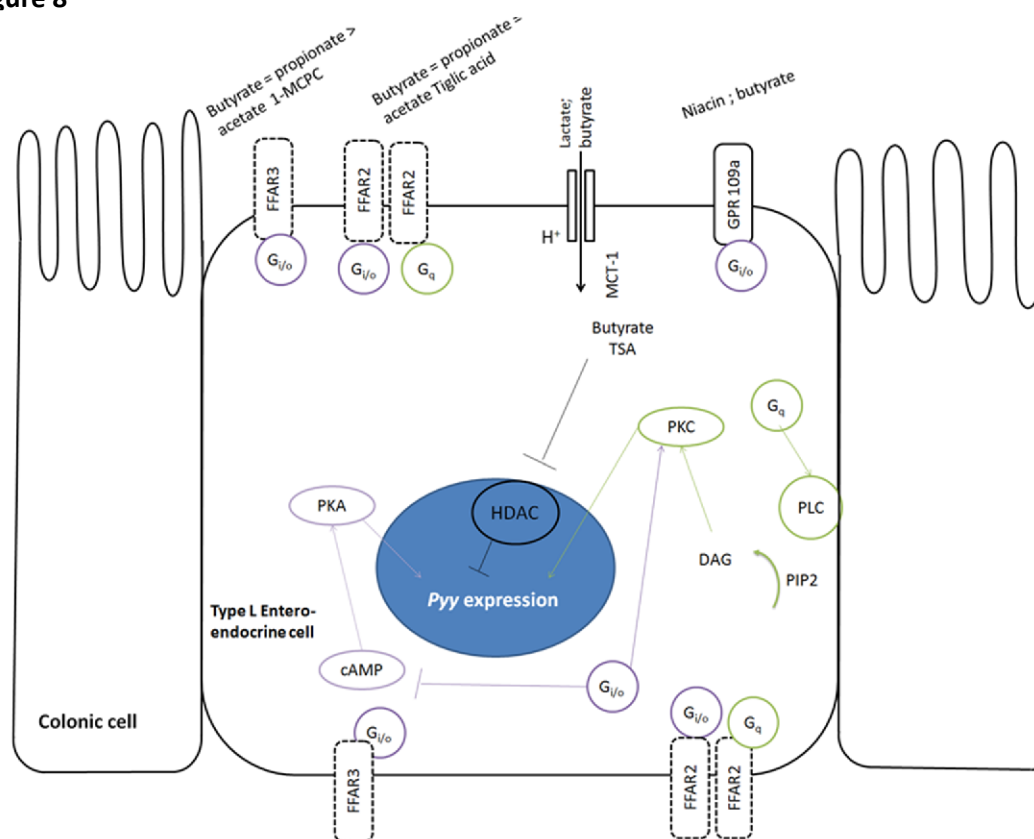
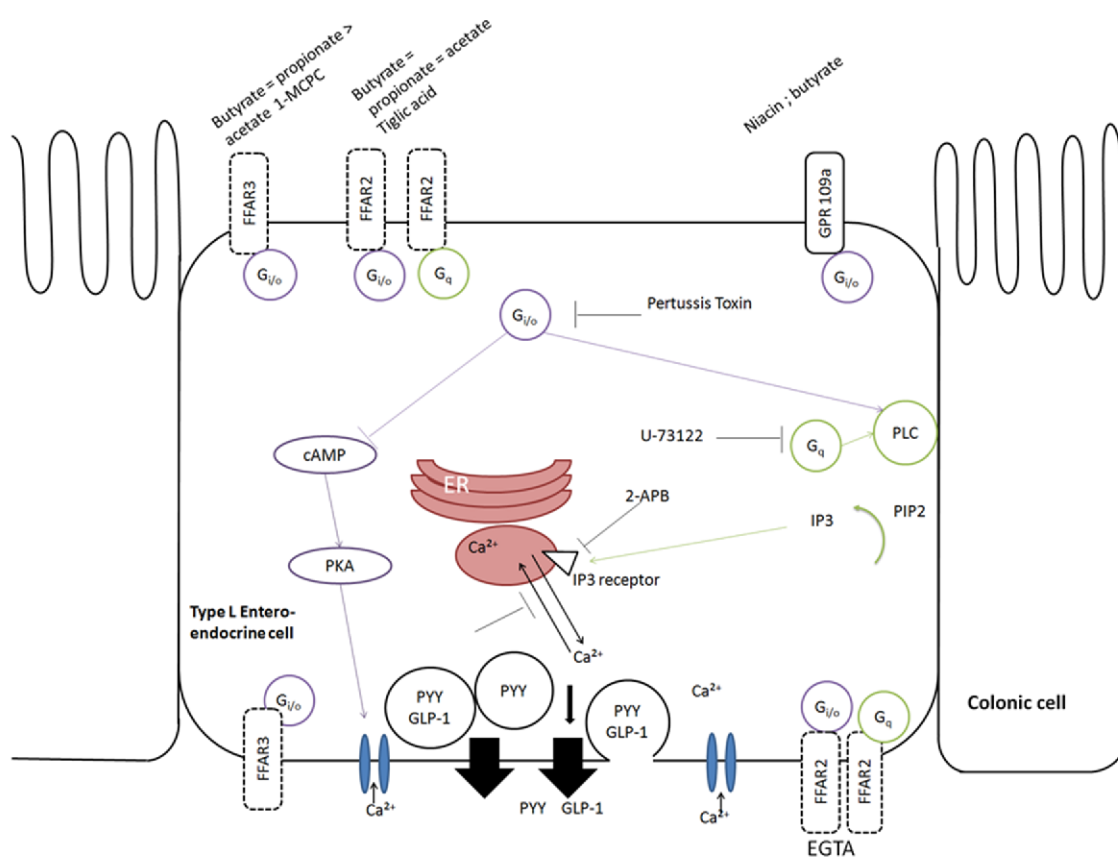


Figure 8

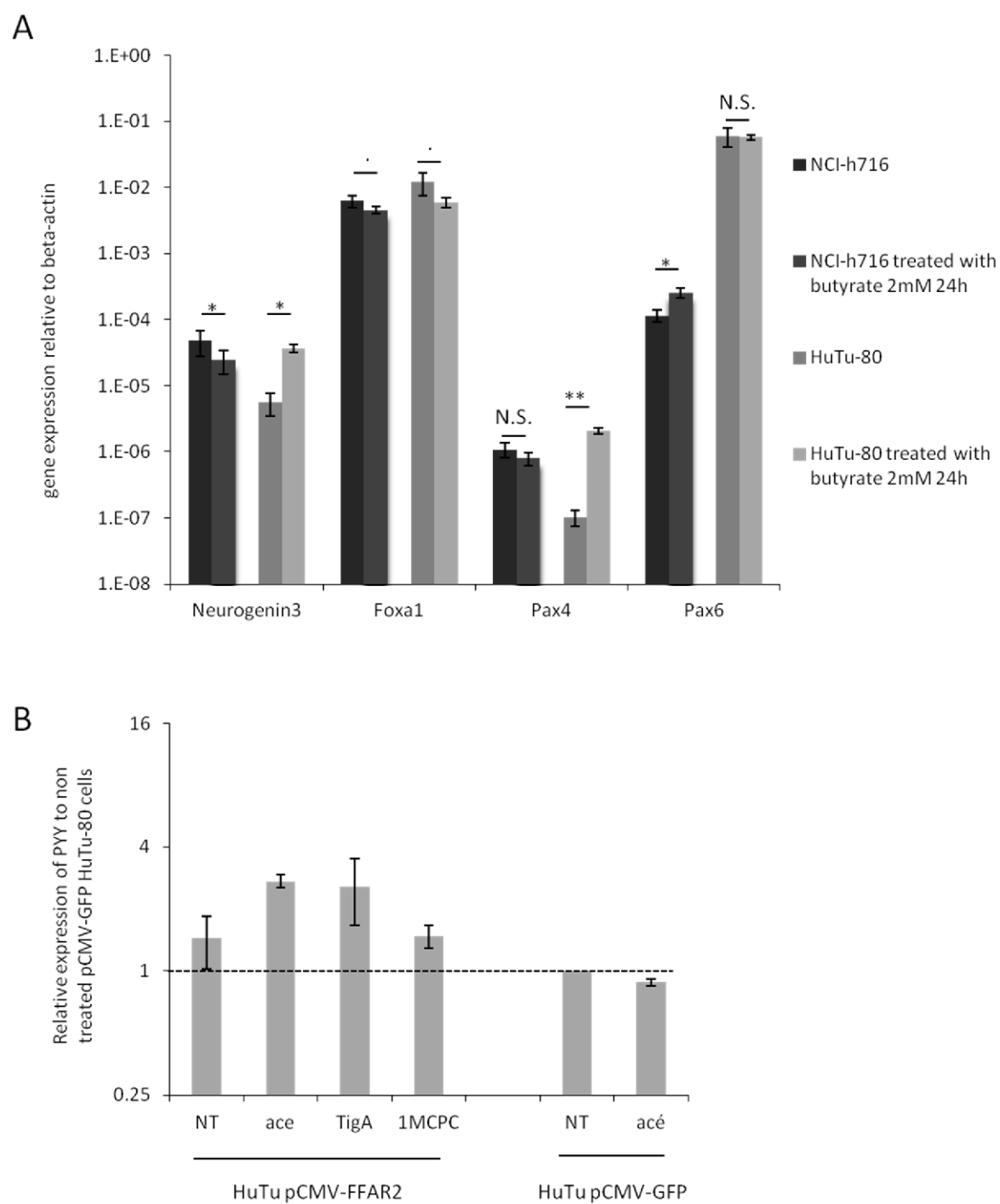


Pyy expression Regulation

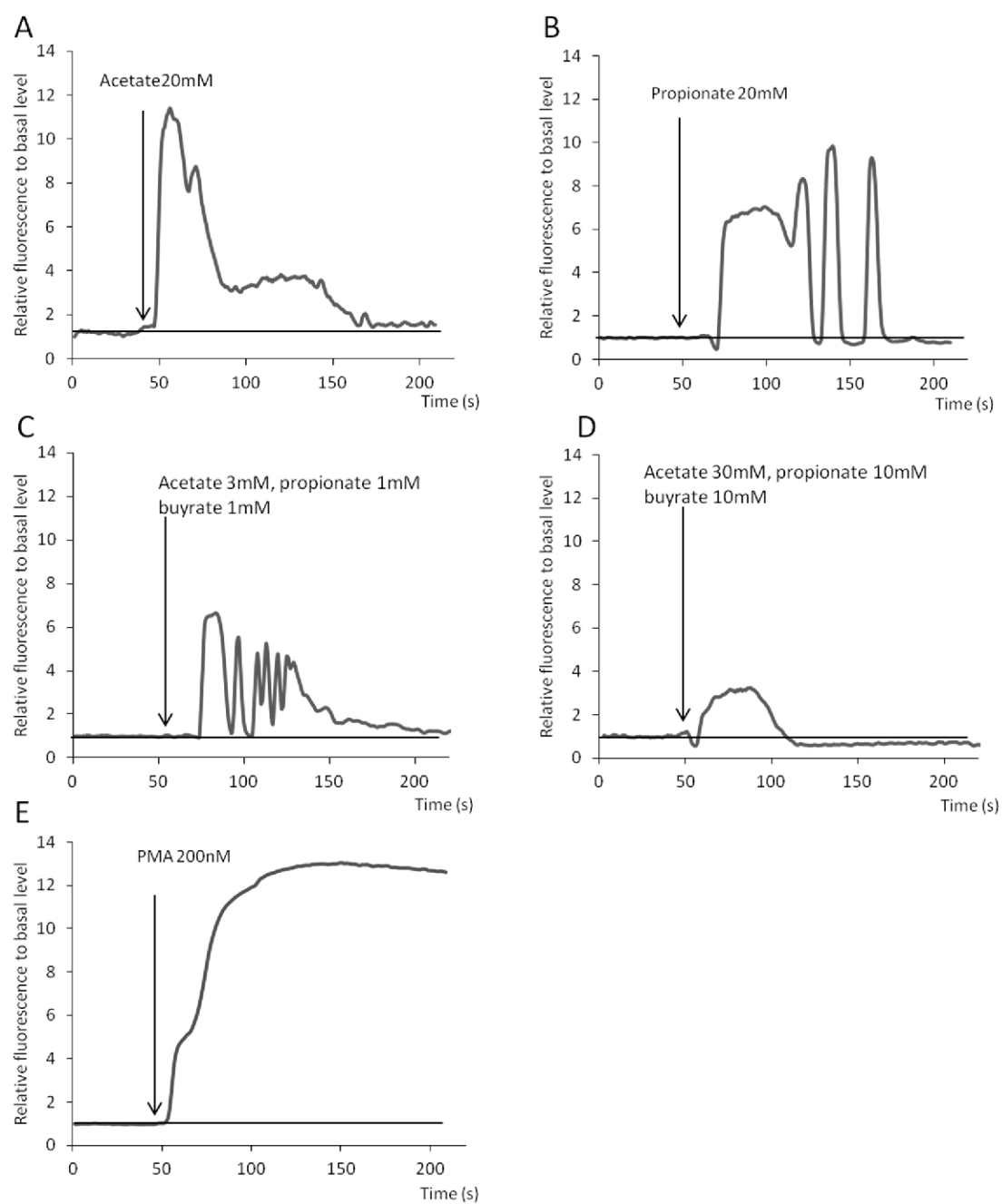


Secretion Regulation

**Supplementary Figure 1**



**Supplementary Figure 2**





**Supplementary Table 1**

GAPDH	Hs02758991_g1
cyclophilin A	Hs04194521_s1
$\beta$ -actin	Hs01060665_g1
PYY	Hs00373890_g1
proglucagon	Hs01031536_m1
FFAR3	Hs02519193_g1
FFAR2	Hs00271142_s1
GPR93	Hs00252675_s1
MCT1	Hs00161826_m1
ChromograninA	Hs00900375_m1
GPR109a	Hs02341584_s1
SLC5A8	Hs00377618_m1
Neurogenin3	Hs01875204_s1
FoxA1	Hs04187555_m1
Pax6	Hs00240871_m1
Pax4	Hs00173014_m1

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# Microbial sensing by TLRs in L-cells is increased by butyrate and modulates Pyy expression

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*Abbreviations used in this paper:* cytosolic calcium concentration: [Ca<sup>2+</sup>]<sub>i</sub>; EEC: enteroendocrine cells; GLP-1 glucagon-like peptide-1; LPS: Lipopolysaccharide; MAMPs: Microbial Associated Molecular Patterns; PYY: peptide YY; SCFAs: short chain fatty acids; TLRs: Toll-like Receptors

**Abstract:**

The intestinal epithelium is an active barrier between host and microbiota and sense microbial through expression of TLRs. However, expression and function of TLRs in a specific subtype of intestinal cells, enteroendocrine L-cells, implicated in regulation of different gut motility, food intake and insulin secretion has not yet been assessed. Using a human cellular model of L-cells and a reporter system for NF- $\kappa$ B activation pathway, we reported functional expression of all TLRs except TLR4 and TLR8, and increased expression and activity after stimulation with butyrate, one main fermentation product. Stimulation of TLRs by agonists increased expression of *Pyy* but not *Proglucagon* in an additive manner with butyrate. Nucleic acids induced calcium response in these cells that may be associated with secretion. Altogether, our results demonstrated a role of TLRs in the modulation of *Pyy* expression and the importance of butyrate, a product of bacterial fermentation in regulation of microbial TLR-dependent sensing.

## Introduction

Enteroendocrine cells (EEC) are scarce but important cells of the intestinal epithelium that secrete hormones regulating many host functions including digestion, glucose and lipid metabolism and behavior<sup>1-4</sup>. Sensing of luminal content is mediated through expression of a great variety of receptors and transporters, including receptors that recognize fatty acids, peptones, bile acids as well as glucose and ion transporters, leading to secretion<sup>5-8</sup>. Different types of EECs are found along the gut, expressing specific hormones and receptors, depending on their localization and expression of differentiation factors<sup>4,9</sup>. Enteroendocrine L-cells are of main interest as they secrete GLP-1, GLP-2 and PYY which are implicated in regulation of insulin secretion<sup>10</sup>; intestinal permeability and differentiation<sup>11,12</sup>; and food intake and intestinal motility and secretion<sup>13,14</sup> respectively. EECs represent less than 1% of intestinal epithelial cells, limiting access to pure enteroendocrine populations. Only a few cell lines issued from human adenocarcinoma, corresponding to different enteroendocrine types have been developed so far. NCI-h716 cells have been first described to secrete GLP-1<sup>15</sup>, as well as PYY and express specific differentiation factors, and have been used as a human model to study enteroendocrine L-cells response to different luminal nutrients<sup>16-18</sup>.

Intestine harbors an important microbial community including bacteria, archaea, yeasts and viruses, mainly in the colon, named microbiota and that participates to the regulation of host immunity and in fiber digestion leading to the production of short chain fatty acids (SCFAs). SCFAs, mainly acetate, propionate and butyrate are at high concentration in the colon lumen and can be used by the host as energetic substrate and as signaling molecules regulating different functions<sup>19,20</sup>. Part of the cross talk between microbiota and intestinal cells is mediated through recognition of conserved microbial associated molecular patterns (MAMPs) by a super-family of receptors called Pattern Recognition Receptors present in epithelial cells and immune cells<sup>21</sup>. Among them, Toll-Like-Receptors have been described for their important role in the innate immune response, mainly by activating NF- $\kappa$ B signaling and inducing pro-inflammatory gene expression<sup>22</sup>. Expression of TLRs in intestinal epithelium cells has been described, and has been implicated in the regulation of barrier functions and Paneth cell degranulation<sup>23-26</sup>. Expression of some TLRs, TLR1, 2, 4, 5, 6 and 9, has been described in the murine enteroendocrine cell line STC-1<sup>27,28</sup> and their stimulation increased secretion of cholecystokinin, a hormone secreted by enteroendocrine I-cells, a cell type mainly present in the duodenum. TLRs are also expressed in enterochromaffin cells, a particular type of enteroendocrine cells, and their stimulation induced serotonin secretion<sup>27,29</sup>. L-cells are mainly present in the colon where microbial density and consequently concentration and diversity of MAMPs are the highest. However, expression of TLRs in this cell type has not been addressed to our knowledge and potential functions of TLR activation on PYY and GLP-1 production and secretion is unknown. In this study, we reported the expression of TLRs in human NCI-h716 cell line and the effect of another bacterial product, butyrate, on their expression. We questioned their implication in the regulation of gut hormone production and release, as they may participate in the cross talk between microbiota and host in the regulation of gut functions and food intake.



## Results

### *NCI-h716 cells express functional Tlrs at the exception of Tlr4 and Tlr8*

By qRT-PCR, we tested the expression of TLRs in NCI-h716 cells, and detected expression of all human *Tlrs* except *Tlr4* and *Tlr8* (Fig1A). *Tlr* expression was much lower than in THP-1 cells, a monocyte cell line, except for TLR6, highly expressed in both cell lines and TLR3 and TLR7, receptors for virus RNA which have low levels of expression in both cell lines (data not shown). Surprisingly, whereas TLR6 is supposed to function only in heterodimers with TLR2, its expression was much higher than *Tlr2* in NCI-h716 cells.

Then, we used NCI-h716 cells stably expressing a reporter system for NF- $\kappa$ B activity to test if these TLRs were functional in this cell line. Stimulation of most TLRs by specific agonists induced an increase of NF- $\kappa$ B activity in these cells. The highest responses were by activation of the TLR2/6 dimer by Pam2CSK, followed by activation of TLR1/2 dimer and TLR5 at the dose tested (see Material and Methods) (Fig1B). However TLR9 activation had very little effect and TLR3 and TLR7 agonists had no significant effect on NF- $\kappa$ B activation in NCI-h716, similarly to the results found in THP1 cells (data not shown and Invivogene data). Agonists of TLR4 and TLR8 had no effect, in accordance to the lack of expression of these receptors in these cells. NCI-h716 cells also responded to IL-1 $\beta$  and TNF- $\alpha$  by increasing NF- $\kappa$ B activity, indicating that they may also express receptors to these cytokines. We could therefore demonstrate that NCI-h716 cells express TLRs that are functional and induce an NF- $\kappa$ B activation when stimulated with specific agonists.

### *Butyrate increases TLR expression and activity*

Butyrate, a major fermentation product present at high doses (~20mM) in the colon lumen has been shown to modify expression of several genes, including receptors FFAR2 and GPR93 and *Pyy* in NCI-h716 (Larraufie et al, submitted, unpublished data). We tested if *Tlr* expression was also modified by butyrate stimulation. We highlighted that most *Tlrs* gene expression levels were increased by butyrate, except *Tlr6* which expression was already high, and *Tlr8* which was not expressed (Fig 2A). Moreover, we detected *Tlr4* expression after butyrate treatment whereas it was not detectable in untreated cells. Then, difference of expression prompted us to test TLR responses in cells treated with butyrate. As expected, we showed that in these cells, butyrate stimulation increased responses to TLR agonists except for agonists to TLR2/6 and TLR8 consistently with the unaffected expression of these receptors observed upon butyrate stimulation (Fig2B). Interestingly, stimulation with TLR3 and TLR7 agonists induced statistically significant activation of NF- $\kappa$ B after butyrate treatment whereas no activation was detected in untreated cells despite detectable expression of these *Tlrs*. Moreover, butyrate treated cells became sensitive to TLR4 agonist LPS, confirming functional expression of *Tlr4* after butyrate treatment.

Altogether, our results demonstrate that NCI-h716 cells, a model of colonic EEC L-cells, express functional TLRs except TLR8 and TLR4, and TLR expression and the TLR dependent responses are increased by butyrate, a compound usually found at high concentration in the lumen of the colon.

### *Stimulation of TLR increases Pyy expression in an additive manner with butyrate*

Promoter gene analysis revealed that *Pyy* promoter contains two putative binding domains to NF- $\kappa$ B in position -832 and -866 (data not shown). Thus, we hypothesized that *Pyy* expression may be increased consequently to NF- $\kappa$ B activation. By qRT-PCR, we showed that *Pyy* expression was increased by 80%-100% after treatment with TLR agonists that also activated NF- $\kappa$ B (Fig3A). Interestingly, TLR2/6 activation induced a similar *Pyy* expression as other TLR stimulations, indicating that increased activation of NF- $\kappa$ B did not have higher effect on *Pyy* expression. TLR3 and TLR4 activation which induced no NF- $\kappa$ B activation had no effect on *Pyy* expression. Surprisingly, TLR7 and TLR8 agonists induced no NF- $\kappa$ B response in these cells but they increased *Pyy* expression similarly as other TLRs agonists. Moreover, IL-1 increased *Pyy* expression, but TNF- $\alpha$  had no effect, which may be explained by different signaling pathways. Interestingly, TLR stimulation did not increase expression of *proglucagon*, another gene coding for two hormonal peptides secreted by L-type EEC, which could be anticipated as no NF- $\kappa$ B binding site was detected in its promoter (Fig 3C).

As butyrate increased functionally *Tlr* expression and had strong effect on *Pyy* expression (Larraufie et al, submitted), we tested if both stimulations were cumulative. Butyrate alone increased *Pyy* expression by a fold of about 300 after 48h of stimulation, and addition of TLR agonist for the last 24h of treatment with butyrate increased *Pyy* expression to a fold of about 500 compared to untreated cells (Fig3B). Interestingly, stimulation of TLR3 and TLR4 both increased *Pyy* expression after butyrate stimulation whereas they had no effect on untreated cells. We therefore concluded that a cumulative effect between butyrate and TLR activation increased *Pyy* expression which might mimic physiological colon environment.

### *Only Nucleic acids related TLR agonists induced calcium fluxes*

Important feature of enteroendocrine cells is to secrete hormones in response to different stimulations. As calcium signaling is necessary for secretion, we assessed secretion indirectly using calcium imaging in NCI-h716 stably expressing GCaMP-5G, a calcium concentration dependent fluorescent protein. Only agonists of endosomal TLRs recognizing nucleic acids, TLR3, TLR7, TLR8 and TLR9 induced a small and brief calcium concentration increase, which was only observed in a third of the cells (Fig 3B). Stimulation of all the other TLRs had no detectable effect on calcium signaling indicating that stimulation of TLRs recognizing bacterial and yeast membrane patterns (TLR1/2, 2, 4, 5 and 2/6) may not induce directly secretion in these cells. We therefore concluded that TLR stimulation may not directly induce secretion in these cells, but may have a long term effect by increasing its expression, and therefore the quantity of PYY produced and secreted.

## Material and methods:

### Cell culture:

NCI-h716 (a kind gift from C. Roche, Lyon, France) were cultivated in RPMI (Fischer Scientific) supplemented with 10% SVF, 2mM L-glutamine and 50IU/mL penicillin and 50ug/mL streptomycin in a humidified incubator at 37°C, 5% CO<sub>2</sub>. Cells stably expressing the calcium probe CMV-GCaMP-5G (a gift from D. Kim and L. Looger (Addgene plasmid #31788)) and the NF-κB reporter system pNifty2 SEAP (Invivogene) were selected from a clonal population and maintained using respectively G418 (500μg/mL) and Zeocin (50μg/mL).

### TLR agonists and cytokines:

All TLRs ligands were purchased from Invivogene and were used at recommended concentration: Pam3CSK4 (TLR1/2) : 500ng/mL ; Poly(I:C) (TLR3) : 10ug/mL ; LPS (TLR4) : 1ug/mL ; Flagellin (TLR5) : 100ng/mL ; Pam2CSK4 (TLR2/6) : 10ng/mL ; Imiquimod (TLR7) : 1ug/mL ; Cl075 (TLR8) : 1ug/mL, ODN2395 (TLR9) : 5ug/mL. Human recombinant IL-1β (10ng/mL) and human recombinant TNFα (10ng/mL) were purchased from Peprotech.

### RNA extraction and qRT-PCR

10<sup>6</sup> NCI-h716 cells were seeded 48 hours before lysis in 12 well-plates. TLR agonists were added 24h before RNA extraction if not indicated otherwise. RNA was extracted using a Qiagen RNeasy minikit with a DNase treatment (Qiagen) according to manufacturer's instructions. 2ug of RNA were used for RT using High capacity cDNA Reverse transcription kit (Applied Biosystems). qPCR was performed on a AbiPrism 7000 system with Taqman gene expression assay probes for TLR1-9, Pyy, proglucagon, β-actin and GAPDH (resp Hs00413978\_m1 ; Hs01872448\_s1 ; Hs01551078\_m1 ; Hs00152939\_m1 ; Hs01920773\_s1 ; Hs01039989\_s1 ; Hs01933259\_s1 ; Hs00152972\_m1 ; Hs00370913\_s1 ; Hs00373890\_g1 ; Hs01031536\_m1 ; Hs01060665\_g1 ; Hs02758991\_g1 (Lifetechnologies)), and Taqman gene expression master mix (Lifetechnologies). Data was analyzed using 7000 System SDS software (Applied Biosystems) normalized to β-actin and Student nonpaired t-test analysis was used for statistical analysis.

### NF-κB activity

NCI-h716 pNiftySEAP2 cells were seeded at 5.10<sup>4</sup> per well in 96 well-plates and incubated for 24h in 100uL RPMI with TLR agonist or control. 20μL supernatant was used for revelation in 200μL Quantibblue reagent (Invivogene), and 655nm OD was measured after 6h incubation at 37°C using a microplate reader (Infinite 200, Tecan). Data were normalized to non-treated cells for each experiment and analyzed using Student's t test.

### Calcium Imaging

NCI-h716 pCMV-GCaMP5G cells were seeded at 5.10<sup>4</sup> two hours before stimulation cells per well in saline solution (NaCl 140mM, KCl 5mM, MgCl<sub>2</sub> 2mM, CaCl<sub>2</sub> 2mM, Hepes 10mM, sucrose 6mM and glucose 10mM, pH adjusted to 7.35). Cells were observed on a Zeiss LSM700 confocal laser scanning microscope (MIMA2 platform, INRA) at 25°C and filmed for 4 minutes at 1 image per second. Reagents were added volume/volume 50s after beginning of acquisition unless specified. Image analysis and cell fluorescence measurement were achieved using Fiji software<sup>30</sup>, data analysis was performed using R.

## Discussion:

PYY is an important hormone implicated in the regulation of food intake and gut motility, requiring tight regulation of its expression and secretion. In this study, we could demonstrate that the cells secreting PYY in the gut, the enteroendocrine L-cells, can sense microbial molecules through the expression of a variety of TLRs. *Tlr* Expression was similar to intestinal epithelial cells and enterochromaffin cells except for *Tlr4* which could not be detected by qRT-PCR in NCI-h716 cells<sup>23,27,28</sup>. Moreover, we showed that these cells are also stimulated by another bacterial product, butyrate, present at high concentration in the colon. In addition, we showed a butyrate dependent increase of TLR expression leading to higher NF- $\kappa$ B activity. This indicate a potential process in which L-cells responded to microbial fermentation by increasing receptors and sensing of the microbiota. Moreover, butyrate modified the cellular responses to MAMPs. Indeed, we observed a butyrate-dependent *Tlr4* expression, and a butyrate-dependent TLR3 and TLR7 upregulation leading to a NF- $\kappa$ B activation. We therefore proposed that butyrate can participate in immune regulation in L-cells by increasing microbial sensitivity for a range of patterns. Effect of butyrate on *Tlr* expression in other intestinal epithelial cells is not known, but comparison between expression of *Tlrs* in small intestine and colon, where butyrate concentration is higher, shows that colonic epithelial cells expressed a broader range of *Tlrs*. Small intestinal and colonic cells have different stem cell origin, but their environment and in particular the presence of butyrate could be an interesting hypothesis to explain differences in *Tlr* expression<sup>24</sup>. However, L-cells have been mainly studied for PYY and GLP-1 secretion, but their role in immunity is not known. The scarcity of EECs in the intestinal epithelium makes difficult the study of cytokine expression and secretion by these cells. However, in enterochromaffin cellular models, TLR stimulation induced an increase of expression of pro-inflammatory genes, indicating that enteroendocrine cells may participate in immune response<sup>31</sup>.

As in our model, cells were not polarized, we could not assess the TLR localization precisely which would have gave us clues of their role in these cells: to sense commensals microorganisms in the colon lumen or microorganisms that have crossed the epithelial barrier and are potential pathogens for the host. Indeed, the two localizations have not the same role as the first one participate in the sensing and regulation of a commensal population which activates continuously the TLRs and should not induce a strong inflammatory response whereas the second participates in sensing of potential infection and therefore should induce an inflammatory response. Moreover, stimulation of the same TLR can induce different responses depending of its localization<sup>32</sup>. *In vivo* studies are therefore required to validate expression of TLRs in human L-cells and determine their localization. Moreover, more functional studies may decipher precise role of expression of TLRs in these cells.

Promoter analysis revealed potential NF- $\kappa$ B binding domains in *Pyy* promoter but not in *proglucagon* promoter suggesting that *Pyy* may be regulated by TLR stimulation. Our results confirmed partially this hypothesis as *Pyy* but not *proglucagon* expression was increased after TLR stimulation leading to activation of NF- $\kappa$ B in these cells. Interestingly, TLR3 and TLR4 stimulation did not induce NF- $\kappa$ B activation and had no effect on *Pyy* expression. Butyrate treatment increased *Tlr* expression, leading to a NF- $\kappa$ B activation and higher expression of *Pyy* by their agonist. This suggest that NF- $\kappa$ B activation upregulates *Pyy*. However, TLR7 and TLR8 agonists did not induce detectable activation of NF- $\kappa$ B but increased *Pyy* expression. TLR7 and TLR8 stimulation did not induce detectable NF- $\kappa$ B activation in our conditions, however, it may induce a weak activation that did not activate our reporter system, but may be sufficient to increase *Pyy* expression. TLR7 and TLR8 agonists may also activate other pathways that may also induce increased *Pyy* expression. Effect of TLR8 agonist was surprising as we could not detect expression of *Tlr8* in these cells nor associated to NF- $\kappa$ B activation. However, human TLR8 and TLR7 recognize similar MAMPs, single strand RNAs<sup>33</sup>. We therefore could not exclude that agonists for

TLR8 can also activate with lower potency TLR7, and thus effects described may be mediated through TLR7. Moreover, increased expression of *Pyy* was not dependent on NF- $\kappa$ B activation level as all agonists increased *Pyy* expression similarly whereas they had a wide range of effects on the activation of NF- $\kappa$ B profiles. We therefore hypothesized that *Pyy* expression may be regulated depending on a threshold of NF- $\kappa$ B activation in a binary manner, and TLR7 stimulation induced a sufficient NF- $\kappa$ B activation, even if not detectable in our conditions, to increase *Pyy* expression. Moreover, we highlighted that effect of TLR stimulation on *Pyy* expression was additive to butyrate effect, highlighting the important role of microbiota in increasing PYY production.

Interestingly, only *Pyy* but not *Proglucagon* expression was increased by MAMPs, indicating a potential unique feature of PYY as a sensor of microbiota among gut peptides. Butyrate also increased *Pyy* but not *proglucagon* expression (Larraufie et al, submitted). Indeed, PYY is the hormone for which pattern of expression correlate to the density of microbiota<sup>34</sup> and its functions may be important to regulate microbiota as it modulates food intake and small intestine and colonic motility revealing a potential important crosstalk between microbiota and host through this hormone<sup>35,36</sup>.

Increased expression of *Pyy* during colonic infection due to TLR stimulation of L-cells could participate in host response by decreasing food intake and modulating intestinal motility, two important functions of PYY. PYY production has been reported to be increased after sepsis<sup>37</sup> or in some infections<sup>38-41</sup>. Anorexia associated to infection has been shown to be an important mechanism of host defense, and increased PYY circulating levels could participate in the regulation of food intake during colonic infection<sup>42,43</sup>. Moreover, PYY has different roles on motility due to different receptor activation after peptidic maturation. PYY matured form, PYY 3-36 decreases motility by inhibiting neuronal activity<sup>13</sup> whereas PYY1-36 increases motility directly increasing muscle contractions<sup>36,44</sup>. Therefore, increasing PYY may lead to a local increased colonic motility, a physical way for expelling pathogens and limiting nutrient availability in the concerned area by impairing transit upstream, and therefore permitting increased nutrient absorption in non-pathogen infected area. Therefore, we could hypothesized that PYY may be an actor of host response to colonic infection as production is increased by TLR stimulation and potential other pathways.

L-cells are described as sensors of luminal content and secrete PYY and GLP-1 in response to different signaling molecules, with increased secretion after food intake. PYY mainly regulates ileal brake after chyme entering into the duodenum in order to promote nutrient absorption by higher transit time and regulating enzyme and electrolyte secretion<sup>45,46</sup> and satiety by decreasing food intake<sup>47</sup>. Regulation of basal secretion of gut hormones is less studied as secretion is mainly monitored by modification of luminal nutrient composition. However, due to its short half-life, PYY must be secreted continuously. We believed that it could be partially explained by a constant microbiota dependent stimulation. Moreover, it can be hypothesized that increased production of PYY by L-cells impacts basal PYY levels, and therefore increase satiety.

Rapid rise of PYY plasma levels is due to stimulation of regulated secretion, releasing PYY stored in secretory granules. Regulated secretion is dependent on calcium concentration rise. We showed that only stimulation of TLRs recognizing nucleic acids induced a small transient calcium concentration increase in about a third of cells. However, as all ligands induced this response, even agonists for TLR8 which is not detected, we could not conclude if this effect was directly mediated by TLR stimulation. Indeed, nucleic acids are also recognized by other pattern recognition receptors including Retinoic Acid Inducible Protein I (RIG-I), Melanoma Differentiation Associate Gene 5 (MDA-5), IFN $\beta$  promoter stimulator 1 (IPS-1) or DNA-dependent activator of IFN regulatory factors (DAI)<sup>48</sup>. Moreover, These TLRs are endoplasmic, and therefore necessitate the endocytosis of their agonists to induce their stimulation, but the responses we detected were very rapid, which may not correspond to kinetics

observed for such response. Different studies reported the role of TLR activation on secretion or degranulation in other intestinal cells, in models of small intestine enteroendocrine cells STC-1<sup>27,28</sup>, enterochromaffin cells<sup>29,31</sup> as well as Paneth cells in the small intestine<sup>26,49</sup>. However, we could not reproduce these effects in NCI-h716, indicating that pathways may be activated differently depending on the cell type.

Altogether, our results highlighted a role of TLR expression in enteroendocrine cells by regulating *Pyy* expression, which may be an important mechanism implicated in regulation of microbiota and in host defense during colonic infection. However, *in vivo* studies are required to confirm these *in vitro* results and precise the mechanisms implicated. A few studies described a role of TLR in metabolism. In mice depleted for TLR5 had important microbiota modifications and presented phenotype similar to metabolic syndrome. Increased food intake was also found in these mice compared to wild type mice<sup>50</sup>. Implication of PYY was not assessed in this study, but it is possible that PYY plasma levels may be decreased due to lack of TLR5 induced *Pyy* expression. Modification of microbiota has also been associated with intestinal motility modifications, and stimulation of TLR4 and TLR5 inhibited ileal induced motility in a mechanism similar to PYY<sup>51,52</sup>. Considering our results, it may be hypothesized that PYY regulates these functions by sensing gut microbiota and therefore modulates host responses. Interestingly, TLR-dependent effects were modified by another bacterial product, butyrate, indicating a relationship between different bacterial products.

Figure 1

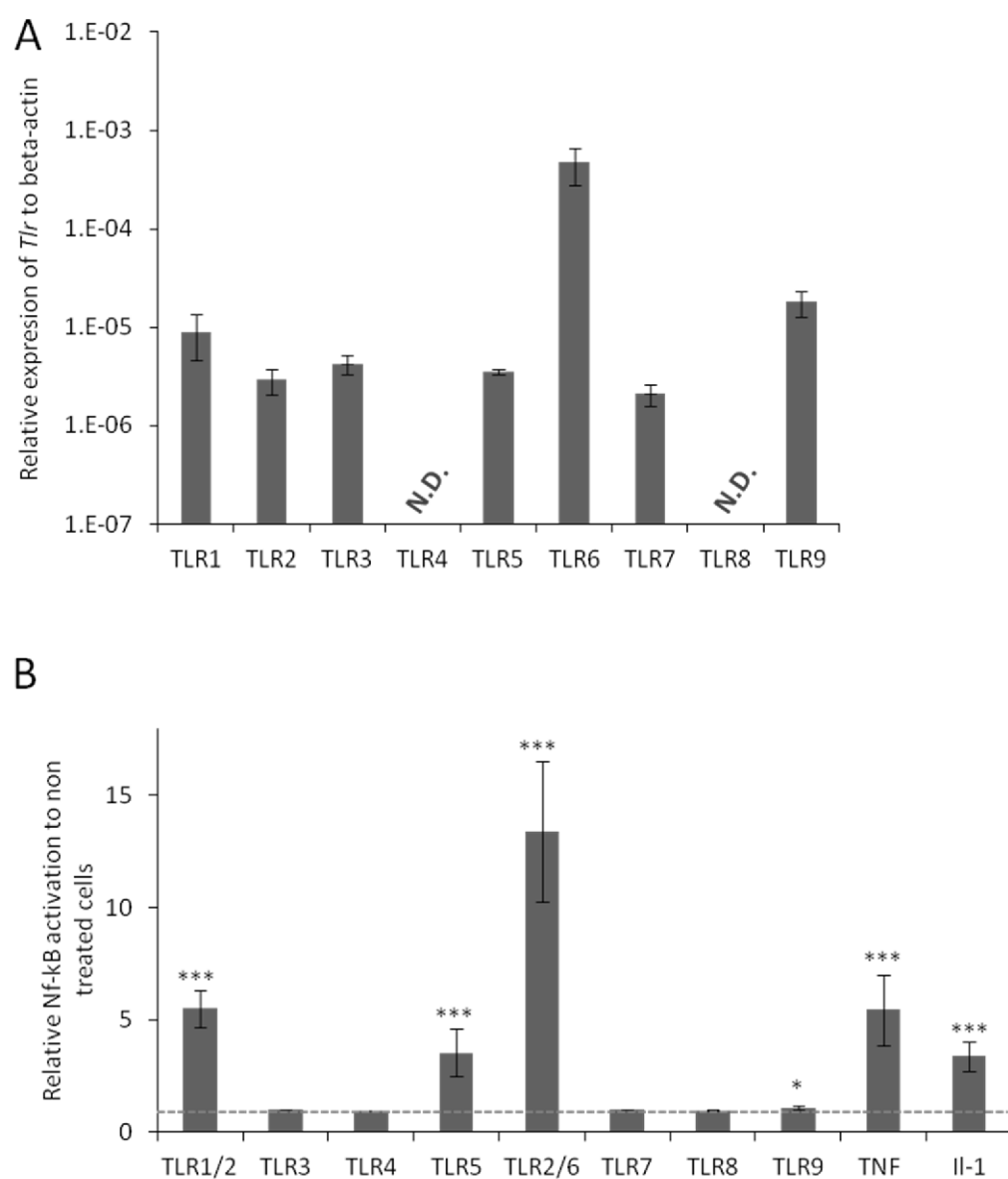


Figure 2

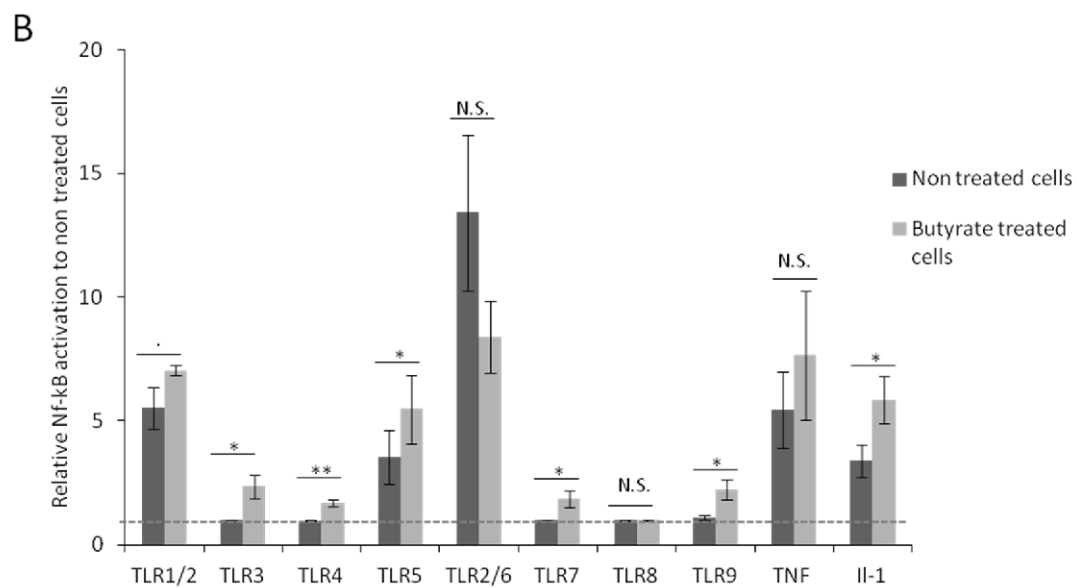
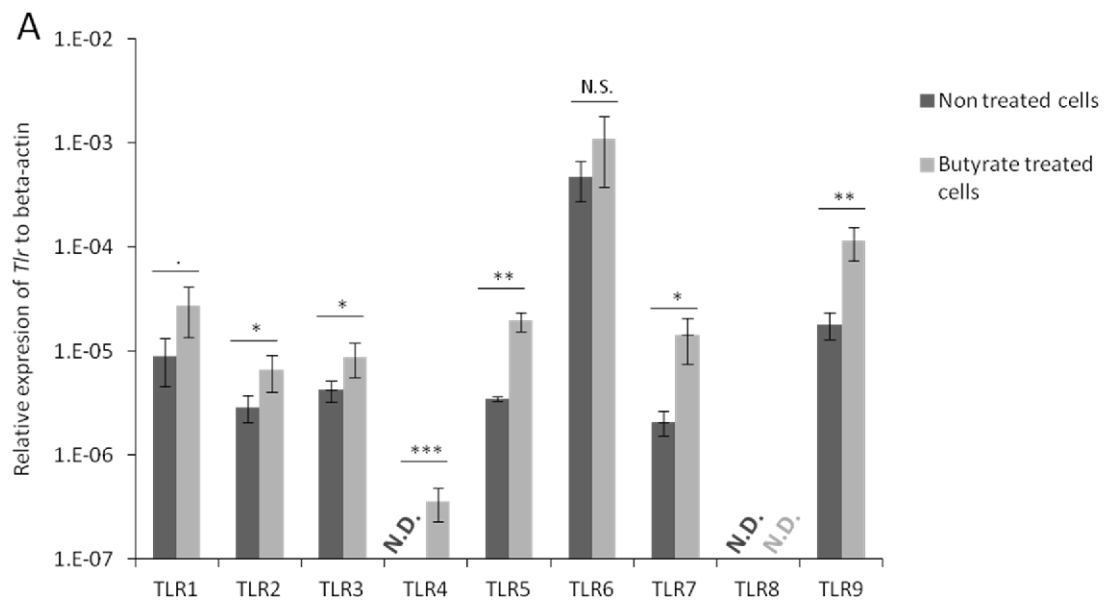
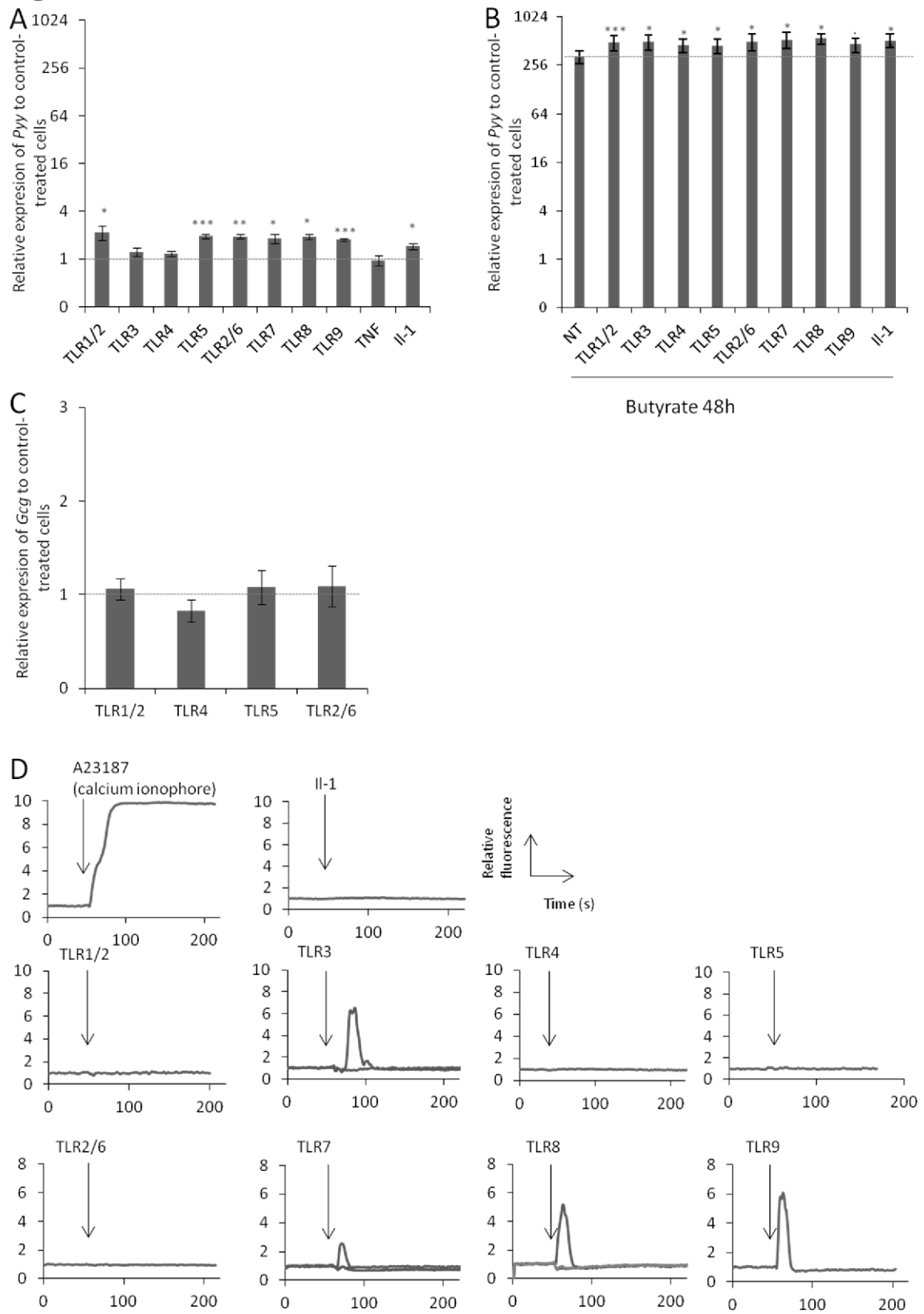




Figure 3



## Figure legends

### Figure 1: NCI-h716 express functional TLRs

(A): Expression of *Tlr* 1-9 was detected by qRT-PCR in NCI-h716 cells. Data are expression relative to  $\beta$ -actin determined by the  $2^{-\Delta Ct}$  method, represented as means  $\pm$  sem on at least four distinct experiments performed in duplicate. N.D.: Not Detected (B): Activation of NF- $\kappa$ B in NCI-h716 Nifty2SEAP by TLR agonists relative to non-treated cells measured by quantification of SEAP secreted after 24h stimulation. Data are means  $\pm$  sem of at least three distinct experiments performed in duplicate.

### Figure 2: Butyrate increases TLR expression and increase response to TLR agonists

(A): Expression of *Tlr* 1-9 was determined by qRT-PCR in NCI-h716 treated with butyrate 2mM for 24h (grey bars) or not (black bars). Data are expression relative to  $\beta$ -actin determined by the  $2^{-\Delta Ct}$  method, represented as means  $\pm$  sem on at least 3 distinct experiments performed in duplicate, N.D. : Not Detected. (\*\*\*:  $p < 0.001$  ; \*\*:  $p < 0.01$  ; \*:  $p < 0.05$ ). (B): Activation of NF- $\kappa$ B in NCI-h716 cells treated with butyrate (grey bars) or not (black bars) by TLR agonists relative to non-treated cells measured by quantification of SEAP secreted after 24h stimulation. Data are relative means  $\pm$  sem of at least three distinct experiments. (\*\*\*:  $p < 0.001$  ; \*\*:  $p < 0.01$  ; \*:  $p < 0.05$ ).

### Figure 3: TLR activation increase *Pyy* expression, effects are additive with butyrate

A-C: Effect of stimulation during 24h with TLR agonists on *Pyy* expression in non-treated NCI-h716 cells (A) and treated cells for 24h with butyrate 2mM before stimulation (B). (C): Effect of stimulation of NCI-h716 stimulation during 24h with TLR agonists on *Proglucagon* (*Gcg*) expression in non-treated NCI-h716 cells. Relative expression of *Pyy* and *Gcg* is determined by the  $2^{-\Delta\Delta Ct}$  method using  $\beta$ -actin as control gene. Data are means  $\pm$  sem of at least three distinct experiments. (\*\*\*:  $p < 0.001$  ; \*\*:  $p < 0.01$  ; \*:  $p < 0.05$ ). (D): Calcium response of NCI-h716 GCaMP-5G cells to TLR agonists by measurement of fluorescence variation in single cells. Curve represents relative fluorescence to mean fluorescence before drug addition in one representative cell of 50-100 cells acquired per experiment, repeated at least four times. Stimulation by adding TLR agonist is indicated by a vertical arrow.

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# Discussion



## In vitro cellular models to study PYY release by L-cells

### Modulation of PYY plasma levels

Regulation of circulating levels of hormones is important to ensure their functions at the right place and at the right time. PYY is present at low concentration during fasting, and its plasma concentration increases and stay high for several hours after food intake. It has functions in several organs including gut, hypothalamus, pancreas, adipose tissue and bone. However, little is known on its precise local concentration as it is supposed to be rapidly degraded (Addison 2011). Regulation of its plasma level is dependent on many different parameters, from increased number of producing cells to degradation. PYY levels can be regulated by the modulation of the number of enteroendocrine cells through proliferation, differentiation or apoptosis, the modulation of gene expression, of post translational regulations, of secretion, of maturation into PYY 3-36 and of peptide degradation. *In vivo* models only permit measurement of effects of elements (molecules, modification of microbiota by diet or antibiotics, inoculation of specific bacterial strain, surgery...) on PYY plasma levels and eventually number of L-cells and PYY content in intestinal epithelial tissue but cannot decipher the precise mechanisms implicated in the modulation of PYY plasma levels. PYY secretion can be divided in three parts. During fasting, secretion is low maintaining basal plasma PYY concentration. Mechanisms regulating this secretion have not been described and can depend on autocrine, paracrine, endocrine signaling as well as in sensing of luminal content. Just after food intake, nutrients are sensed proximally by duodenal enteroendocrine cells that signal through afferent neurons, mainly by secreting CCK, to induce an increase of PYY secretion, resulting in increased PYY plasma concentration (Degen 2007, Lin 2000). This mechanism allows to regulate digestive functions and to induce satiation by increasing rapidly levels of PYY in response to food intake. Finally, PYY plasma levels remain high for several hours after food intake due to direct sensing of molecules like secondary bile acids or peptones reaching the colon a few hours after food intake.

Most studies focused on PYY plasma concentration variations during one or two hours after administration of a specific compound. Thus they could only assess rapid mechanisms, mainly secretion and degradation (Adrian 1993, Chambers 2014, Freeland 2010, Fu-Cheng 1995, Geraedts 2011). Long term modulation of PYY was also studied after diet modification or surgery, thus assessing the result of all possible modification described (Batterham 2006, Cani 2009, Chandarana 2011, Delzenne 2005, Pfluger 2007, Reimer 2012,). A few studies could also demonstrate that the number of L-cells could be regulated in the long term (Cani 2007, Mumphrey 2013). These results indicated potential regulations of PYY, but that could be explained through different mechanisms. *Ex vivo* and *in vitro* models have been used to demonstrate specific mechanisms implicated in the response to molecules. Secretion was mainly studied as an important mechanism to modulate rapidly PYY plasma levels (Cordier-Bussat 1998, Hand 2012, Psichas 2015, Svendsen 2014). Many studies focused on modulation of GLP-1 secretion (Reimer 2001, Reimer 2006, Simpson 2007, Tolhurst 2012), due to a high interest in regulation of GLP-1 plasma levels for type II diabetes treatment. Results from GLP-1 secretion studies are thought to be extended to PYY secretion as both hormones are present in same secretory granules (Nilsson 1991). Moreover, secretion could be assessed using calcium imaging to demonstrate direct effect of molecule on secretion as a rise of calcium concentration increase is required for secretion. However, all these effects corresponded to rapid response to a stimulation, which may mimic nutrient or food sensing in the colon after food intake, but may not represent well physiological conditions nor regulation of PYY secretion in fasting state. Direct effect of molecules on differentiation or proliferation could be recently assessed using models recently developed, gut



organoids (Petersen 2014). Indeed, gut organoids consist in culturing *in vitro* intestinal stem cells that reproduce proliferation and differentiation, producing all type of intestinal cells (Sato 2009), and modification in cell type proportion and expression of differentiation factors can be easily measured. Moreover, use of transgenic mice expressing a fluorescent protein specifically in L-cells enabled precise studies on pure L-cell populations, but this method cannot be used in humans (Reimann 2008).

### Importance of gene expression regulation

To our knowledge, regulation of expression of human *Pyy* has not been studied probably because of the difficulty to sort L-cells and only HuTu-80 and NCI-h716 cell lines have been reported to express and secrete PYY (Kim 2014, Rozengurt 2006). It is noteworthy that only secretion was assessed in these cells. Similarly, regulation of rodent *pyy* expression has been described in only a few studies (Hand 2012, Zhou 2006, Zhou 2008), as studies mainly focused on GLP-1 production, the other main hormone secreted by L-cells (Nian 1999, Zhou 2006, Petersen 2014). However, conclusions are limited as one study used STC-1 cell line to assess effects of fatty acids only on production and secretion of PYY, and did not look at modulation of gene expression (Hand 2012). In another article, activation of *pyy* promoter was tested in human HEK cells, an unexpected model to study regulation in intestinal enteroendocrine cells (Zhou 2008). Nevertheless, regulation of expression of *Pyy* may be of particular importance as it modulates quantity of PYY that can be secreted upon stimulation. Indeed, quantity of PYY released after stimulation of secretion in single L-cells is dependent on two parameters: intensity of secretory signal, which regulates approximately number of secretory granules that exocytose; and quantity of PYY stored in secretory granules, depending on PYY production. Our results showed that increased expression of *Pyy* by butyrate increased quantity of PYY in non-stimulated cells. We therefore proposed that, along with increased number of producing cells which leads to similar effects by an overall increase of PYY quantity in the epithelium, increased production of PYY per cell may be a strategy to increase PYY plasma levels of obese patients with low fasting levels of PYY.

### Differences between human and rodent *Pyy* encoding gene

Gene analysis revealed important differences between human and rodent genes encoding for *Pyy*, as discussed in the introduction. It is surprising to find such a large intronic sequence between first exons in the sequence encoding for 96aa preproprotein cleaved in a single 36aa peptide, even if other large introns can be described in human genome (Sakharkar 2004). They indicate potentially important regulatory sequences or can be consequence of DNA duplication. Further analyses should be done to determine precisely the role of its structure, and comparative analysis to highlight potential differences in regulation of human and mouse *Pyy* gene and determine the important regions regulating *Pyy* expression. Moreover, C-terminal and N-terminal parts of preproPYY are poorly conserved. These sequences are mainly important for the sorting of the preproprotein to secretory granules for the maturation of the hormone. These differences may indicate differences in regulation of post translational modification and sorting to different types of secretory granules between human and mouse PYY, or simply reveal different mechanisms of sorting between the two species. Another observation which has been poorly addressed to our knowledge is the maturation of PYY1-36 to its cleaved form PYY 3-36 by DPP-IV. The third amino localized just after cleavage site is not conserved between human and mouse PYY and seems to be a variable amino acid as it is also different in rabbit

(Grandt 1994). DPP-IV cleaves the two first amino-acids of proteins and peptides when second amino acid is a proline (Mentlein 1993, Mentlein 1999). However, effect of modification of the third amino acid might impact kinetic or cleavage efficiency and therefore the potential ratio between the two forms of PYY. As the two forms have different activities by binding to different receptors, it would be interesting to know if these ratio and total quantity of each form are conserved between species. Moreover, DPP-IV activity is an important parameter in the regulation of PYY activity, but only a few studies addressed it. Inhibitors of DPP-IV have been used in order to decrease GLP-1 degradation in treatment for type 2 diabetes. These treatments affect PYY cleavage and further degradation (Aaboe 2010), but precise effects on functions mediated by different forms of PYY must be further analyzed, as well as general effects of these inhibitors as DPP-IV cleaves many different peptides. Regulation of this protease may be important in the context of different pathologies, as studies indicated a decreased expression of DPP-IV associated with Crohn disease (Moran 2012).

### PYY secretion in humans and rodents

Divergent regulation of *Pyy* may be associated with different effects of plasma PYY increase between the two species. Indeed, rodent and humans have different feeding cycles as humans are dependent on separated meals whereas rodents have a continuous feeding behavior during active period. Only a few studies reported 24h profile of circulating PYY, but all showed that PYY peaks after meals in humans (Hill 2011, Hill 2013). On the contrary, levels of PYY increased throughout the active period in mice fed *ad libitum* either during a fiber rich diet or a normal chow in one studies (Zhou 2008). However, to our knowledge, no other study tested PYY level profile over time in rodents fed *ad libitum*. Indeed, most *in vivo* studies measured *Pyy* plasma levels in fasted animals and/or after a single defined meal or nutrient ingestion, to mimic human feeding behavior. Moreover, it can be noted that PYY concentration rise is much slower in mice than in humans, which may reflect difference of energy uptake as daily energy uptake is divided in three or four meals in humans whereas it is nearly continuous in rodents. Therefore, based on these physiological remarks, we can think that *Pyy* expression may be regulated differently to respond to different patterns of feeding. In rodents, L-cells are nearly always stimulated by nutrient ingestion during active phase and therefore PYY is continuously secreted at high levels during active phase, whereas in humans, PYY secretion is much variable, requiring different production of PYY over time. More generally, using rodents as models for human behavior can be questioned due to this important difference: in humans, rise of PYY plasma levels as well as other gut hormones in response to food intake participates in the satiation mechanisms ending food intake. On the contrary, in mice, response to food intake does not induce a satiation feeling similarly as humans, but may participate in the regulation of caloric amount ingested. Other animal models have been used, mainly dogs, which may represent better models of human feeding behavior (Greeley 1988, Lin 2003, Yoshinaga 1992). However, rodent models have many advantages including easy laboratory handling, genetic manipulation to deplete or increase expression of specific genes or tag cell populations, and used as human disease models. Moreover, functions of PYY are similar between species as it is recognized by the same family of receptors in the same tissues.

Another important difference that can influence differential secretion of PYY is related to receptor expression or affinities between human and rodents. Indeed, human and rodents have many heterologous receptors, but some species may have specific receptors not found or not functional in other species. Family of Y receptors, recognizing NPY/PYY/PP is composed of five members in mice,  $y6$  which role remains uncertain but may participate in responses to PYY as it is expressed in the

hypothalamus (Berglund 2003, Larhammar 2004). In a work comparing intestinal Y receptors expression between rats and human, major differences in the expression of the different receptors was observed (Ferrier 2001). TLR family shows equally different members between species as human have ten TLRs (1-10) whereas mice have 12 TLRs with only 11 being functional (1-7, 9, 11, 12 and 13) as TLR8 is not functional (Kawasaki 2014). Therefore, scope of microbial sensing may differ between these species, and specific microbes may induce a response in mice and not in humans or inversely. Another important example of difference between human and rodent receptors can be described based on their affinity or their tissue expression. We described importance of FFAR2 and FFAR3 in responses to SCFAs. However, despite important homology, they are not activated with same affinities and order of potency in humans and in mice. Indeed, acetate is selective for FFAR2 in humans, *i.e.* has much higher affinity for FFAR2 than for FFAR3 but not in mice, whereas propionate is not selective for FFAR2 or FFAR3 in humans but is selective for FFAR3 in mice (Hudson 2012). Moreover, FFAR2 affinity is higher for SCFAs in humans than in rodents. These differences of affinities may explain, among other hypothesis, difference of sensitivity of L-cells in response to SCFAs in human and rodents. Indeed, two publications were recently published assessing effect of propionate on PYY and GLP-1 secretion in colonic primary cell cultures using similar protocols for culture preparations, and performed in the same laboratory. Secretion was only significantly increased when crypts were stimulated with concentration higher than 100mM of propionate in humans, whereas concentration as low as 1mM induced secretion in mice and rats (Chambers 2014, Psichas 2015). A last consideration that must be taken into account regarding different regulations of secretion between species is the differences of compounds and their concentration that can be found in the colonic lumen. A typical example is bile acids which are differently modified in humans compared to mice. Conjugation with taurine is increased in mice whereas majority of bile acids are conjugated with glycine in humans (Garcia-Cañaveras 2012). Signaling of bile acids in L-cells is mainly mediated by the GPCR TGR5 (Bala 2014, Parker 2012), and TGR5 has been shown to have increased sensitivity to taurine-conjugated bile acids (Kawamata 2003), indicating potential different sensing and activation in L-cells. Altogether, conclusions from studies using different species models to assess effect of elements should be confirmed in human or humanized models to validate that similar pathways are implicated in humans.

## Human cell lines modeling L-cells

In our project, we took advantage of human cell lines modeling enteroendocrine L-cells to assess two different potential regulation of PYY that can be easily measured and of particular interest for PYY activity: gene expression by measuring mRNA levels in cells and secretion by calcium imaging and measure of total PYY secreted during of short period (<2h) as both regulation can be considered to be important regarding PYY plasma levels.

We chose to use human cell lines NCI-h716 and HuTu-80 as they represented in our opinion the best models to characterize modulation of PYY expression and secretion in humans, NCI-h716 being the best model for enteroendocrine L-cells described. Using these cell lines allowed us to directly assess effect of molecules on PYY expression and secretion in an *in vitro* model. We could assume that, as cells were in controlled conditions, effects on Pyy mRNA levels or PYY concentration in cell supernatant was only regulated at the cellular level by gene expression modulation and/or mRNA stabilization or secretion respectively. Indeed, these cell lines are unique tools to assess effect of molecules on a specific type of cells that is sparse giving direct access to them, without requiring use of transgenic markers only available in animal models and cell sorting. Indeed, pure population allows direct

monitoring of effects, whereas in mixed population, a “noise” signal can occur due to regulation of the activity by other cells. Use of pure population can therefore decrease this “noise” and increase responses. However, this “noise” may also be important as it may represent indirect regulation due to paracrine activities from other cell types.

An interesting feature of *in vitro* experiments is the ability to engineer them to express reporter system or modify gene expression. Moreover, cell lines can be stably modified to ensure reproducible measures, an important feature when screening for different molecules or drugs as all cells will present similar feature. We took advantage of this possibility to integrate reporter systems for NF- $\kappa$ B activity and for calcium flux in cell lines, and attempted without success so far to integrate a reporter gene in the gene encoding for PYY to measure indirectly PYY production. Moreover, deletion of genes are possible using Crispr/cas9 or Talen making possible to directly modify human genome similarly to what has been done *in vivo* in rodent models, but in *in vitro* models, limiting ethical issues of these modifications (Le Cong 2013). We started to use this approach to target genes of interest to confirm implication of receptors. Furthermore, cell lines have a great advantage compared to other models as they can be used in large scale experiments and can be expended to high quantities easily, at low cost and without raising ethical issues, on the contrary to animal models. They are therefore greatly adapted to perform large scale screening.

### Limitation of cell lines

However, cell lines have many drawbacks and NCI-h716 and HuTu-80 cells are still very different from human L-cells. Cell lines are mostly tumor cells; they therefore express specific genes due to mutations or chromosomal rearrangements that allow them to cycle and proliferate, a process inhibited in differentiated cells. Modification of genes expression can induce different responses. It is important to validate in the used model the expression of important genes in the implicated regulatory pathways and compare them with *in vivo* results from the literature or experiments when possible. Concerning human L-cells, most data come from analysis of colonoscopies/biopsies associated with colonic tumors, even if samples come from parts that present healthy histology it is not known how they may be affected by the pathology. However, expression of specific genes in L-cells remains difficult to address because of their low number in tissues and the absence of external markers to sort them, and expression therefore relies on co-immunostaining of protein of interest with markers of L-cells.

Another major drawback of our models is the absence of polarization, as intestinal epithelial cells are polarized, which represent an important aspect. Indeed, they separate two very different environments: the gut lumen and the lamina propria. Polarization is very important in sensing as depending on the localization of the receptors, different molecules or concentrations can be sensed. Whereas SCFAs concentration are very high near the apical membrane (or at least in the colon lumen), they are several orders of magnitude lower in the lamina propria. Thus, a receptor, depending on its localization, may be activated or not in the same cell conditions. Moreover, polarization induces different regulation of signaling pathways by recruiting specific effectors at one side specifically, and activation of same receptor may induce different responses in the cell (Lee 2006). Development of polarized models for enteroendocrine cells would be of great interest to address these issues. Moreover, study of enteroendocrine cells in pure culture may not be considered as physiologically relevant as *in vivo*, they are mainly in contact with other type of cells, which may have paracrine effects and be important in the regulation of L-cells functions. Therefore, models of polarized cells in which L-cell are in contact with other epithelial cells should be more representative.

### Organoids, a future in *in vitro* studies?

Recent advances regarding stem cells allowed development of very promising *in vitro* models of intestinal epithelial, namely organoids (Sato 2009). Organoids can be seen as a good intermediate between *in vivo* studies and cell lines as they reproduce functional, polarized intestinal epithelium with all cell types present in similar proportion as *in vivo*, but allow precise control of environmental conditions and measure of specific features. Moreover, organoids can be cultivated for a much longer period than primary tissues and passed several times, and even can be expanded in a long term manner (Sato 2011). However, organoids may not reproduce all intestinal epithelial cells features, as they are cultivated isolated, and therefore are not influenced by host matrix, including afferent neuron signaling, immune cells nor complex lumen composition including mucus, microbiota and digested foodstuffs. Engineering of organoids is currently tested, but use of modern techniques should allow use of genetically modified organoids to allow functional analysis (Matano 2015), even if it may imply higher ethical issues due to modification of stem cell genome and experimental conditions for they culture may not be adaptable for large screening.

Moreover, most studies to assess effect of a molecule rely on binary responses: addition of molecule at different concentration in a simple defined condition. However, cells may not be stimulated that way but by variations of concentration due to local modification of the environment. Indeed, many molecules are present in the gut lumen, but depending of food intake and other stimuli, their variation may change. Moreover, other products may also interact in the responses by modifying cellular response, as we could demonstrate effect of butyrate on acetate or MAMPs sensing. Nevertheless, we considered that NCI-h176 and HuTu-80 cell lines were the best models to decipher precise mechanisms in humans regarding the direct modulation of microbial products on PYY expression and secretion and decipher the mechanisms implicated as well as to screen for large number of bacterial products to find new potential regulators.

## Importance of microbial sensing by L-cells

Taking advantage of cell lines modeling human L-cells, our results demonstrated different molecules and pathways implicated in the regulation of PYY expression and secretion in human L-cells by microorganisms, but confirmation of these mechanisms in *in vivo* models or *in vitro* models that better represent human intestinal epithelial is required, as well as determination of physiological conditions inducing observed responses. Microbiota, and in particular butyrate, seems to be an important regulator of L-cells by increasing PYY production, regulating expression of several receptors and modulating secretion in enteroendocrine cells through different mechanisms.

### Sensing of microbial products

We described for the first time expression of all TLR except TLR4 and TLR8 in L-cells and could demonstrate that they are functional. We also confirmed published results that L-cells express FFAR2, FFAR3 and GPR109a, and the expression of these receptors was similar to data from literature (Tazoe 2009). Interestingly, we reported that butyrate modulated expression of receptors, increasing expression of *Tlr* and decreasing expression of *Ffar2*. Therefore, we could demonstrate that L-cells may adapt in response to sensing of bacterial compounds by increasing its sensitivity to some elements but decrease its response to others, and in particular itself. Butyrate may therefore represent a primary signal from microbiota resulting in an adaptation of L-cells that are close to microbiota to enhance response in these cells.

### Regulation of PYY expression by SCFAs

Our results revealed a strong increase of *Pyy* expression after incubation with propionate or butyrate which could be reproduced by using a K/HDAC specific inhibitor, TSA. SCFAs may therefore represent important signaling molecules to increase *Pyy* expression. Interestingly they had no effect or even inhibitory effects on *proglucagon* expression. These data may explain partially the localization of L-cells and the specific density pattern of PYY. Indeed, PYY content of intestinal epithelium can be correlated with increased concentrations of SCFAs on the contrary to other hormones as PYY concentration is very low in proximal intestine and increases in the ileum to reach maximum in the colon. Moreover, effect of SCFAs on *Pyy* expression could be related to increased PYY plasma levels in a sustained manner associated with consumption of SCFAs-producing dietary fibers extensively described in the literature and in the introduction. Moreover, we could describe that SCFAs induced different effects depending on their chain length. Studies using different fibers showed that modulation of PYY was dependent on type of fibers (Arora 2012, Delzenne 2005, Parnell 2009, Zhou 2008). Fiber fermentation produces different ratio and amount of SCFAs, therefore we can hypothesize that different effects of fiber diet can be explained by differential production of butyrate and propionate and therefore different increased expression of *Pyy*. Other mechanisms may also be implicated as SCFAs regulate many other functions including number of L-cells, and fibers also impact on microbiota and thus potential range of microbial products affecting the host. Indeed, in organoid models, SCFA treatment increased number of L-cells and secretion, but effects were different between human organoids and mouse organoid (Petersen 2014). Interestingly, SCFAs did not increase late

enteroendocrine differentiation factors expression per cell, which are transcription factors inducing *Pyy* and *Proglucagon* expression in mouse organoids, but an increase was observed in the whole organoid due to increased number of differentiated cells. This indicated that SCFAs may not modulate expression of differentiation factor per cell, a result we confirmed assessing their expression in L-cells after butyrate treatment in human cells and for which we described significant but weak effects in NCI-h716 cells.

## Mechanisms implicated in the response to SCFAs in the modulation of gene expression

Interestingly, the main effect of propionate and butyrate was independent of receptors but of internalization of the SCFAs into the cell where they inhibit K/HDAC. We did not demonstrate directly that increased *Pyy* mRNA quantity was dependent on K/HDAC inhibition but only that a drug that also inhibits K/HDAC had a similar effect. Previous works have already demonstrated this effect of butyrate (Siavoshian 2000). Moreover, we did not assess if the effect was direct on *Pyy* transcription by modulating histone acetylation on *Pyy* gene or dependent on modulation of expression of another gene as done previously for p21/Kip1 (Siavoshian 2000). However, the effect of butyrate on gene expression was rapid as significant increase of *Pyy* expression was detected within two hours, suggesting a direct effect. Levels of *Pyy* mRNA still increased for 48h, indicating an accumulation of *Pyy* transcripts over time. We could therefore hypothesize that in cells treated with butyrate, *Pyy* transcription rate was higher than degradation, leading to a time dependent increase of expression; or that *Pyy* transcription kept increasing. We judged first hypothesis more probable as in cells treated with butyrate for 24h then incubated for another 24h without butyrate, the expression of *Pyy* was higher than in cell just treated for 24h with butyrate. This loss of balance between *Pyy* mRNA production and degradation can be quite surprising has potentially inducing a very important effect on the long-term. However, L-cells like other intestinal cells have a rapid turnover of about five days. Therefore, unbalanced transcription can be a mechanism to ensure maximum of production of PYY at the top of crypts just before anoikis. L-cells at the top of the crypts will be in contact with maximum of luminal content and therefore will be in first line to sense modifications of luminal compounds. Thus, PYY cell content should increase gradually from bottom to top of crypts. However, as L-cells are scarce in the epithelium and less than one enteroendocrine cell is found per crypt in general, it may be difficult to assess this hypothesis directly *in vivo* requiring live system assessing *Pyy* transcript levels to follow evolution of *Pyy*.

Transporters implicated in SCFAs uptake have been extensively described, and we could find expression of MCT-1 but not SLC5A8 in these cells. These two transporters are classically implicated in butyrate uptake and are normally localized on the apical membrane of intestinal cells (Gonçalves 2011, Ritzhaup 1998). Therefore, question of sensing luminal or basolateral content was not an issue regarding this regulation on the contrary to regulations dependent on receptors.

## Regulation of *Pyy* by microbial products through activation of receptors

Localization of receptors for which we demonstrated the expression in these cells is not known in colonic enteroendocrine cells. Thus, it is not known if luminal content, lamina propria content or both



are sensed by these receptors. Localization of membrane TLRs is of main importance in intestinal cells as they should not detect similar events and therefore induce similar responses to maintain host homeostasis. Lumen contents is composed of a lot of microorganisms and thus has high concentrations of MAMPs, thus TLRs localized on the apical membrane are supposed to be stimulated most of the time and should not induce important inflammatory response as sensing commensal bacteria. On the contrary, microorganisms in the lamina propria can mainly be associated with pathogen conditions and must induce an inflammatory response to resorb infection. We could show that *Pyy* expression was increased in NCI-h716 when TLRs stimulated. Implication of NF- $\kappa$ B activation and potential beneficial effects of increased production of PYY in response to infection has been discussed in the article about TLR expression in L-cells. However, it would be important to better characterize TLR expression and activation *in vivo* to understand under which situation they are activated and further analyze the effects of their activation on L-cells.

Similarly, localization of FFAR2, FFAR3 and GPR109a is important as SCFAs concentration in the gut lumen is more important than receptors selectivity, and at luminal concentrations, even acetate stimulates FFAR3 activation (concentration of 60mM vs EC<sub>50</sub> of 2mM). SCFA concentrations in the lamina propria however are not sufficient to activate GPR109a, and are of same order of magnitude as FFAR2 and FFAR3 EC<sub>50</sub> (~50 $\mu$ M) except acetate, which is not recognized by FFAR3 in these conditions (Brown 2003, Cummings 1987, Le Poul 2003, Thangaraju 2009). Thus, considering these SCFAs concentration, it may be hypothesized that FFAR2 and FFAR3 main functions will be in the basolateral membrane. Indeed, localization at the apical membrane would be associated with permanent activation if concentration of SCFAs in proximity to intestinal epithelial cells is the same as concentration measured in the lumen, as discussed further. Localization of receptor at the membrane is regulated by internalization after activation. Indeed, activation of most GPCRs signals for membrane rearrangement and endocytosis of the activated receptor. Different signalization pathways have been described that regulate recycling of receptors at the cell surface or degradation. These mechanisms therefore decrease localization at the cell surface of receptors that are activated permanently (Ferguson 2001, Hanyaloglu 2008) limiting extended activation. On the contrary, as SCFAs concentrations in the lamina propria are of the same order of magnitude as FFAR EC<sub>50</sub>, low modulations of SCFAs concentration would induce modification of activation of these receptors. Therefore, FFAR2 and FFAR3 would have a more important physiological function by sensing SCFAs that are released in the lamina propria and thus to the circulation. However, intravenous administration of acetate did not increase PYY plasma levels on the contrary to rectal administration (Freeland 2010), which would indicate a localization of FFAR2 at the apical membrane. However, acetate can be rapidly absorbed and released in the lamina propria and circulating acetate may not increase acetate concentration in the lamina propria, thus indicating that further analysis with more precise methodology are required to decipher the precise localization of FFAR receptors in the L-cells. On the contrary, GPR109a localization in basolateral membrane would not be associated with any activation, whereas it may be a sensor of variations of butyrate concentration in lumen when localized at the apical membrane due to its low affinity for butyrate.

Both stimulations of FFAR2 and FFAR3 increased *Pyy* expression but not stimulation of GPR109a. We could not identify precise signaling pathways implicated in these responses as inhibitors of G $\alpha$ q and G $\alpha$ i/o pathways both affected *Pyy* expression in our conditions, and could not decipher effects of SCFAs compared to effect of inhibitors on gene expression. Interestingly, only NCI-h716 cells and not HuTu-80 cells were sensitive to receptor activation regarding *Pyy* expression. We could explain that HuTu-80 cells were not sensitive to FFAR2 activation as this receptor is expressed at much lower levels in these cells than in NCI-h716 cells. Moreover, increased expression of FFAR2 induced a response to this receptor activation. This demonstrated that activation of FFAR2 directly induced *Pyy* expression.



However, whereas FFAR3 expression level is similar in both cell lines, only activation in NCI-h716 was associated with increased *Pyy* expression, which may question the effect of activation of this receptor. Moreover, as butyrate and propionate also stimulate these receptors, it would be interesting to test if part of their effect on *Pyy* was dependent on FFAR expression. A first indication that it may be the case is that butyrate and propionate have much lower effects in HuTu-80 cells than in NCI-h716 cells. Testing effect of butyrate and propionate in HuTu-80 cells overexpressing FFAR2 similarly to acetate would give a good indication, as well as depletion of FFAR2 and FFAR3 in NCI-h716 cells.

Concerning the stimulation of TLRs increased *Pyy* expression, we may hypothesize that this mechanism is mediated by NF- $\kappa$ B pathway activation. Indeed, TLR stimulation associated with an NF- $\kappa$ B activation induced an increase of *Pyy* expression whereas stimulation of some TLRs that did not activate NF- $\kappa$ B had no effect on *Pyy* expression. Moreover, hypothetical NF- $\kappa$ B binding domains were found in *Pyy* promoter gene. However, some stimulation of TLRs had no detectable effect on NF- $\kappa$ B activation but increased *Pyy* expression whereas TNF- $\alpha$  did not increase *Pyy* expression whereas it activated NF- $\kappa$ B. Use of inhibitors of NF- $\kappa$ B pathways would be important to prove implication of this pathway in regulation of *Pyy* expression.

### Concentration of SCFAs sensed by L-cells

We could not use concentrations of SCFAs similar to concentrations found in the colon on cell lines for long term due to inhibitory effects on proliferation and decreased viability of cells. However, butyrate induced an increase of expression of *Pyy* in a dose dependent manner and we could not detect saturating response, indicating that cells may be sensible to variations of SCFAs concentration. Precise concentrations in the colon could be measured only in human victims of sudden death to describe normal physiological concentrations in the colon (Cummings 1987). However, little is known about variations of SCFA concentration in the colon in response to food ingestion. Impact of diets is measured ever directly when animals are sacrificed by measuring concentration in the caecum, or indirectly by measuring SCFAs concentration in feces. However, concentration in the feces depends on two parameters, production by gut microbiota and absorption by epithelium. As epithelium absorbs majority of SCFAs and rate of absorption can be affected by different conditions including pH, SCFAs concentration in feces is not a good indicator for colonic concentration. Moreover, local concentration of SCFAs in proximity to L-cells is not known, as epithelial cells rapidly absorb SCFAs through transporters (Moschen 2012, Ritzhaupt 1998). Thus, local concentrations should be lower than concentrations found in the lumen due to the epithelium acting as negative source of SCFAs. Therefore, we could suppose the establishment of a gradient concentration of SCFAs between the lumen, where they are mainly produced and epithelium where they are absorbed. Moreover, depending on their localization in the crypt or at the surface of crypts, SCFAs concentration may vary, being higher at the top of crypts in this gradient hypothesis. Therefore, it can be assumed that enteroendocrine cells sense increased concentrations of butyrate during migration, resulting in increased production rate of PYY. On the contrary, in the lamina propria where SCFAs may activate basolateral receptors, SCFAs concentration are higher close to epithelial cells as they represent a positive source of SCFAs in that case, and SCFAs diffuse in the lamina propria before they enter circulation. Concentrations in proximity to the L-cells should therefore be more important than mean concentration in the lamina propria.

Temporal variations of SCFAs concentration in the lumen or in the proximity of intestinal epithelium are not known either. In humans, circulating concentrations were dependent on food intake and increased rapidly after food intake (Den Besten 2013, Lappi 2014, Wolever 1997). However, this

concentration increase cannot be induced by fiber fermentation as transit time for foodstuff to reach ileon and colon where most of fermentation takes place is much longer. Thus, other mechanisms may regulate SCFAs plasma concentration such as direct absorption of SCFAs present in foodstuff or increased transport of colonic SCFAs but may not be associated with variations of SCFAs concentrations close to L-cells. In the colon lumen, SCFAs concentrations are supposed to remain high overtime due to continuous fiber degradation and fermentation activity by bacteria. Small temporal concentration modification may occur due to increased availability of fibers or increased fermentative activity due other regulations or environmental modifications, but they may not be directly related to food intake but rather to intestinal transit.

### Regulation of PYY production by SCFAs

We could confirm effect of increased *Pyy* expression on production indirectly assessing cell secretion without stimulation in glucose saline buffer, and could show that increased expression of *Pyy* after butyrate treatment could be correlated with increased PYY release independently of secretion conditions. However, increase of released PYY was much lower than increase of expression, with a fold of 4 and 120 respectively. Two different hypotheses can be brought to explain this important difference. Firstly, increased expression does not necessarily induce similar increased production as many mechanisms are involved in regulation of production, implicating translation and post-translational modifications involved in maturation of preproPYY and sorting to secretory granules. Moreover regulation of transcription can be very rapid, whereas translation and sorting into secretory granules takes more time. Therefore, quantity of PYY released after 24h butyrate treatment should not be compared with quantity of *Pyy* mRNA after 24h butyrate treatment, but after less time, corresponding to the time required for translation, sorting and maturation. As effect of butyrate on *Pyy* transcription was time dependent, this difference may be important. Moreover, we could show that SCFAs only slightly increased ChromograninA expression. As ChromograninA is required for sorting of preproPYY in secretory granules, sorting into secretory granules can become a limiting step and overproduced preproPYY that may not be sorted to the right vesicles and be addressed to the lysosomes, resulting in a loss of production compared to transcriptional activity. Secondly, released PYY is only an indirect estimation of intracellular content, and only reflects quantity of PYY in secretory granules that go endocytosis without external stimulation, which may represent only a partial quantity of secretory granules. Ratio of PYY sorted in these granules may be dependent on its production. Direct assessment of PYY at the protein levels in these cells should be measured, however, we could not find satisfactory experimental conditions to measure this as evaluations of PYY intracellular content after lysis by ELISA were not reproducible and were not correlated to an increase of PYY expression or secretion. Moreover, measure of induced secretion in butyrate-treated cells compared to untreated cells may not reflect stored PYY as well. Indeed, butyrate also modulates secretion by modulating expression of receptors, including receptors to SCFAs, but also TLRs as well as GPR93, a GPCR recognizing peptones (data not shown). Therefore, stimulation of these receptors to induce secretion in butyrate-treated cells may be different from untreated cells and dependents on two mechanisms, modulation of PYY intracellular content and modulation of receptor activity as discussed further, and will not reflect only modulation of PYY content.

### Regulation of secretion by SCFAs

Calcium signaling is an interesting tool to assess indirectly secretion and allow efficient analysis of the regulation of secretion and can be easily used to decipher pathways implicated. Using ELISA assays to

measure secreted PYY during 2h, we showed that quantity of released PYY was increased when cells were incubated with each SCFA, confirming calcium-imaging results that indicated that all SCFAs induced a secretory response. However, calcium imaging indicating different responses as acetate and propionate induced a strong oscillatory response by stimulation of FFAR2 that we associated with important secretion whereas butyrate induced two responses: first a rapid and weaker oscillatory response than acetate or propionate which may be responsible for observed secretion and then a long term calcium hyperpolarization which we associated with inhibition of calcium rises due to lower cytosolic calcium concentrations. Indeed, addition of butyrate during acetate induced oscillations inhibited these oscillations. We could therefore associate two phenotypes in response to butyrate (and to a lesser extent propionate when used at higher concentrations): the calcium oscillatory response depending on  $G\alpha_q$  signaling that was associated with secretion and the cell hyperpolarization depending on  $G\alpha_i/o$  activation that inhibited secretion. These pathways corresponded to known FFAR coupling as only FFAR2 is coupled to  $G\alpha_q$  whereas all three receptors FFAR2, FFAR3 and GPR109a are coupled to  $G\alpha_i/o$ . As only butyrate activates these three receptors, it can be thought that activation of all of them was necessary to induce strong decrease of cytosolic calcium concentration whereas propionate, which only stimulates FFAR2 and FFAR3, had a much lower effect. Interestingly, it can be assumed that at lower doses, propionate and butyrate may have similar effects as butyrate no longer activates GPR109a but still activates FFAR2 and FFAR3 similarly to propionate in humans. We confirmed that butyrate inhibited secretion because secreted levels of PYY were increased when butyrate was removed from the media after long term incubation.

Altogether, activation of secretion in L-cells may be dependent on increase of SCFA concentration in the lamina propria whereas effect of hyperpolarization is dependent on increased butyrate production both at the basolateral and the apical membrane. We thus hypothesized that increased luminal butyrate concentration would signal for inhibition of secretion by L-cells and decreased concentration would be associated with a release of inhibition of secretion, thus increasing PYY secretion. Inhibition of secretion in a long term manner by butyrate would be a mechanism to limit its own effect on PYY production to limit high PYY plasma levels. Then, high butyrate concentration may increase storage of PYY due to the additive effects of increased PYY production and decreased secretion, and thus may increase the quantity of PYY released when cells are strongly stimulated.

### SCFAs modulate sensing in L-cells

Butyrate and propionate also modulate sensing of other molecules by regulating expression of some receptors. We could show a decrease of FFAR2 expression, but an increase of expression of most of TLRs as well as another GPCR, such as GPR93 which recognizes peptones. Butyrate decreases sensitivity and response to SCFA stimulation, but increases response to TLRs ligands and may increase response to peptones by increasing GPR93 expression by a fold of  $22 \pm 3.7$ , even if we could not associate directly TLR stimulation with secretion and did not test the response to GPR93 stimulation in NCI-h716 cells. Thus, butyrate may indirectly regulate PYY plasma levels by regulating expression of receptors expressed by L-cells, and therefore their response to luminal content. These important effects highlight the importance of these molecules and can be explained by the variety of cellular signaling pathway they may modulate, including GPCR activation, K/HDAC inhibition, cellular energetic and lipid metabolism...

SCFAs gained again interest recently due to characterization of their effects based on known cellular pathways implicated in their response (Den Besten 2013, Macia 2015), but demonstration of their

physiological importance had already been demonstrated (Cherbut 1998, Cummings 1987, Ropert 1996). Interest was also renewed by new techniques to study microbiota, mainly based on meta-“omics”, and implication of microbiota in several diseases. SCFAs luminal production can easily be modulated by diet as fibers are the main sources of SCFAs in the gut, and many studies revealed beneficial effects for host of fiber-rich diets. However, ingestion of fibers does not only increase SCFAs production, but induces a more general modification of microbiota and may have other effects than increased production of SCFAs (Tachon 2012, Cotillard 2013).

### SCFAs and metabolism in L-cells

SCFAs are also known to be an important source of energy for colonocytes by  $\beta$ -oxidation of butyrate. It is not known if colonic enteroendocrine cells are also able to use butyrate similarly to other colonocytes. It would therefore be also interesting to assess the role of butyrate as an energetic substrate in these cells and how it may impact on the energy-consuming hormone production.

## Beneficial effects of regulation of PYY production and secretion

### PYY in germ-free animals

Our results demonstrated that in humans, SCFA and TLR stimulation should increase plasma concentrations of PYY. However, different studies reported that circulating levels of PYY and number of L-cells were increased in germ free rodents, *i.e.* deprived of a microbiota and its fermentative capacities compared to conventional rodents (Arantes 1997, Goodlad 1989). Increased PYY plasma concentrations in germ-free rodents may result from adaptation of intestinal epithelium. Indeed, whereas germ-free animals may represent a good model to study role of microbiota or specific bacterial species, these animals present several physiological adaptations, including decreased cell proliferation, increased colon length, intestinal maturation and structure and impaired immunity development (Arantes 2001, McCulloch 1998, Round 2009, Wostmann 1983) that may participate in increasing PYY production and secretion indirectly. Surprisingly, despite high levels of PYY, germfree animals had higher food intake that compensated decreased energy absorption, indicating important other mechanisms regulating food intake based on sensing of absorbed nutrient and energy and not based on luminal content (Wostmann 1983). Moreover, colonization of germ free animals in models of infection or to induce fermentation increased PYY plasma levels (Khosravi 2015, Samuel 2008). On the contrary, whereas GLP-1 levels were high in germ free animals, colonization to induce fermentation decreased *proglucagon* expression, confirming our observation that butyrate and propionate decreased *proglucagon* in human cells (Wichmann 2013). Modification of gut hormone plasma levels in germ free models have been associated with an adaptation as intestinal epithelium is not matured by microbiota. These results in germ free animals can be associated with observation that neonates have increased plasma PYY levels (Adrian 1986, Wojciki 2012), and decreased plasma levels correspond to stabilization of microbiota and modification of feed behavior.

Altogether, these results indicate that maturation of intestinal epithelium by microbiota may decrease PYY production, corresponding to different role of PYY and different feeding behavior during early ages.

### Genetics of PYY and obesity

Genetic alteration of expression of PYY either by increased expression or depletion had little effects on food intake and obesity. Mice over expressing *pyy* gained less weight and did not develop obesity in genetic obese background or in response to high fat diet inducing obesity, but no statistical difference in food intake and weight gain were found when fed a normal chow. However, study was limited by low survival of embryos and therefore limited number of mice and potential selection of the surviving ones (Boey 2008), indicating a main function of PYY during embryo development. Similarly, depletion of *pyy* did not increase food intake in mice but had little effect on adiposity and bone metabolism (Boey 2006, Wong 2012). Genetic analysis searching for mutations of *Pyy* in humans revealed no mutation associated with obesity except one rare variant that may be associated with obesity, but further analysis and larger cohorts would be required to determine its importance (Ahituv 2006). Interestingly, in this study, they also found a mutation inducing a nonsense mutation. Heterozygote bearers of this mutation had slightly decreased circulating levels of PYY, but mutation was not associated with increased food intake or BMI. Once again, results were limited by the low

number of individuals and familial relationship of bearers of this mutation. Altogether, these results indicated that depletion of *pyy* or overexpression may not affect food intake due to many other pathways implicated in regulation of food intake and potential long term adaptation. However, one important way PYY regulates food intake is due to variations of its concentrations, which cannot be assessed in these experiments. Indeed, one important feature of PYY is variations of its levels after food intake, and decreased levels while fasting. In the ARC, PYY mostly inhibits NPY neurons by activation of Y2 receptors. NPY neurons themselves have orexigenic actions by inhibiting second order neurons in the PBN and the PVN as presented in the introduction. Sustained activation of receptors can induce its desensitization. Therefore, PYY may induce desensitization of Y2R on the long term in the ARC when continuously present at high doses and similarly, sustained secretion of NPY and AgRP may also desensitize their receptors and therefore their effects when PYY no longer inhibits their secretion. Therefore, better models to study effect of PYY on food intake would use inducible promoters to modulate its plasma levels. Indeed, variations of its concentrations in the range of physiological concentrations by intravenous injection or stimulation of its secretion modified food intake in many studies (Adrian 2012, Batterham 2003, Chambers 2014) whereas supraphysiological concentrations did not increase effect on food intake but induced nausea and increased fullness feeling, participating also in the regulation of feeding behavior (Le Roux 2008). To confirm these results, it would be interesting to infuse continuously PYY, as a resistance mechanism may be hypothesized to happen.

### Effect of SCFAs to modulate secretion for its anorexigenic effects

Therefore, long term effects of PYY on food intake may depend on variations of concentrations of PYY which necessitate time dependent regulations of secretion. Variation of concentration of SCFAs in the colon are not clearly known, but it can be supposed that they do not vary directly in response to food intake and variations are dependent on chime arrival several hours after food intake and regulated by intestinal transit. However, an interesting feature of butyrate is, in addition to increase PYY production, to reduce secretion potentially by decreasing cAMP levels through activation of  $G_{\alpha i}$ . Therefore, L-cells require either decrease of butyrate concentration or strong stimulatory signals to secrete PYY. As storage of PYY would have been increased, quantity of PYY released would be more important. Role of butyrate can thus be understood as a molecule that decreases L-cells response to low stimulatory signal and increases response for higher signals, resulting in increased variations of PYY secreted upon time. An important parameter moreover may be the variations of extracellular butyrate concentration as a decrease of butyrate concentration would result in an increase of sensitivity of L-cells and therefore increased secretion when stimulated. On the contrary, acetate which effect is mainly to induce secretion but has little effect on PYY production has a less clear function due to unknown regulation of its local concentration regarding food intake regulation. Continuous long term activation of secretion is potentially negative for PYY function on food intake, but variations of acetate concentration could be a potent strong stimulatory signal to increase PYY secretion. Moreover, SCFAs in the lamina propria correspond to SCFAs that are absorbed and not metabolized in epithelial cells but released to enter the circulation and serve as energetic substrate in other tissues. Therefore, increased levels of SCFAs in the lamina propria may signal for increased *Pyy* expression and secretion to reduce food intake by stimulating FFAR2 and FFAR3 receptors localized on the basolateral membrane.

Altogether, our results demonstrated part of the mechanisms that may be implicated in these observations and the importance of SCFAs, and especially propionate and butyrate in regulation of L-cells, by increasing production of PYY, increasing its secretion as well as increasing sensing of other molecules that are also implicated in the regulation of PYY secretion. However, we only described here a partial mechanism assessing effect of a few molecules in conditioned environment and isolated cells. However, we could decipher some mechanisms involved in the interesting effects of fibers in decreasing food intake in obese.

### Obesity, microbiota, SCFAs and TLRs

In human adults, obesity is associated with decreased PYY plasma level and decreased response to food intake, but obese individuals responded to administration of PYY by decreased food intake (Batterham 2003, Le Roux 2006). Moreover, obesity is also associated with a modification of microbiota, which can mainly be seen as a loss of genetic diversity and modification of functions. Microbiota from obese individuals has decreased number of genes associated with butyrate and methane production, but increased hydrogen sulfate production (Le Chatelier 2013, Turnbaugh 2009), indicating that microbiota from obese individuals may produce less SCFAs. Moreover, obesity is also associated with western diet: decreased consumption of fibers and increased consumption of lipids and non-complex carbohydrates, therefore reducing potential production of SCFAs by limiting substrates and increase prevalence of microbial genes implicated in metabolism of fibers (De Filippo 2010, Ou 2013). However experiments from gnotobiotic mice assumed that obesity could be associated with increased energy harvest, including from SCFAs and therefore SCFAs production could be increased in obese patients (Turnbaugh 2006). Moreover, different ratio of acetate: propionate: butyrate may be considered, as decrease of butyrate producers was mainly found, but it is possible that microbiota from obese individuals increases production of acetate and propionate (Lecomte 2015). We therefore could propose that reduced production of propionate and butyrate decreases production of PYY and as well as limit inhibitory effect of butyrate on secretion, two mechanisms we hypothesized to be important for PYY function regarding food intake. Moreover, obesity is also associated with low grade inflammation due to a more permissive intestinal barrier. We could show that sensing microbial products through TLRs by L-cells increased expression of PYY. Interestingly, PYY has been implicated in reduction of inflammation through Y receptors expressed on white adipose tissue macrophages (Macia 2012, Dimitrijevic 2005). However, TLRs sensing was increased after butyrate treatment and in our cells. We could therefore hypothesize that butyrate may also have an anti-inflammatory effect by increasing PYY production along with MAMPs and therefore could increase its effect on macrophages. However, this effect of butyrate may not be specific of L-cells, but butyrate may regulate more generally TLRs expression in intestinal epithelium and therefore indirectly modulate inflammatory response, which remain to be determined.

### Microbiota and PYY interrelations

Altogether, our results confirmed our hypothesis that L-cells can sense microbial products, at least MAMPs and SCFAs, resulting in modulation of Pyy expression and its secretion in human cell lines modeling enteroendocrine L-cells. Moreover, we could decipher precisely some of the cellular pathways implicated in the responses to SCFAs and we reinforced the beneficial role of fiber fermentation product, butyrate. However, due to the numerous limitations of our models, only hypothesis about the physiological responses associated with these mechanisms could be developed



as we could not assess directly important parameters such as localization of receptors or temporal and variations of SCFAs concentrations. Moreover, our model lacked interactions with other molecules implicated in regulation of PYY as well as proliferative and differentiation effects, which may result in much more complex effects. Altogether, we highlighted some mechanisms that main be implicated in the beneficial effects of fibers found *in vivo*. Interestingly, we also highlighted an important difference between PYY and GLP-1 as microbial products had no or inhibitory effect on *proglucagon* expression.

Knowledge about physiological functions of PYY indicates that PYY may also modulate microbiota through food intake and gut motility. Indeed, modulation of gut motility directly modulates microbiota (El Oufir 1996, Vandeputte 2015) in a physical way and by modulating nutrient availability, a function shared with regulation of food intake. Therefore, we could hypothesize that L-cells participate in the cross talk between microbiota and the host by sensing some of its products and by secreting hormones that regulate host functions modulating microbiota. We focused on two families of products that were known to have an effect on L-cells but for which precise mechanisms in humans remained to be deciphered or with known family of receptors. However, other pathways can be implicated such as stimulation of TGR5 by secondary bile acids that induces secretion in L-cells in a mechanism sensitive to hydrogen sulfate production by microbiota (Bala 2014, Adrian 2012) and some may remain to be discovered and characterized.

To conclude, using in vitro cell lines as model of L-cells, we found that microbes from the gut through production of SCFAs and MAMPs can impact on *Pyy* gene expression and PYY secretion. We believe that other microbial compounds are also involved in these regulations. The tools that were developed in the present work will be used in the near future through the functional metagenomic approach. Taking into account the important relationship between gut microbiota and human cells is needed for a complete understanding of human physiology, and a comprehensive analysis of human diseases.





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# Annexes



## Conference on 'Diet, gut microbiology and human health'

### Symposium 1: The gut microbiome: forming the microbiome and modern approaches to its study

#### Functional metagenomics to decipher food–microbe–host crosstalk

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The recent developments of metagenomics permit an extremely high-resolution molecular scan of the intestinal microbiota giving new insights and opening perspectives for clinical applications. Beyond the unprecedented vision of the intestinal microbiota given by large-scale quantitative metagenomics studies, such as the EU MetaHIT project, functional metagenomics tools allow the exploration of fine interactions between food constituents, microbiota and host, leading to the identification of signals and intimate mechanisms of crosstalk, especially between bacteria and human cells. Cloning of large genome fragments, either from complex intestinal communities or from selected bacteria, allows the screening of these biological resources for bioactivity towards complex plant polymers or functional food such as prebiotics. This permitted identification of novel carbohydrate-active enzyme families involved in dietary fibre and host glycan breakdown, and highlighted unsuspected bacterial players at the top of the intestinal microbial food chain. Similarly, exposure of fractions from genomic and metagenomic clones onto human cells engineered with reporter systems to track modulation of immune response, cell proliferation or cell metabolism has allowed the identification of bioactive clones modulating key cell signalling pathways or the induction of specific genes. This opens the possibility to decipher mechanisms by which commensal bacteria or candidate probiotics can modulate the activity of cells in the intestinal epithelium or even in distal organs such as the liver, adipose tissue or the brain. Hence, in spite of our inability to culture many of the dominant microbes of the human intestine, functional metagenomics open a new window for the exploration of food–microbe–host crosstalk.

Metagenomics: Gut microbiota: Glycoside hydrolases: Bacterial functions

Immune-mediated, chronic diseases of modern societies, steadily increasing worldwide since the 1950s, are most often associated with an alteration of the intestinal microbiota. The relevance of this observation is often challenged by the question of causality; i.e. the chicken or egg question. It nevertheless appears quite plausible that whether dysbiosis comes as a primary event or as a consequence of disease onset, in most pathophysiological conditions

alteration of intestinal ecology will promote stress signals for the immune system while aggravation of the inflammatory tone will deliver oxidative stress signals to the gut microbiota, such that a vicious circle is most likely to establish<sup>(1)</sup>. Preventing or tackling this context will hence require combining measures of immune and microbiota modulation. Both necessitate a finer understanding of food–microbe and microbe–host crosstalk.

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In a context where a large fraction of dominant gut microbes has remained inaccessible by classical culture methods<sup>(2)</sup>, functional metagenomics is providing the best approach to decipher food–microbe–host crosstalk. Shotgun sequencing of genetic information extracted from the intestinal environment allowed us to broadly revisit our view of the ecosystem in terms of phylogenetic structure and yielded massive datasets that may inform on functional potentials. At the same time, it has become possible to extract and clone large metagenomic fragments and directly explore their functionality with respect to food–microbe and microbe–host interfaces. Heterologous gene expression hence became a key for the culture-independent functional exploration of complex ecosystems relying on the extraction of environmental genomic DNA and its cloning in well-known cultivable hosts via fosmids, cosmids or bacterial artificial chromosomes. This exploration of functionalities of uncultivable bacteria within the metagenome of complex ecosystems is commonly referred to as activity-based functional metagenomics, as opposed to sequence-based phylogenetic or metagenomic characterisation of microbial ecosystems<sup>(3)</sup>.

#### Functional metagenomics and the food–microbe interface

One of the first applications of functional metagenomics gave access to formerly unknown fibrolytic enzymes from the microbiota of a thermophilic digester. Using *Escherichia coli* (a Gram-negative bacterium) as a host, even genes from anaerobic Gram-positive bacteria could be expressed<sup>(4)</sup>. Still using an *E. coli* host and a bacterial artificial chromosome library, Walter *et al.* screened a mouse intestinal metagenome for  $\beta$ -glucanase activity<sup>(5)</sup>. Out of a total of 5760 clones (representing 320 Mb genomic DNA, 55 kb per clone) they identified three clones encoding enzymes of interest. Similarly, Ferrer *et al.* screened a metagenome derived from rumen contents<sup>(6)</sup>. Within a metagenomic library of 14 000 clones (representing 77 Mb genomic DNA, up to 8 kb per clone) they identified and characterised twenty-two clones with distinct hydrolytic activities. In these two studies, the coverage of the actual metagenome was extremely limited owing to the size of the library. They nonetheless highlighted the ability of the functional to yield access to full operons and operational gene clusters.

Jones *et al.* screened about 90 000 fosmid clones derived from a human faecal sample (representing 3.6 Gb large inserts metagenomic DNA) for bile salt hydrolase activity<sup>(7)</sup>. They observed these functions in all major gut bacterial divisions. In the same way, Tasse *et al.* screened 156 000 clones from a human faecal sample (representing 5.5 Gb metagenomic DNA) for hydrolytic activities towards different polysaccharides<sup>(8)</sup>. This exhaustive analysis allowed the identification of seventy-three novel carbohydrate-active enzymes, belonging to thirty-five known and nine totally novel glycoside-hydrolase families involved in the catabolism of complex

glycans in the human intestinal tract. Yet unknown mechanisms and three-dimensional structures have been pointed out for twelve families and fifteen families, respectively. This allowed the first discovery of novel functions of carbohydrate breakdown by using metagenomics<sup>(9)</sup>. Following a similar strategy, prebiotic-catabolising metagenomic clones were selected both from faecal and ileal samples, highlighting the potential of still unknown gut bacteria to metabolise functional foods<sup>(10)</sup>.

#### Functional metagenomics and the microbe–cell interface

Another important function of microbiota is its interaction or crosstalk with its host through intestinal epithelial cells. The use of reporter cell lines that allow quantification of specific functions such as cell proliferation, transcription factor activity or modulation of gene expression makes the screening of intestinal microbiota for these functions possible in a high-throughput format.

Interaction between microbes and eukaryotic cells can be explored using cultured commensal bacteria. Kaci *et al.* used a reporter system for a key transcription factor involved in the inflammatory response, NF  $\kappa$ B, to study the anti-inflammatory effects of different strains of *Streptococcus salivarius*<sup>(11)</sup>. Using human epithelial cells that were stably transfected with a reporter gene (in this case *Luciferase*) expressed under the control of a NF  $\kappa$ B binding domain, strains were tested for their capacity to down-regulate TNF  $\alpha$ -induced NF  $\kappa$ B activation simply by measuring reporter gene activity. Strains can be differentiated for their anti-inflammatory capabilities and characterisation of cellular mechanisms involved can be further implemented. This strategy has been used to test different bacterial strains of the species *Lactobacillus delbrueckii*<sup>(12)</sup>, questioning their anti-inflammatory properties as well as some pathogen activities<sup>(13)</sup>.

However, since culturable bacteria only represent a minority of total bacterial species of the intestinal ecosystem, metagenomic libraries may be used to find functions of unknown genes towards epithelial reporter cell lines in order to overpass this limitation, in analogy to their use to study enzymatic activities (Fig. 1). A first approach was to screen for cellular growth using crystal violet. In the present study, Gloux *et al.* screened over 20 000 metagenomic clones for modulation of proliferation of two human cell lines, HT-29 (modelling intestinal epithelial cells) and CV1 (a kidney fibroblast)<sup>(14)</sup>. This approach resulted in the identification of over fifty clones modulating cell proliferation. Interestingly, clones came from all major phyla represented in the two libraries tested in similar proportion, and a majority of genes belonged to unculturable bacteria, showing the possibilities offered by this technique<sup>(14)</sup>.

We subsequently used the NF  $\kappa$ B reporter system in two different human intestinal cell lines for functional metagenomic studies, and revealed new clones involved in modulation of this immunomodulatory pathway<sup>(15)</sup>. Screening of a library of mutants resulting from the random insertion of transposons in clones of interest was



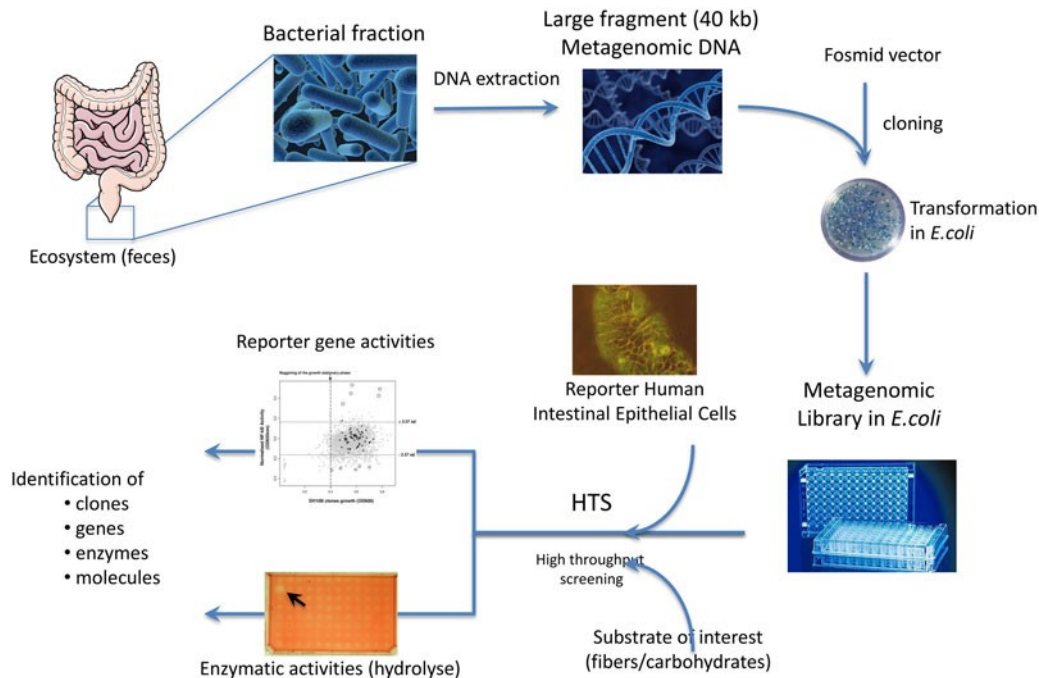


Fig. 1. (Colour online) Schematic overview of the functional metagenomic approach developed to search for bacterial genes able to hydrolyse substrates of interest or to activate cell-signalling pathways in reporter human intestinal epithelial cells. Briefly, after isolation of faecal microbiota and DNA extraction, metagenomic DNA of about 40 kb are separated by pulse-field gel electrophoresis before cloning in a fosmid vector and infection of *E. coli* carrier bacteria to obtain metagenomic libraries. High-throughput screening (HTS) is then performed using robotic liquid-handling equipment to identify clones presenting the activity of interest, i.e. degradation of selected carbohydrates or modulation of reporter gene expression in human cells.

used to identify which genes of the clones were responsible for the activity. Use of reporter system with easily measurable activity such as luminescence, absorbance or fluorescence enables automation of screening, making possible use of larger libraries, representing a better coverage of the gut metagenome. Moreover, to decipher new microbe–host interactions, other screening models and advanced methods have been developed to study transcription factor activity such as activator protein 1 or PPAR $\gamma$  or gene expression related to the production of transforming growth factor  $\beta$ , indoleamine 2,3-dioxygenase, thymic stromal lymphopoietin and others<sup>[16]</sup>. Recent advances in eukaryotic genome engineering with development of TALEN or Crispr/Cas9 technologies may be used for the development of new reporter systems by tagging endogenous genes. The development of these new tools will allow the discovery of new functionally bioactive clones, for which mechanistic studies will further expand our knowledge of interactions between the microbiota and its host.

However, one major drawback of functional metagenomics is that genes encoded in clones may not be expressed functionally, as they may require specific maturation or secretion systems. Moreover, bacterial genes are often expressed in functional clusters. This is why most screenings published so far have used large inserts, from 10 to 50 kb, to allow expression of potentially full operons required for the expression of a gene and

observation of its associated activity. Still, whole organism and not only adjacent genes may be important for their functional expression. Until now, *E. coli* has been the only model of bacteria used for gut microbiota screening, but other host models should also be developed. Even though functional genomics using *E. coli* as the host strain made it possible to discover bioactive genes from Gram-positive bacteria, only about 40 % of heterologous genes are functionally expressed in this strain<sup>[17]</sup>. Different efforts to use a Gram-positive host strain have been made, as most probiotics (which may by definition confer beneficial effects on the host) are Gram-positive, and this kind of bacteria has specific secretion and cell-wall anchoring systems. Dobrijevic *et al.* recently developed a strategy to use a *Bacillus subtilis* strain with low basal activity as a vector of expression<sup>[18]</sup>. However, the use of this model for high throughput screening has not been published yet.

As a conclusion, metagenomics allows a good and exhaustive characterisation of the human intestinal ecosystem going from who is there to what do they do, towards an ecological understanding of the gut<sup>[1]</sup>. The functional metagenomic approach is an interesting complement to full sequencing. Deeper characterisation of the functions of the microbiome in a healthy state as well as in pathological conditions in which microbiota dysbiosis is observed remains to be investigated further.

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### Conflicts of Interest

None.

### Authorship

All authors contributed to the writing and editing processes.

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## Annex 2: Measure of cell intensity over time, a script for automatical images

This script works with a time stack of images acquired on a Zeiss LSM700 confocal saved as .czi using Fiji software without additional plugging.

// This function only requires the folder the stacks are stored in, the folder to save result and the name of the file, and saves a table containing the mean intensity for each cell detected over time, as well as the masks used saved as region of interest (ROI). If all files of a folder have to be similarly analyzed, the function getFilelist can be used to create a list of all filenames, and this function may be run in a loop for each element of the list.

```
function moyenne_cell (input, output, filename) {

//open the stack and in case of hyperstacks if different colors are tested, it selects the second stack
(corresponding to GFP in our case)

run("Bio-Formats Importer", " open=["+ input + filename + "]" color_mode=Grayscale open_files
split_channels view=Hyperstack stack_order=XYCZT use_virtual_stack");

if (isOpen(filename + " - C=1") ) {

selectWindow(filename + " - C=1");

close();

selectWindow(filename + " - C=0");

}

//Reduce stack to a single image averaging each pixel intensity over time

run("Z Project...", "start=0 stop=180 projection=[Average Intensity]");

saveAs("Tiff", output + "avgbef"+ filename + ".czi");


//The unsharpmask increases contrast and border delimitations, increasing efficiency of the threshold.
A test to determine best automatic threshold method for specific cell type or depending on cell density
should be done as different methods can give different results.

run("Unsharp Mask...", "radius=5 mask=0.60");

setAutoThreshold("Huang");

// A watershed algorithm is applied to discriminate different structures, and final image is saved for
the user to control result.

run("Make Binary");
```

```

run("Watershed");

saveAs("Tiff", output + "mask" + filename + ".czi");

selectWindow(
"mask" + filename + ".tif");

// based on the binary image obtained, particles are determined as positive continuous structures
// respecting size and circularity conditions (depending on cell type, these parameters should adapted).
// The particles are saved as ROI, allowing their use on other analyses

run("Analyze Particles...", "size=50.00-Infinity circularity=0.20-1.00 show=Nothing clear include add");

roiManager("Save", output + "ROI" + filename + ".zip");

saveAs("Tiff", output + "ROI" + filename);

close();

// The stack is open again and ROI corresponding to cells are used to measure on each time point
// fluorescence, result is saved as a table with single cells and time point in entries and intensity are the
// values

selectWindow(filename + " - C=0");

roiManager("Multi Measure");

saveAs ("Results", output + filename + ".txt" );

close();

run("Close All");

}

```

## Annex 3: From a table of values to graphs and representative values

This script was developed on R software to calculate from a table of intensity per cell and per time interesting features to compare results together and draw the curbs representing variations of fluorescence per cell. It requires some specific libraries: clusterSim, FactoMineR, ade4, gplots, lattice, ggplot2, sm and MASS

### Construction de la fonction normalisation : moyenne des trois valeurs adjacentes avec un poids double pour la valeur centrale divisée par la valeur basale calculée comme la moyenne des valeurs prises entre la 5 et 40eme image, pour une série de mesure, retourne le vecteur correspondant. Les valeurs limites sont calculées uniquement en moyennant avec leur unique voisin###

```
normalization<- function(x) {

  l=length(x)
  vec=rep(0,l)
  length(vec)<- l
  basal<-mean(x[5:40])
  vec[1] <- mean(x[1:2])/basal
  vec[l] <- mean(x[(l-1):l])/basal
  for (i in 2: l-1) ( vec[i]<-mean(c(x[(i-1):(i+1)], x[i])))/basal)
  invisible(vec)
}

# choix du dossier de travail et récupération des fichiers du dossier
setwd("C:/Users/plarraufie/Desktop/films gcamp/20150413 Htu NCI GPR43/Results mcherry/ ")
dir.create("normalize")
fichiers=list.files(, pattern=".txt")

# Pour chaque fichier du dossier
for( k in 1 : length(fichiers))
{
  datatest=read.table(file=fichiers[k])
```

```

norm<- matrix(0 , nrow(datatest), ncol(datatest), dimnames= list(rownames(datatest),
colnames(datatest)))

## #applique la fonction normalisation à chaque courbe et forme la matrice des courbes normalisées
et trace l'ensemble des courbes sur un même graphe. Enregistre le graphique en PDF et le tableau
des valeurs normalisées ###

for (j in 1 : ncol(datatest))
{
norm[,j]<-normalization(datatest[,j])
}

normtxt=paste("normalize/", sub(".dzi.txt", "", fichiers[k]), "norm.txt")
normpdf= paste("normalize/", sub(".dzi.txt", "", fichiers[k]), "norm.pdf")
file.create(normtxt)
write.table(norm, normtxt, sep="\t")
pdf(normpdf)
matplot(norm, type="l")
dev.off()
}

#définition du maximum local : valeur supérieure à moyenne sur les quatre seconde autour * 1.1 et
#valeur max sur +-2s

#période = temps entre deux max successifs

#temps d'activation = temps pour lesquels valeurs > (max -1)/exp(1/2)

#sur les fichiers normalisés

setwd("C:/Users/plarraufie/Desktop/films gcamp/20150413 Htu NCI GPR43/Results
mcherry/normalize")

fichiersnorm=list.files(pattern="norm.txt")

### Pour chaque fichier normalisé, construit deux tableaux, un d'analyse et un de résumé
comprenant 8 variables d'intérêt : le min, le max, la moyenne, le nombre de pics, la période des pics,
la sd de la période des pics, la durée des pics et la sd de la durée des pics ###

for (p in 1 : length(fichiersnorm) )
{
test=read.table(fichiersnorm[p])
pfile=sub(".txt", "", fichiersnorm[p])
pfileanalysis=paste(pfile, "analyzed.txt")
pfilesummary= paste(pfile, "summary.txt")

```

```

listcurbanalysis= list()

nb= ncol(test)

interestvariables <- c("min", "max", "mean", "nbpeaks" , "period", "periodsd", "peakduration",
"peakdurationsd")

curbanalysis<- matrix (0, nrow=8, ncol=nb, dimnames = list(interestvariables, NULL))


for ( a in 1:nb)
{
ntest=(test[,a])
l=length(ntest)

#le min, le max et la moyenne sont calculés sur la période entre 70s et la fin
curbanalysis[1,a] = min(ntest[70 :l])
curbanalysis[2,a] =max(ntest[70:l])
curbanalysis[3,a] =mean(ntest[70:l])


#### recherche les maximums relatifs, définis comme des maxima sur la période -4s ; +4s et dont la
valeur est supérieure à 1.1*la valeur moyenne sur cette même période ; associe à la position du
maximum sa valeur et calcul le temps pendant lequel la courbe (décalée de 1) est supérieure à la
valeur du maximum * e^(-1/2) (durée du pic) pour chaque courbe d'une expérience #####

z<-0

maxrel<-matrix(0,nrow=3)

for(i in 11:(l-10))
{
if ( (ntest [i]==max(ntest [(i-2):(i+2)])) & (ntest[i]>(1.1* mean(ntest[(i-4):(i+4)] ) ) ) )
{
k=0 ; j=0 ; z=z+1
while( (ntest[i-k]-1) >= ( (ntest[i]-1) /exp(1/2)) ) k=k+1
while( (ntest[i+j]-1) >= ( (ntest[i]-1) / exp(1/2)) & (i+j<l) ) j= j+1

x=( (ntest[i]-1)/(exp(1/2)) - ntest[i-k]+1 ) /(ntest[i-k+1]-ntest[i-k])
y=( (ntest[i]-1)/exp(1/2) - ntest[i+j] +1 ) /(ntest[i+j-1]-ntest[i+j])

```

```

res<-matrix(c(i,ntest [i], (k+j-x-y)), nrow=3)

maxrel<-cbind(maxrel,res)

}

}

#obliger d'enlevée la première colonne des data sur les max relatifs (0)

maxrel<-maxrel[,-1]

tempo<-c(1)

curbanalysis[4,a] <- z

#### si au moins trois pics, calcule une période moyenne comme la moyenne des écarts de temps
entre deux maxima relatifs consécutifs, en détermine également l'écart type (caractérisation des
oscillations pour savoir si la fréquence à un sens ou non. Calcul également la moyenne des durées
des pics et encore la sd ####

if (z>2)
{
for(i in 1 : (z-1)) tempo[i]=(maxrel[1,i+1]-maxrel[1,i])

curbanalysis[5,a] = mean(tempo)
curbanalysis[6,a] = sd(tempo)
curbanalysis[7,a] = mean (maxrel[3,])
curbanalysis[8,a] = sd(maxrel[3,])
}
}

### sur l'ensemble des courbes d'une expérience, calcul la moyenne des min, des max, des
moyennes, le pourcentage de cellules oscillantes, le nombre d'oscillations moyen pour ces cellules, la
moyenne des périodes et des durées de pic calculés précédemment. ###

meanresults=matrix(0,nrow=7, ncol=1, dimname=list(c("min", "max", "mean", "%respondingcells",
"nbpeak", "period", "peakduration"), NULL))

meanresults[1,1] = mean( curbanalysis ["min",])
meanresults[2,1] = mean( curbanalysis ["max",])
meanresults[3,1] = mean( curbanalysis ["mean",])

```



```
## tri les courbes par période pour déterminer si certaines courbes oscillent ou pas. Si oscillations,
calcule le nombre de courbes avec oscillations et les moyennes sur les courbes oscillantes###
```

```
sortcurb=curbanalysis[,order(curbanalysis["period",], decreasing=T)]
```

```
if (sortcurb["period",1]>0)
```

```
{
```

```
o=1
```

```
while ( (sortcurb["period", o]>0) & (o<ncol(sortcurb)) ) o=o+1
```

```
meanresults[4,1] = (o-1)/ncol(curbanalysis)
```

```
meanresults[5,1] = mean(sortcurb["nbpeaks",1:(o-1)])
```

```
meanresults[6,1] = mean(sortcurb["period",1:(o-1)])
```

```
meanresults[7,1] = mean(sortcurb["peakduration",1:(o-1)])
```

```
}
```

```
write.table(curbanalysis, file = pfileanalysis)
```

```
write.table(meanresults, file=pfilessummary)
```

```
}
```

```
####Construit la matrice des résultats précédents ( variables) pour l'ensemble des expériences du
dossier###
```

```
variablesname = c("min", "max", "mean", "%respondingcells", "nbpeak", "period", "peakduration")
```

```
fichierssum=list.files(pattern="summary.txt")
```

```
bilan=matrix(0,nrow=7, ncol=length(fichierssum) )
```

```
expe=list()
```

```
for (q in 1:length(fichierssum) )
```

```
{
```

```
bilanq=read.table(fichierssum[q])
```

```
for( s in 1 : 7) bilan[s,q]=bilanq[s,]
```

```
expe = c(expe, fichierssum[q])
```

```
}
```

```
dimnames(bilan) = list(variablesname, expe)
```

```
write.table(bilan, file= "resultscompilation.txt")
```











## Résumé de la thèse

L'intestin est un organe majeur de l'organisme de par ses fonctions et sa localisation, établissant une barrière active avec un environnement complexe composé du microbiote intestinal, des aliments digérés et d'éléments sécrétés par l'hôte. Outre ses fonctions digestives, absorbatives et immuno-modulatrices, l'intestin est également un important organe endocrinien, sécrétant une vingtaine d'hormones régulant des fonctions physiologiques telles que la prise alimentaire, le métabolisme énergétique ou la digestion et le transit intestinal. Ces hormones sont produites par une famille de cellules épithéliales, les cellules entéroendocrines, et sécrétées en réponse à l'activation de récepteurs reconnaissant des éléments du contenu intestinal. En particulier, les cellules entéroendocrines de type L sécrètent GLP-1 et Peptide YY (PYY), impliqués respectivement dans le contrôle de la sécrétion d'insuline et dans la régulation de la prise alimentaire ainsi que le contrôle du transit intestinal. Elles sont majoritairement localisées dans l'iléon et le côlon, là où le microbiote intestinal est le plus dense. Le microbiote intestinal permet notamment la fermentation des fibres en acides gras à chaîne courte (AGCC), la production de vitamines, la maturation du système immunitaire de l'hôte et joue lui-même un rôle de barrière contre les pathogènes. Un dialogue entre le microbiote intestinal et l'hôte est nécessaire dans le maintien de l'homéostasie intestinale, nécessitant la reconnaissance par l'hôte de produits bactériens. En particulier, les récepteurs Toll-Like (TLR) permettent la reconnaissance de motifs moléculaires microbiens conservés et sont impliqués dans l'immunité innée de l'hôte. Certains produits bactériens ont également un rôle physiologique tels que les AGCC qui sont une source d'énergie importante pour les colonocytes, en plus d'activer des voies de signalisation. Il a été montré que des régimes riches en fibres, et donc permettant une production accrue d'AGCC, ou plus directement l'administration d'AGCC dans le colon, induit chez l'Homme ou la souris une augmentation des concentrations plasmatiques de PYY, par des mécanismes encore peu compris. En utilisant des lignées cellulaires humaines modèles de cellules entéroendocrines, nous avons caractérisé les effets des AGCC et des motifs bactériens reconnus par les TLR sur l'expression et la sécrétion de PYY et les réponses calciques dans ces cellules. Nous avons pu démontrer que les TLR sont exprimés de manière fonctionnelle, à l'exception de TLR4 et TLR8 dans ces cellules, et que le butyrate augmente leur expression et leur activité. De plus, la stimulation des TLR augmente l'expression de Pyy d'un rapport de 2, mais a peu d'effet sur la sécrétion dans ces cellules. Les AGCC ont des effets divers sur l'expression et la sécrétion de PYY. Alors que le butyrate et le propionate augmentent très fortement l'expression de Pyy, par des rapports respectivement de 120 et 40, par un mécanisme d'inhibition des déacétylases d'histone et de lysine, l'acétate augmente l'expression de Pyy plus modestement par l'activation des récepteurs aux AGCC FFAR2 et FFAR3. L'activation de FFAR2 par les AGCC induit une forte réponse calcique oscillatoire induisant la sécrétion de PYY alors que l'activation de FFAR3 et de GPR109a par le butyrate diminue la concentration calcique cellulaire et réduit les réponses sécrétoires. Ainsi, les AGCC augmentent la production de PYY et régulent sa sécrétion, mais avec et par des effets différents. Ces travaux ont permis de montrer le rôle des cellules entéroendocrines humaines de type L dans la reconnaissance de produits bactériens par l'expression de TLR et par leurs réponses aux AGCCs modulant l'expression et de la sécrétion de PYY. De plus, ces résultats ont déterminés en partie les mécanismes impliqués dans la réponse bénéfique de l'hôte à la consommation de fibres et l'augmentation de la production d'AGCC.

## Summary of the thesis:

The human gut exerts major functions, mainly due to its localization and by forming an active barrier between a complex environment made of the gut microbiota, digested food products and secreted elements by the host. The main functions of the gut are digestion and absorption of nutrients and it is the first pool of immune cells and a barrier against pathogens, but the gut is also a main endocrine organ secreting more than twenty different hormones. These hormones regulate a wide range physiological functions including food intake, energy metabolism or digestion. Enteroendocrine cells, a sparse family of intestinal epithelial cells, produce and secrete these hormones in response to the activation of a variety of receptors that sense luminal content. Among them, L-cells secrete GLP-1 and Peptide YY (PYY) that are implicated in the regulation of insulin secretion, food intake and intestinal motility. They are mainly found in the distal ileum and in the colon where the microbiota is the densest. Gut microbiota ferments fibers into short chain fatty acids (SCFAs), produces vitamins, participates in regulation of host immune system and is a barrier against pathogens. The cross talk between microbiota and intestinal epithelium is important to maintain the local homeostasis, and is mediated by host receptors recognizing microbial products. Among them, Toll-like receptors (TLRs) recognize conserved microbial associate molecular patterns (MAMPs) and participate to the host innate immunity. Some microbial products also have important functions for the host such as SCFAs that are an important energy substrate for colonocytes and can also activate different signaling pathways. It was shown that fiber-rich diets, increasing production of SCFAs, as well as direct administration of SCFAs in the colon in humans or mice increased PYY plasma levels through mechanisms still undeciphered. Taking advantage of human cell lines as L-cell models, we assessed the different effects of SCFAs and TLR stimulation on PYY expression and secretion and calcium signaling in these cells. We showed that TLRs are functionally expressed in these cells at the exception of TLR4 and TLR8, and that butyrate, one of the three main SCFAs produced by the microbiota increases cell sensitivity to TLR stimulation by increasing their expression. Moreover, TLR stimulation increases Pyy expression by a fold of two but has little effect on secretion. SCFAs differently regulate Pyy expression. Propionate and butyrate highly increase Pyy expression by a fold of 40 and 120 respectively, and their effects are mainly mediated by inhibition of lysine/histone deacetylases whereas acetate increases expression of Pyy by a fold of 1.8 through stimulation of FFAR2 and FFAR3. SCFAs also induce a strong FFAR2-dependent oscillatory response monitoring PYY secretion whereas butyrate via FFAR3 and GPR109a decreases cytosolic calcium concentration and consequently reduces secretory responses. Thus, SCFAs differently increase PYY production and secretion depending of their chain length. Altogether, these results highlight the role human L-cells in microbiota-host crosstalk by sensing microbial products through expression of TLRs and their responses to SCFAs modulating PYY production and secretion. Furthermore, we deciphered some of the mechanisms implicated in beneficial host response to enriched fiber diets and increased production of SCFAs.